A STUDY OF PLATELET AGGREGATION IN WHOLE BLOOD FROM NORMAL AND PROTHROMBOTIC SUBJECTS

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PhD Thesis

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ABSTRACT

The aim of this thesis was to study platelet function in whole blood, in an environment close to normal physiology. It was anticipated that such studies in whole blood would be more sensitive to in vivo changes than studies on isolated platelets, since all of the formed elements are present, there is less risk of in vitro activation, and labile substances should still be present. This approach was thought to be particularly applicable to platelet studies in blood from patients with prothrombotic states where the mechanism of thrombosis is unclear, and for examining anti-platelet drug efficacy. Existing methods were inadequate and it was first necessary to develop new techniques using electrical impedance.

Sensitive and reproducible impedance methods were developed, and these were especially useful for blood samples with platelet counts below $50*10^9/l$. A particular type of aggregate was detected, requiring irreversible aggregation, thromboxane A_2 generation, but not ADP secretion. Measurements in non-anticoagulated blood allowed a global view of haemostasis, reflecting interactions of platelets, neutrophils, thrombin, and fibrin.

In the presence of calcium^{++}, neutrophils and thrombin potentiated the aggregation response. There was excessive aggregation in non-anticoagulated blood from patients with multi-organ failure. This was related to increased neutrophil count, enhanced thrombin generation, and reciprocal cell activation. Such patients may benefit from eicosanoid antagonists and protease inhibitors to prevent unwarranted

activation of coagulation and cellular defense mechanisms.

Chronic arteriopaths showed reduced aggregation compatible with in vivo activation. Prostacyclin analogue infusions were less effective as judged ex vivo in whole blood compared to traditional techniques; continuous infusion resulted in progressively decreasing platelet sensitivity, rebound hyperaggregability, and increased serum thromboxane B_2. Cigarette smoking caused increased platelet aggregation and adherence to vascular endothelium.

Increased aggregation was seen in multi-organ failure, which traditional methods would not have detected. There appear to be multiple interactions between leucocytes and platelets.

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CHAPTER 1 - GENERAL INTRODUCTION

The aim of this thesis was to study platelets in their natural milieu, ie. whole blood, so that the influence of red cells and leucocytes on platelet function could be investigated in a variety of blood samples, in particular those from patients with a prothrombotic state. This term refers to a disorder or imbalance in haemostasis which predisposes to, or increases the chances of a thrombotic event. This situation could arise due to an abnormality of one of the control systems of haemostasis, or an abnormal concentration of one or more haemostatic components. The situation remains hypothetical since it is difficult to be certain whether any "prothrombotic" or hypercoagulable changes observed in blood are an in vitro artifact, the cause of thrombosis, or a consequence of the inappropriate activation of haemostasis. However, certain conditions in this rather broad and heterogeneous group are generally accepted as representing a prothrombotic state. In this thesis a number of these conditions will be investigated as well as the action of certain anti-platelet drugs. The pathophysiology of conditions associated with a prothrombotic state will be discussed in more detail later in Chapter 1, and results are presented in later chapters.

In order to study platelets in whole blood, it was first necessary to develop new techniques. Little is known about the interactions between platelets and the other formed elements of blood, either at the cellular level, or in terms of substances released or secreted. Most studies of platelets have previously used isolated or washed

preparations, thus eliminating any possible interactions between platelets and the other blood cells.

In order to understand the interactions and behaviour of platelets in whole blood, we must first review the current knowledge of platelet structure, biochemistry and function. In order to select and develop suitable methods, the available techniques for studying platelets will then be analysed.

1.1 PLATELET STRUCTURE, BIOCHEMISTRY AND FUNCTION

1.1.1 PLATELET MORPHOLOGY AND INTERNAL STRUCTURE

In their normal, non-activated state, platelets are small, discoid cells, devoid of a nucleus, that are derived from bone marrow megakaryocytes, and normally circulate in blood for 9-10 days at a count of $150\text{-}400*10^9/l$ (Burstein & Harker, 1983). They display a complex internal morphology (Nichols et al, 1981; White, 1983; White et al, 1981; Ulutin, 1976), with four areas or zones recognised on the basis of ultrastructural observations from scanning and transmission electron microscopy:- the peripheral zone, the sol-gel zone, the organelle zone; and the membrane systems (Figs. 1.1 - 1.4).

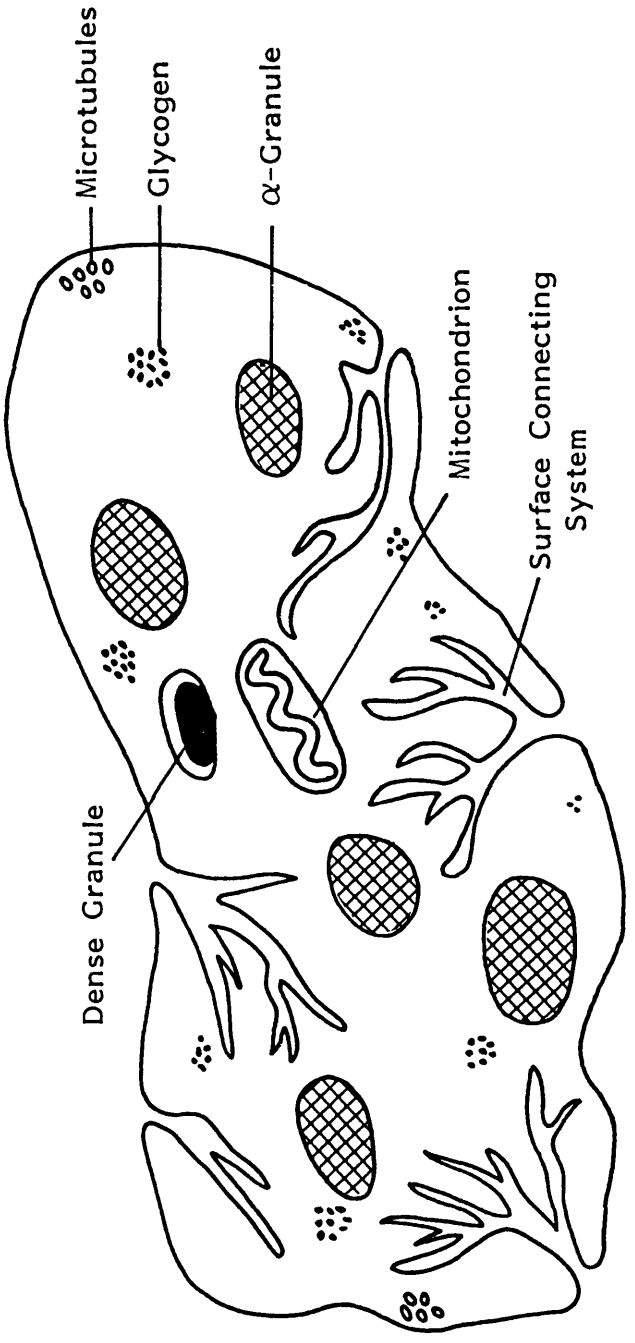

Fig. 1.1. - Diagram of a typical non-activated platelet (cross section).

The peripheral zone is composed of the limiting membrane and the submembrane filaments. The outer surface of the platelet is generally smooth with occasional indentations where openings of the surface connecting system (SCS) occur. The exterior coat or glycocalyx is thicker than on other cells and is rich in glycoproteins; it contains receptors for prostanoids, coagulation factors, immunoglobulins, complement and other proteins. The precise function of many of the membrane glycoproteins is unknown, but some act as receptors or binding sites for cytoadhesins and proteins involved in platelet cohesion or aggregation. They can be separated by SDS polyacrylamide gel electrophoresis (Ginsberg & Jaques, 1983), and have been classified (Table 1.1) according to the order of the bands on the gels (George et al, 1981). The functional importance of some of these glycoproteins is illustrated by the diseases which arise from inherited deficiencies. Glycoprotein (Gp) Ib is decreased in Bernard Soulier Syndrome, a haemorrhagic diathesis where there is a failure of platelet adhesion to subendothelial tissues, and abnormal platelet von Willebrand factor binding. GpIIb, GpIIIa, and to a lesser extent, GpV and GpIX, are decreased in thrombasthenia, a disease characterised by haemorrhage, and a failure of platelets to aggregate to collagen or ADP.

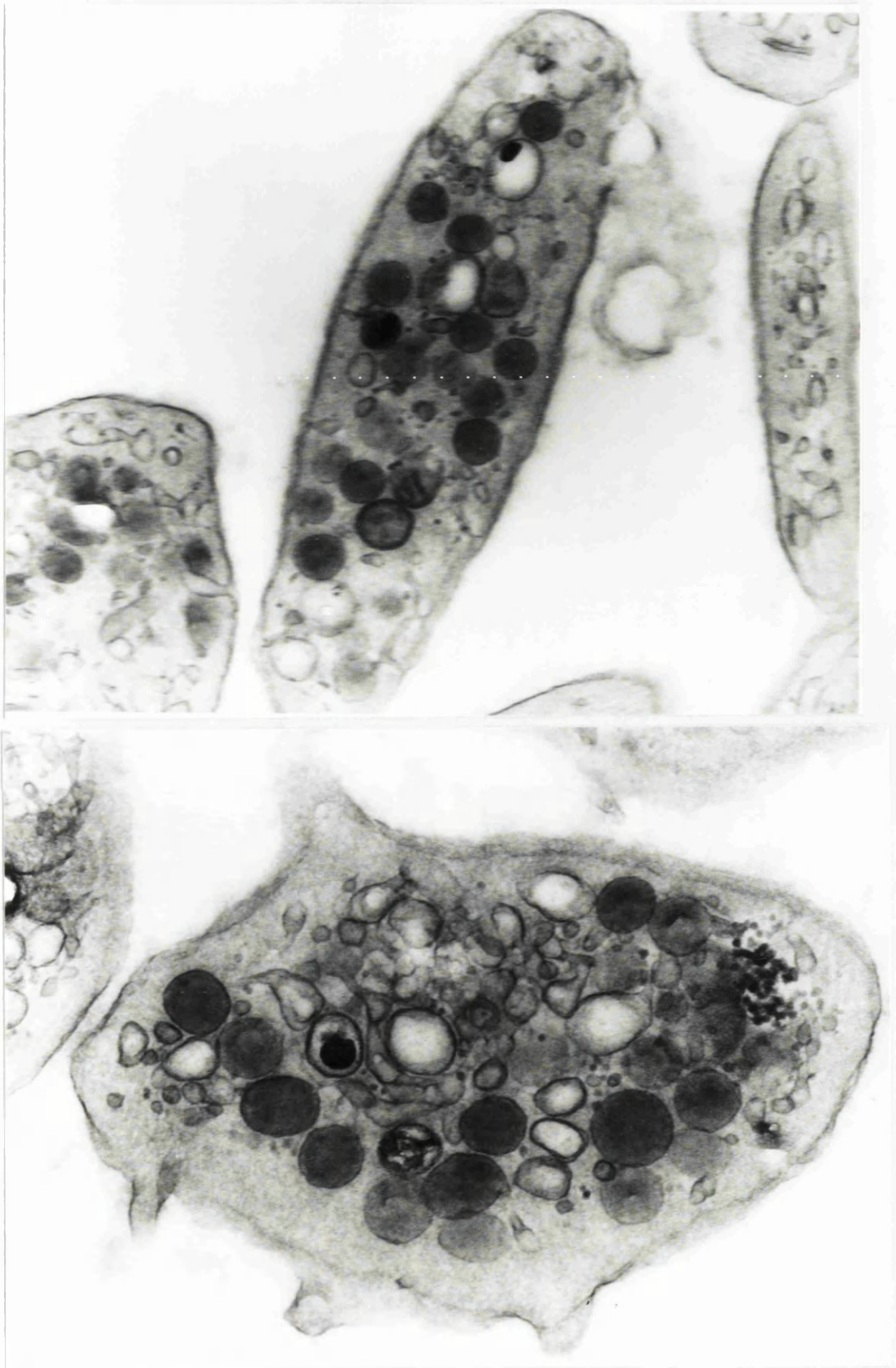

Fig. 1.2. Transmission electron micrograph of platelets: a) non-activated; b) after minimal activation, pseudopods starting to form. Scale bars represent 1um.

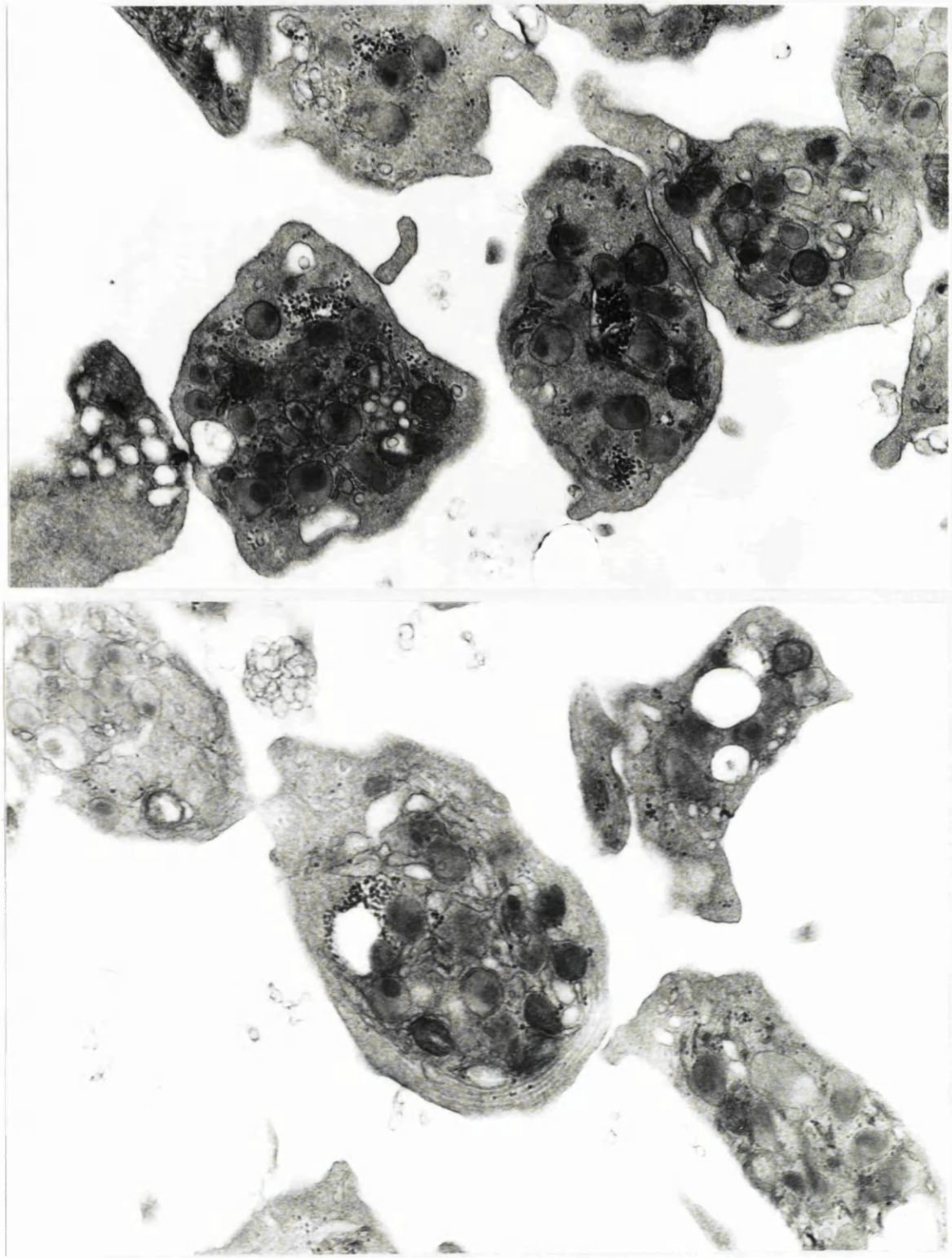

Fig. 1.3. Transmission electron micrograph of platelets: a) activated, pseudopods present and interplatelet contact points developing; b) activated, showing circumferantial bands of microtubules and granule centralization. Scale bars represent 1um.

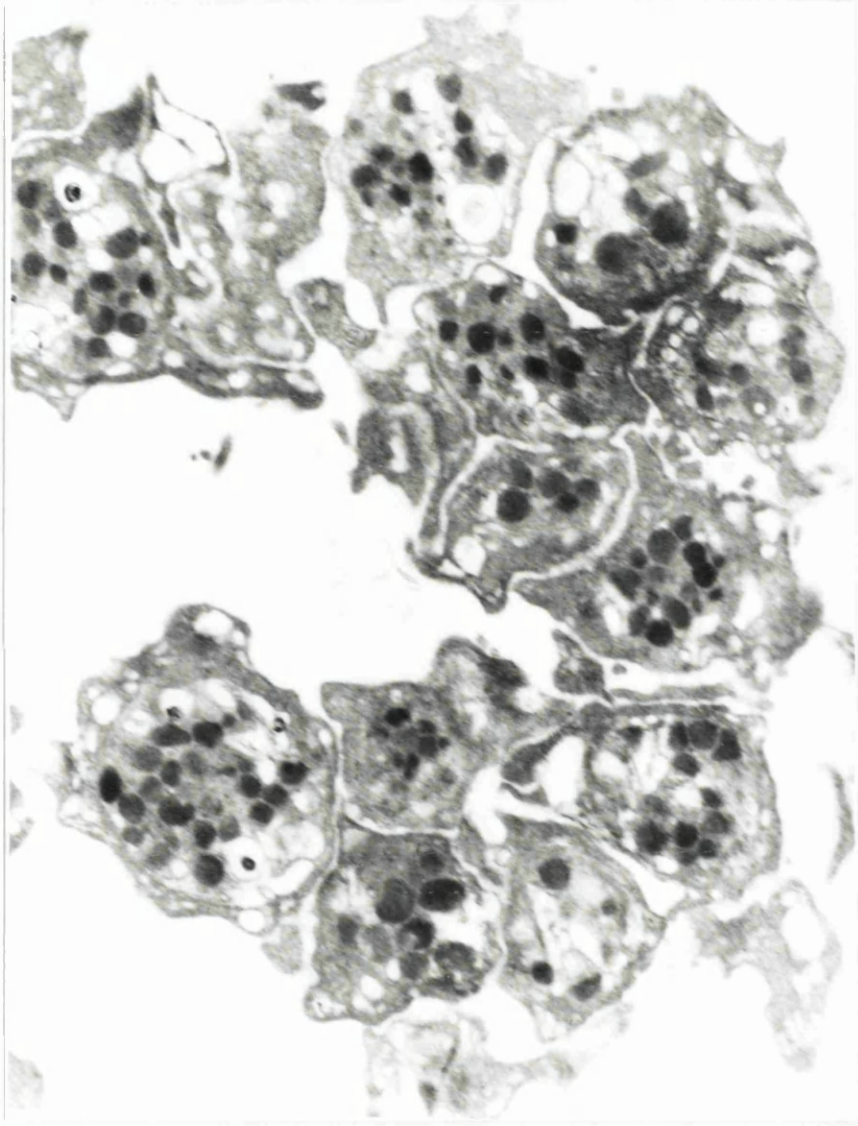

Fig. 1.4. Transmission electron micrograph of a platelet aggregate. Scale bars represent 1um.

The platelet limiting membrane is similar in structure to other cell membranes, containing a variety of anion and cation pumps eg. Na/K ATPase. The membrane contains a phospholipid bilayer with an asymmetrical distribution of phospholipid types. The acidic phospholipids: phosphatidyl serine, phosphatidyl inositol, and phosphatidyl ethanolamine, are preferentially concentrated in the inner leaflet of the bilayer. The functional significance of this will be discussed later.

GLYCOPROTEIN	**FUNCTION**
Ia	Collagen receptor
Ib	(with GpIX) receptor for vWF.
Ic	(with GpIIa) fibronectin receptor.
IIa	(with GpIc) fibronectin receptor.
IIb	(with Gp IIIa) receptor for fibrinogen, VWF, and fibronectin.
IIIa	(with GpIIb) receptor for fibrinogen, vWF, and fibronectin.
IV	Thrombospondin receptor.
V	Thrombin binding site.
IX	(with GpIb) vWF receptor.

Table 1.1 - Platelet Membrane Glycoproteins. The Roman numerals and subscript letters refer to the position of protein bands on polyacrylamide gel electrophoresis.

The sol-gel zone contains the matrix of the cytoplasm and is composed of 3 fibrous systems, microfilaments, microtubules, and the submembrane filaments. These fibrillar systems exist in various states of polymerisation and support the discoid shape as well as providing the contractile system. Below the platelet limiting membrane are the submembrane filaments which exist in close association with the cell membrane and the circumferential band of microtubules. In activated cells the microtubules contract into a tight ring around the centrally clumped organelles

(Fig. 1.3), however, the tubules themselves do not deliver the contractile force. They are polymers of the macromolecular subunit tubulin and their formation can occur very rapidly but is reversible.

Microfilaments are so dense that they can only be resolved in spread platelets or pseudopodia. They consist predominantly of filamentous actin (F-actin) and are interspersed with myosin filaments which represent 1% of platelet microfilaments. Alpha-actinin, a transmembrane protein anchors F-actin to the cytoplasmic side of the platelet membrane. The contractile protein exists in the resting platelet as a non polymerised form (G-actin) and polymerisation of this to F-actin is a pre requisite for contraction, which results in platelet shape change and centralisation of granules, with encircling by microtubules and a web of microfilaments.

Typical mitochondria, Golgi bodies, ribosomes and peroxisomes are usually found within platelets. In addition, three types of membrane enclosed granule have been described: lysosomes, dense bodies, and alpha granules. Platelet lysosomes are not dissimilar to those of other cells, and contain a variety of enzymes active at acid pH, eg. beta glucuronidase, beta galactosidase, acid phosphatase, cathepsins, and nucleases. These enzymes probably have a role in cell debris degradation and wound healing. Alpha granules contain many haemostatic proteins, some specific to platelets. Dense bodies are much smaller and less numerous than alpha granules, they contain adenine nucleotides, serotonin, calcium and pyrophosphate, which are

involved in platelet aggregation and vessel wall tone. Dense granules are named because of the electron dense nature of their contents which probably exist as a complex. Both dense bodies and alpha granules can fuse with the SCS and release their contents to the platelet exterior after contraction.

Two principle membrane systems can be distinguished in the platelet, the surface connecting system (SCS) and the dense tubular system (DTS). The SCS forms a network of canaliculi through the platelet that are in contact with the exterior via surface pores, and give the cell a sponge-like consistency. In this way the SCS greatly increases the surface area of the platelet exposed to plasma and provides both a communication route and an exit for substances liberated in the release reaction. Platelets sometimes behave phagocytically engulfing substances such as latex particles via the SCS. Channels of the DTS are found randomly throughout the cytoplasm and interdigitate with the SCS. They are much smaller in diameter and contain amorphous material similar in opacity to the surrounding cytoplasm. The latter finding may be due to the accumulation and storage of calcium since a Ca^{++}/Mg^{++} ATPase is localised in the channels of the DTS. This membrane system is also considered to be the site of synthesis of platelet prostanoids, since it is rich in the enzymes of both cyclo-oxygenase and lipoxygenase pathways. The DTS originates from the smooth endoplasmic reticulum of the parent megakaryocyte and may represent this in the platelet, whereas the SCS is derived from the megakaryocyte limiting membrane.

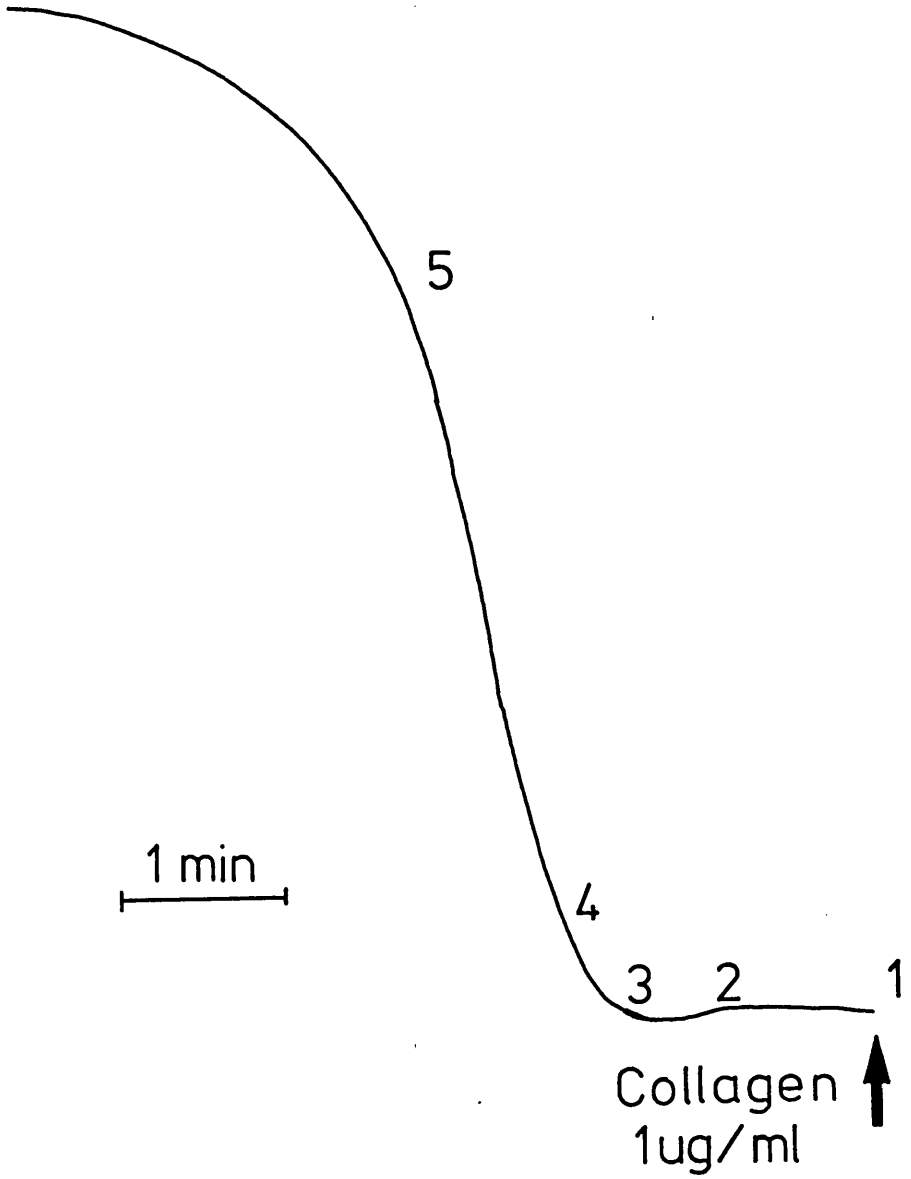

Fig. 1.5. In vitro platelet aggregation. Response to 1ug/ml collagen in an aggregometer. Platelets were removed at various time intervals for TEM. 1=Fig. 1.2a; 2= Fig. 1.2b; 3= Fig. 1.3a; 4= Fig. 1.3b; 5= Fig. 1.4.

1.1.2 PLATELET ACTIVATION

Platelets are very reactive cells, once activated they are able to adhere to subendothelial tissues at sites of damage, release their contents, and aggregate together forming a haemostatic plug. During these processes, platelets assist fibrin formation by providing a surface on which the reactions leading to thrombin generation can occur, releasing coagulation factors, and by protecting activated coagulation factors from their inhibitors. Many active substances are released, including: growth factors which influence smooth muscle cells in the vessel wall; serotonin, which affects vascular integrity; vasoactive materials which modulate local blood flow; and leucocyte chemoattractants.

Platelets may be activated by a variety of particulate and soluble substances (Table 1.2), some of which are physiological, while others occur in pathological states or are used as in vitro tests. After activation platelets display four basic phenomena:- adhesion, shape change, secretion, and aggregation (Figs 1.1-1.6). These may not necessarily occur in the same order, and whether all of them occur depends on the nature and strength of the trigger.

SOLUBLE ACTIVATORS	INSOLUBLE ACTIVATORS
ADP	Collagen
Adrenaline	Glass
Thrombin	Kaolin
Arachidonate	Celite
Thromboxane A_2	Latex Particles
PG Endoperoxides	Viruses
PAF-Acether	Bacteria
Serotonin	Vasopressin
Calcium Ionophores	Immune Complexes
Endotoxin	Proteolytic Enzymes

Table 1.2 - Naturally Occurring And Artificial Activators of Platelets.

When the vascular endothelial cell lining becomes disrupted, the subendothelial connective tissues are exposed and platelets rapidly adhere to them, spreading across the site of damage, and changing shape. They may then secrete their granular contents which modify vascular tone and recruit more platelets to the site, forming an aggregate of cells. Activated platelets also provide a procoagulant surface supporting the reactions leading to thrombin generation and ultimately producing fibrin which adds mechanical strength to the platelet plug.

1.1.3 PLATELET ADHESION

One of the earliest events following blood vascular damage is the adhesion of platelets to areas denuded of endothelial cells (Sixma, 1981). Platelet adhesion requires specific structural components of the subendothelium, plasma proteins, and receptors on the platelet membrane. A number of plasma proteins are candidates as mediators of platelet adhesion to the subendothelium, among these are von Willebrand Factor (VWF), Fibronectin, Fibrinogen, and Thrombospondin, the so called "adhesive proteins" (Packham

& Mustard, 1984).

The best studied of this group is VWF, since deficiency results in one of the most frequently encountered congenital bleeding disorders, von Willebrand's Disease. Patients with typical von Willebrand's Disease have a mild to severe bleeding diathesis characterised by a prolonged bleeding time, decreased platelet adhesion, abnormal ristocetin induced platelet agglutination, and decreased amounts or abnormal molecular forms of VWF (Zimmerman & Ruggeri, 1983).

VWF is a glycoprotein synthesised by endothelial cells and secreted in a controlled fashion. The vascular endothelium secretes VWF directly into the subendothelial matrix as well as into plasma (Sussman & Rand, 1982). Synthesis also occurs in the megakaryocyte with storage in platelet alpha granules, and release on degranulation (Bloom, 1979). It is macromolecular, existing in plasma as a series of multimers (1500-15000 kd), each of which is a polymer composed of protomers (500 kd). The protomers contain 2 identical subunits (250 kd) held together by disulphide bonds (Zimmerman & Ruggeri, 1983). Using monoclonal antibodies and tryptic fragments of VWF, Sixma and coworkers (1984a) have shown that there are discrete functional domains on the molecule; the site responsible for ristocetin induced binding to platelets differing from that involved in the interaction with collagen types I and III. It is not clear whether platelet adhesive activity varies according to multimer size, or if individual protomers are active (Sixma et al, 1984b).

VWF does not interact with unstimulated circulating

platelets, unless ristocetin or desialylated FVIII-VWF are present (De Marco & Shapiro, 1981; Zucker et al, 1977; Schneider-Trip et al, 1979). After secretion by endothelial cells, vWF may bind immediately to the underlying connective tissue matrix, providing an active surface for platelet attachment should the vessel wall be damaged (Rand et al, 1980). Such interactions with subendothelial components may cause Ca^{++} dependent conformational changes in VWF allowing platelet binding, since free ionised calcium mediates platelet adherence in this situation (Sakariassen et al, 1984). Alternatively, changes in the environment of the platelet membrane receptor may occur, perhaps involving activation by proteases, and the redistribution of surface charge so that VWF is no longer electrostatically repelled.

The platelet receptor responsible for VWF binding in the presence of ristocetin is GpIb (Jenkins et al, 1976; Nurden & Caen, 1975), probably in association with GpIX. Owing to its large multimeric structure, VWF binding to GpIb may play a role in platelet aggregation as well as adhesion, by linking adjacent platelets.

Under suitable conditions, VWF also binds to the GpIIb/IIIa complex on the platelet membrane (Fig. 1.6) in response to thrombin or ADP (Ruggeri et al, 1983; Gralnick & Coller, 1983). However in normal plasma, thrombin stimulated platelets do not bind VWF when the fibrinogen level is greater than or equal to 1mg/ml, and VWF binding to washed platelets can be inhibited by purified fibrinogen (Schullek et al, 1984; Pietu et al, 1984). A specific structural domain of VWF protein distinct from the collagen and GpIb

binding sequences has been demonstrated for GpIIb/IIIa binding (Meyer, 1985).

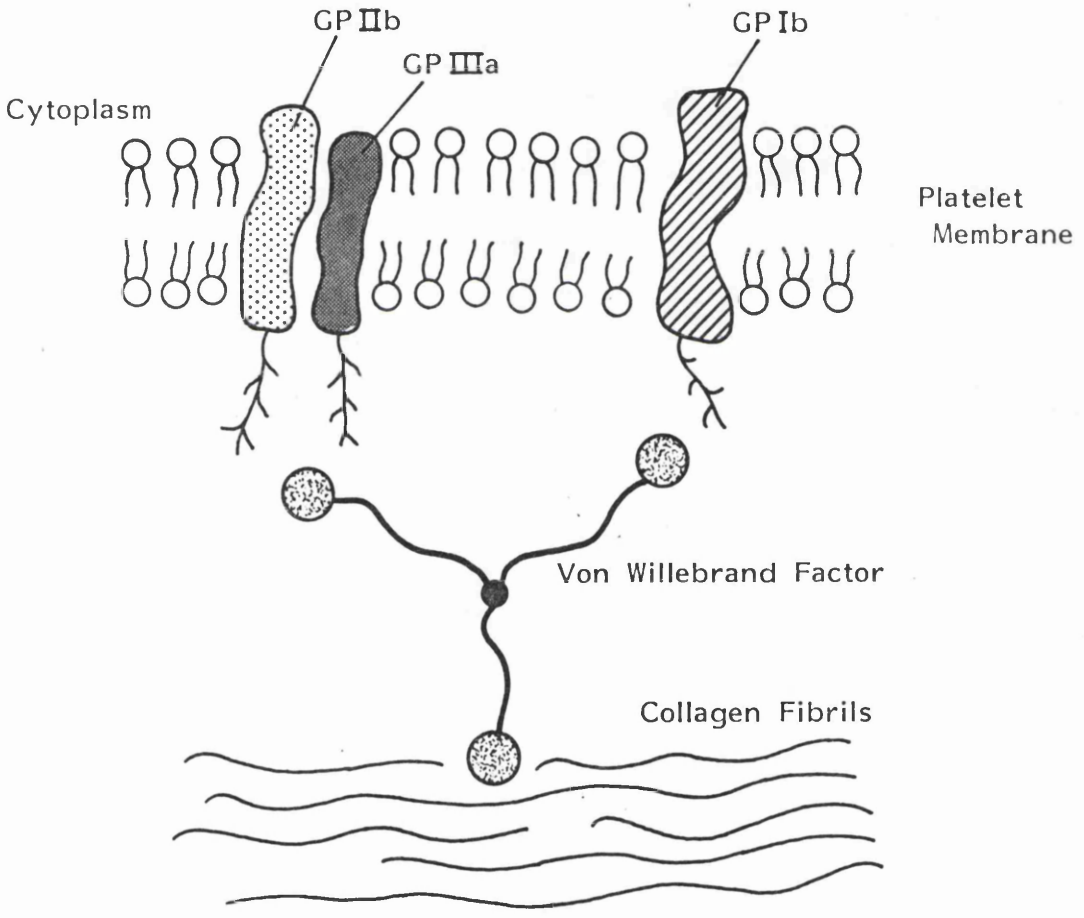

Fig. 1.6. The interactions of von Willebrand factor with platelets and the sub-endothelium. GP = Glycoprotein.

The physiological relevance of this secondary platelet binding site is unknown, since fibrinogen normally exists in molar excess over VWF in plasma. However, at the damaged vessel wall, the presence of large amounts of VWF bound to the subendothelium may cause local concentrations of VWF to exceed fibrinogen, so that VWF can bind to GpIIb/IIIa.

The receptor site for VWF binding to the subendothelium probably involves collagen (Legrand et al, 1978; Santoro, 1978); other vessel wall components, eg. microfibrillar structures, may support adhesion, but are difficult to study in vitro because glycosaminoglycans are present and carry a high electrostatic charge. Collagen Types I, II, and III have been shown to bind VWF (Santoro, 1981).

The contribution of VWF to platelet adhesion appears to be highly dependent on wall shear rate (Turitto & Baumgartner, 1983). At low shear rates comparable to those found in large veins (200^{s-1}) and at arterial wall shear rates ($500\text{-}1000^{s-1}$), adhesion occurs independently of VWF. At high wall shear rates similar to those in small vessels ($>1300^{s-1}$), the residence time of platelets at the subendothelium is short and VWF is essential for platelet adhesion. VWF binding to GpIIb/IIIa appears to be less important than the interaction with GPIb since there is no adhesion defect in Glanzmann's thrombasthenia (an inherited abnormality of GpIIb and GpIIIa) (Tschopp et al, 1975). Platelet number, viscosity and red cell count have a linear relationship to adherence, which reflects the rheology of high shear vessels where red cells occupy a central core

position forcing the platelets to marginate to the periphery of the blood vessel, thus increasing the platelet/vessel wall contact.

Under suitable conditions certain other adhesive proteins can bind to the GpIIb/IIIa complex and to other sites on the platelet membrane, e.g. fibrinogen, fibronectin, vitronectin, thrombospondin. The binding of fibronectin and thrombospondin to GpIIb/IIIa occurs via a tetrapeptide integrin recognition sequence (Arg-Gly-Asp-Ser) (Haverstick et al, 1985). This tetrapeptide is also present in the alpha chain of fibrinogen, but it may not mediate GpIIb/IIIa fibrinogen binding (Kloszewiak et al, 1984), since fibrinogen also has a gamma chain dodecapeptide sequence which can facilitate binding (Plow et al, 1984). Recent evidence suggests that platelet adhesion to collagen types I and III in flowing blood is dependent on both VWF and fibronectin (Houdijk et al, 1985). Alternative mechanisms of adhesion may occur at low wall shear rates and some evidence suggests that fibrinogen may be involved.

1.1.4 PLATELET SHAPE CHANGE

Following adhesion to the subendothelium, platelets spread, and cover the exposed connective tissue matrix. In doing so they change from the circulating discoid form to an irregular shaped, elongated cell with cytoplasmic projections. Platelet pseudopod formation appears to result from contractile activity, which is analogous to that seen in muscle cells and requires energy. Both microfilaments and microtubules are found in pseudopods and it is thought that

the latter control recruitment and dissolution of microfilaments. In the early stages of platelet activation, shape change is reversible, but strong stimuli cause the centralization of organelles, degranulation and release accompanied by irreversible shape change and aggregation (Fig. 1.3-1.5). The microtubules form a dense ring around the organelles which liberate there contents into the channels of the SCS.

1.1.5 PLATELET RELEASE REACTION

Platelet alpha granules contain both platelet specific proteins such as beta thromboglobulin and platelet factor 4 as well as some which are normally present in plasma, e.g. fibrinogen (Table 1.3). Dense core granules sequester a pool of nucleotides which are not interchangeable with those utilised in the general metabolism of the cell. The importance of these two types of granule is illustrated by inherited abnormalities associated with their deficiency or dysfunction (Storage Pool Defect, Grey Platelet Syndrome). Lysosomes contain a variety of acid hydrolases such as lysozyme, acid phosphatase and elastase.

beta Thromboglobulin	Fibrinogen
Platelet Factor 4	Fibronectin
PDGF	Thrombospondin
Antiplasmin	von Willebrand Factor
Alpha-2 Macroglobulin	Factor V
Alpha-1 Antitrypsin	Albumin

Table 1.3 - Platelet Alpha Granule Proteins.

Secretion of the platelet granules is thought to occur by fusion of the granule and SCS membranes, in a process requiring membrane labilization, calcium ions, and probably a calcium-dependent phospholipase. Secretion can be induced by calcium ionophores and it has been suggested that TXA_2 acts in this fashion, however TXA_2 is not essential for secretion. Weak aggregating reagents such as ADP and adrenaline require the synergistic action of TXA_2 to raise the cytoplasmic calcium concentration sufficiently for release. Strong activators such as collagen and thrombin are potent calcium mobilisers and induce secretion independently of TXA_2. Alpha granule contents are released at lower concentrations of agonists than dense bodies, whilst lysosomes only release at high concentrations of thrombin and collagen.

1.1.6 Alpha granule proteins

The most extensively studied alpha granule proteins are platelet Factor 4 (PF4) and beta thromboglobulin (BTG) (reviewed by Kaplan, 1981; Kaplan and Owen, 1983; Walz and Hung, 1985). These have been proposed as indicators of prothrombotic states. However the assays require careful control of pre-analytical variables, and the levels are influenced by renal impairment and heparin therapy as well as disordered platelet function. Both proteins are tetramers of basic polypeptide subunits with extensive sequence homology, complexed to a proteoglycan carrier. PF4 interacts with glycosaminoglycans (GAGS) which are a major component of the endothelial cell surface; the order of binding

potency (highest first) to a variety of GAGS is :- heparin, heparan sulphate, dermatan sulphate, chondroitin 6-sulphate (equal with) chondroitin 4-sulphate. It is not surprising therefore, that PF4 binds to the endothelium, and this occurs in a time dependent and saturable fashion. The short plasma half life (<3 minutes) is likely to be due to vessel wall uptake by GAGS. PF4 has a strong heparin neutralising activity, but when bound to the endothelial cell, it may be released by heparin. PF4 inhibits the activity of skin and leucocyte collagenases, and the binding of low density lipoprotein to its cell surface receptor on fibroblasts, and is chemotactic for monocytes and neutrophils. It may also inactivate the intrinsic system of coagulation, probably by the interaction of a positively charged portion of the molecule with negatively charged polysaccharide sulphates. The binding of PF4 to platelet membrane receptors enhances aggregation and secretion (Capitanio et al, 1983).

The physiological relevance of these properties has yet to be ascertained, but sufficiently high levels of PF4 may be obtained in vitro to achieve these effects when platelets adhere to the vessel wall or form thrombi. PF4 may be involved in controlling the level of natural anticoagulant activity, since it can compete with antithrombin-III, heparin cofactor II, and protein C inhibitor (PAI-3) for heparinoids. Thrombocytopaenic serum contains a factor that causes PF4 synthesis in megakaryocytes, so that platelets containing excessive amounts of PF4 are produced. This PF4 may be liberated on the slightest provocation, and thus provide a further control mechanism, and reset the

haemostatic balance in haemorrhagic diatheses.

BTG and PF4 exist in similar quantities in alpha granules, but their plasma concentrations and half-lives differ. BTG has a longer half life and is metabolised by the kidney; it may therefore show false elevations in plasma from patients with renal failure. BTG has been reported to cause inhibition of PGI_2 production by cultured endothelial cells, but this property is disputed. It also appears to be potent at stimulating chemotaxis of fibroblasts (Senior et al, 1983), which may be important in wound healing.

Plasma levels of these two proteins (approximately 1 - 20ng/ml for PF4, and 6 - 50ng/ml for BTG) have been used as an index of in vivo platelet release. However, their levels are very sensitive to poor sample collection, and BTG may be elevated due to failure of renal catabolism, whilst PF4 may be increased during heparin therapy, due to its displacement from binding sites.

Platelet derived growth factor (PDGF) is mitogenic for smooth muscle cells and when released from platelets at a site of damaged vessel wall, it stimulates smooth muscle migration and proliferation in the intima contributing to the atherosclerotic process (Ross, 1981). PDGF may also influence the proliferation of other cell types, and similar substances have been implicated in tumour cell growth and multiplication.

Thrombospondin (Tsp) is the major alpha granule glycoprotein, but is also secreted by fibroblasts, endothelial and smooth muscle cells (Mosher, 1982; Jaffe, 1983; Raugi et al, 1982) (the latter may be induced by

PDGF). Tsp has a molecular weight of 450kd and is composed of 3 identical disulphide linked polypeptide chains of 160kd (Lawler et al, 1978; Phillips & Agin, 1977; Lawler et al, 1982a). It is a multifunctional protein, and binds heparin (Lawler & Slayter, 1981; Gogstad et al, 1983; Dixit et al, 1984), fibronectin (Lahav et al, 1982; Lahav et al, 1983), fibrinogen (Leung & Nachman, 1982), plasminogen (Silverstein et al, 1984), histidine-rich glycoprotein (HRGP) (Leung et al, 1984), type V collagen (Mumby et al, 1984), and calcium ions (Lawler & Simons, 1983). Tsp associates with cell surfaces and extracellular matrices and facilitates cell-cell and cell-matrix interactions (Phillips et al, 1980; Lawler et al, 1982b; Raugi et al, 1982; Jaffe et al, 1983; Mckeown-Longo et al, 1984). Following platelet activation and release, thrombospondin binds to GpIV on the platelet membrane and behaves as a lectin (Fig. 1.7). It also binds to fibrinogen and promotes or stabilises platelet-platelet interactions (Gartner et al, 1981; Jaffe et al, 1982). Thrombin cleavage liberates a heparin binding domain (Lawler et al, 1985) and increases the affinity for plasminogen and fibrinogen.

Fibronectin (FN) is secreted from the alpha granule (where it is present at 2-4 ug/10^9 platelets) in response to a variety of platelet activators (Packham & Mustard, 1984; Mosher, 1980). It is also synthesised by endothelial cells, occurring in large amounts in the basement membrane; and is present in normal plasma at approximately 300 ug/ml. FN is an adhesive protein which binds to many substances and facilitates cell adhesion and spreading. It has been

implicated as the platelet collagen receptor (Bensusan et al, 1978) since it binds to both collagen fibrils and platelet membranes; however, there is evidence against this receptor theory, (Sochynsky et al, 1980), and antibodies against FN do not inhibit platelet collagen binding (Santoro & Cunningham, 1979). It is most likely that FN is involved in platelet spreading on collagen (Hoffman & Hynes, 1979). Released FN binds immediately to the surface of normal platelets by fibrin-dependent and independent mechanisms (Plow et al, 1985). Activated thrombosthenic platelets bind reduced amounts of FN (Ginsberg et al, 1983), and this suggests that the GpIIb/IIIa complex may form part of the FN binding site. During clot formation, FN is covalently cross linked to fibrin by factor XIIIa, and may have a role in platelet adherence to polymerising fibrin (Plow, 1981).

Fibrinogen plays a fundamental role in platelet aggregation following induction by most agonists. ADP induced aggregation is dependent on the presence of extracellular fibrinogen, which binds to specific membrane receptors on adjacent platelets, thus bringing them into close proximity, causing further platelet activation and aggregate formation. Platelet and plasma fibrinogen appear to be identical; the alpha granule source may provide high local concentrations, or present fibrinogen in a more favourable way for aggregation to occur. Some reports suggest that fibrinogen is the receptor for Tsp on platelet membranes and that Tsp is the natural platelet lectin, causing agglutination after prior activation of platelets

(Gartner et al, 1981; Jaffe et al, 1982).

1.1.7 Dense Body Components

Dense body ADP and ATP comprise approximately two thirds of the total platelet adenine nucleotide content. The pool contains mainly ADP and a smaller concentration of ATP, and is not interchangeable with the cytoplasmic metabolic pool. Stored nucleotides are liberated during the release reaction and play an important role in aggregation responses; inherited abnormalities of dense bodies such as 'Storage Pool Disease', are frequently associated with a haemorrhagic diathesis (Holmsen & Weiss, 1970). A large amount of serotonin is also stored, and can be scavenged from plasma; it is thought to have a role in maintenance of vascular endothelium integrity at peripheral sites (Sweetman et al, 1981). Serotonin acts synergistically with other platelet agonists, and causes vaso- constriction which may limit blood loss. The intra-granular concentration of these substances is so high that they cannot remain in solution, and form high molecular weight aggregates with calcium ions (Da Prada et al, 1981) and pyrophosphate; causing the electron dense nature. Platelet release could also provide high local concentrations of calcium ions, thus potentiating the various reactions of the coagulation system.

1.1.8 PLATELET AGGREGATION

The process of platelet aggregation describes the property of platelets to cohere with one another in a specific process requiring energy, intracellular processes and initiators. A large number of agents (Table 1.2) cause

the aggregation of stirred platelets at 37°C. Aggregation is thought to occur by at least 3 different pathways (Kinlough-Rathbone et al, 1977a). The first is mediated by ADP released from the dense granules, the second requires the generation of PG endoperoxides and TXA_2. The third pathway is suggested by the presence of platelet aggregation to calcium ionophore (A23187) or high levels of thrombin even when the arachidonate and ADP pathways have been blocked. One candidate for the third pathway is platelet activating Factor (PAF-Acether) (Chignard et al, 1979). In vivo, perturbed red cells would probably be the main source of ADP and several other platelet activators would be present, and exert synergistic effects.

ADP plays an important role in secondary aggregation following activation by adrenaline, thrombin, prostaglandin endoperoxides, PAF-acether, calcium ionophore A23187, and high doses of collagen. There is a specific ADP receptor on the platelet surface, through which ADP down regulates adenylate cyclase (Haslam et al, 1978) which thus reduces cAMP levels, allowing a generalised activation of cytoplasmic enzymes favouring aggregation and secretion. ADP binding also stimulates fibrinogen binding (Mustard et al, 1978; Bennett & Vilaire, 1979) by inducing a conformational change in the GpIIb/IIIa receptors on the platelet membrane so that fibrinogen can bind. The latter, along with release of Ca^{++} into the cytoplasm is essential for aggregation. Patients with congenital afibrinogenaemia have absent ADP aggregation which is corrected by the addition of normal fibrinogen (Inceman et al, 1966); and washed platelets

resuspended in buffer without fibrinogen do not aggregate to ADP (Kinlough-Rathbone et al, 1977c). In addition, platelets from patients with Glanzman's thrombasthenia, a disease associated with abnormalities of GpIIb/IIIa (Kunicki et al, 1981a), neither bind fibrinogen nor aggregate in response to ADP (Caen et al, 1966; Mustard et al, 1979). The platelet fibrinogen receptor is a complex of GpIIb and GpIIIa stabilised by calcium ions (Kunicki et al, 1981b; Nachman & Leung, 1982); the major GpIIb/IIIa attachment site on the fibrinogen molecule is on the carboxy terminal dodecapeptide of the gamma chain (Hawiger et al, 1982, 1983; Niewiarowski et al, 1983), although a second, weaker site is located on the alpha chain of fibrinogen. Fibrinogen is the most likely candidate for the formation of interplatelet bridges during aggregation (Fig. 1.7). This interaction may well be stabilised by thrombospondin.

Thromboxane A_2 and the cyclic endoperoxides, PGG_2 and PGH_2 may act on a specific receptor to exert their effects, but this remains unclear, owing to the instability of these compounds. They are thought to act as calcium ionophores, and liberate Ca^{++} from the DTS (Gerrard et al, 1981a). They also inhibit PGE_1 and PGI_2 stimulation of cyclic AMP production (Miller et al, 1977; Gorman et al, 1978), although they have no effect on basal cAMP levels.

Collagen exists in the vascular subendothelium as four different types with varying subunit composition and properties. These forms are designated Types I, III, IV, and V (Packham & Mustard, 1984). Types I and III have been shown to cause platelet adhesion and aggregation in a dose

dependent manner, types IV and V do not promote aggregation as readily. A specific nonapeptide sequence found in collagen has been shown to block collagen binding to platelets (Legrand et al, 1980). Nieuwenhuis and coworkers (1985), have reported a patient with a haemorrhagic diathesis whose platelets lack membrane GpIa, and are completely unresponsive to collagen. This suggests that GpIa may have a receptor or transmitter function for collagen.

In vitro, low doses of collagen cause platelet adhesion, phospholipase activation and liberation of arachidonate, which is converted to TXA_2; as well as granular nucleotide release. TXA_2 and ADP then cause aggregation and the release reaction in non adherent platelets (Kinlough-Rathbone et al, 1979; Kinlough-Rathbone et al, 1977c). Acetyl salicylic acid (ASA) inhibits aggregation and in Storage Pool Disease (an inherited defect characterised by abnormal granular storage of nucleotides), aggregation is also decreased. Higher doses of collagen can overcome ASA inhibition and cause aggregation by TXA_2 independent mechanisms.

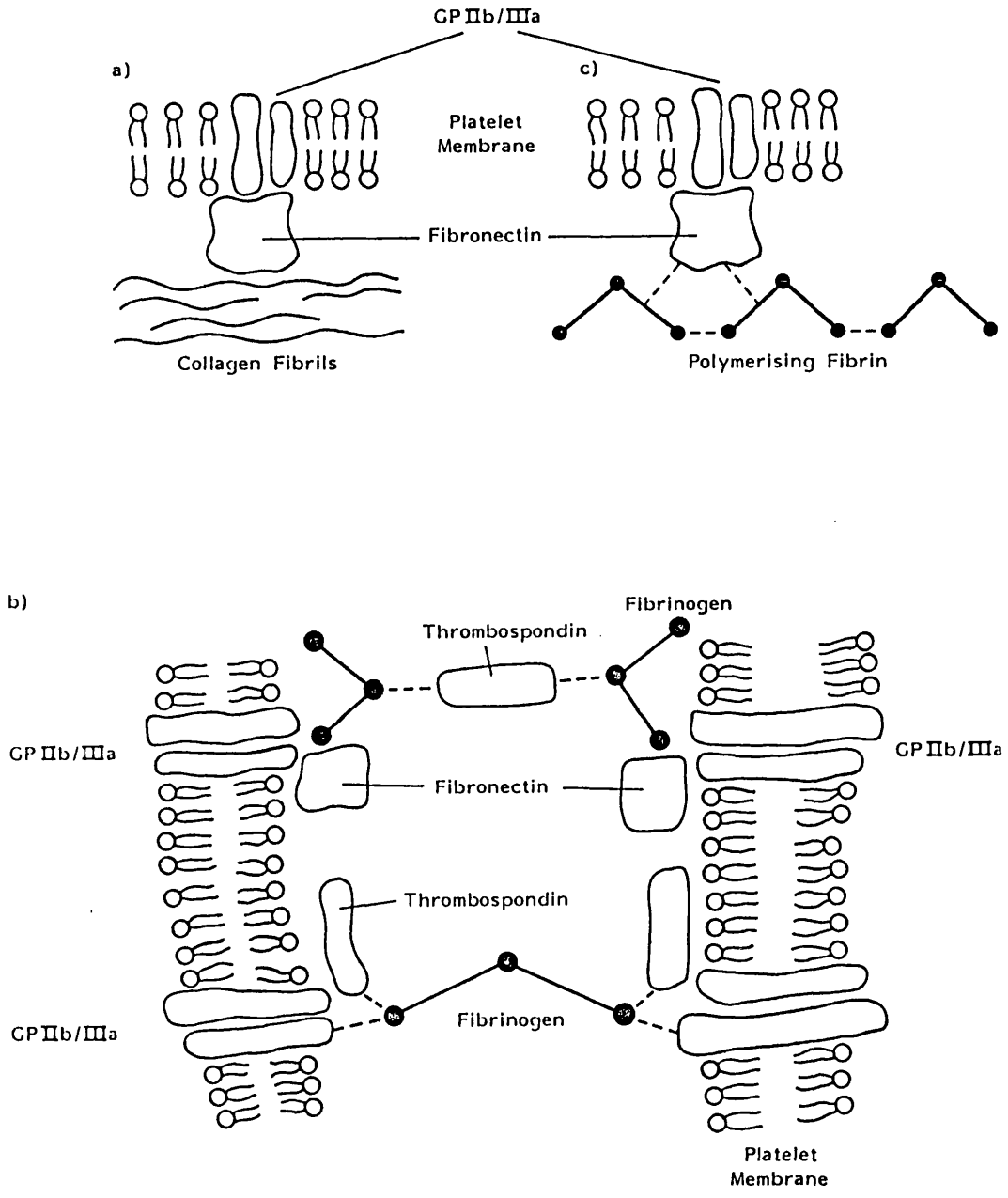

Fig. 1.7. The Role of Fibrinogen, Fibronectin and Thrombospondin in Platelet Aggregate Formation.

Thrombin is the strongest of the physiological activators and causes shape change, stimulation of the PI-cycle, TXA_2 generation, ADP release and ultimately aggregation. Thrombin has at least two receptors on the platelet surface (GpI and GpV are involved), and binding stimulates either phospholipase A_2, or phospholipase C activity (Harmon & Jamieson, 1985).

Adrenaline causes two waves of aggregation in human citrated platelet rich plasma (PRP), the first being reversible aggregation, while the second is associated with granule release and thromboxane generation. PRP prepared from blood anticoagulated with the thrombin inhibitor hirudin, usually shows no response to adrenaline (Lages & Weiss, 1981). Adrenaline as well as serotonin act synergistically with other aggregating reagents (Ardlie et al, 1966; Baumgartner & Born, 1968), but alone are probably not of physiological importance for aggregation.

PAF-acether (1-O alkyl 2-acetyl sn-glyceryl 3-phosphoryl choline) is a substance released from sensitised basophils, which is an extremely potent inducer of platelet aggregation and an important mediator of inflammatory and allergic reactions (Benveniste et al, 1979; Cusack, 1980). A physiological role for PAF-acether remains controversial, Marcus et al, 1981 suggested that it is a strong agonist for aggregation, but a weak inducer of thromboxane, whereas Chesney and colleagues (1982) observed inhibition of aggregation and secretion by indomethacin or ASA, even at high PAF-acether concentrations.

Calcium ionophore A23187 carries divalent cations

across membranes and causes aggregation by directly increasing the cytoplasmic calcium ion concentration. This does not require a membrane receptor mechanism, and leads to thromboxane generation as well as ADP release, which potentiate the direct aggregating effect of the ionophore (Kinlough-Rathbone et al, 1977a).

1.1.9 THE PLATELET CYTOSKELETON

Cytoskeletal and other proteins are responsible for platelet shape change and release reactions. These are mediated by a number of interacting contractile proteins and related enzymes (Fig. 1.8) such as actin (which constitutes 10-15% of total platelet protein) and myosin, as well as complexes of G-actin (profilin), crosslinkers of actin (actin binding protein, alpha-actinin), filament stabilisers (tropomyosin), filament cappers (90Kd protein, and possibly gelsolin) and restrictors of actin length (235Kd protein).

Two cytoskeletal assemblies can be identified, one important for pseudopod extension, the other for the central contractile process which constricts microtubules and squeezes the granules towards the centre of the platelet (Carroll et al, 1982). Actin, actin binding protein and alpha actinin are the principle proteins involved in pseudopod extension (Schollmeyer et al, 1978; Carroll et al, 1982), and the microtubules in the centre of the pseudopod probably add rigidity (Cohen et al, 1982); the mechanism is not fully understood.

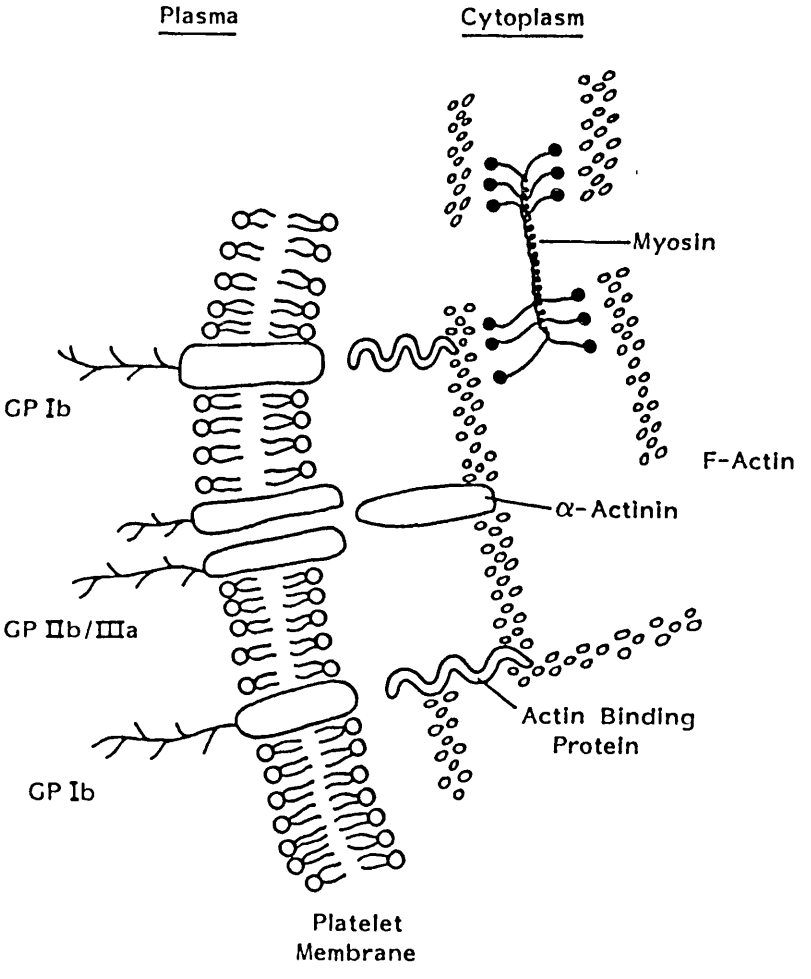

Fig. 1.8. The Platelet Cytoskeleton.
GPIb = Glycoprotein Ib; GPIIb/IIIa = Glycoprotein IIb/IIIa complex.

Actin-myosin contraction is the basis of the process of centralisation (Gerrard & Friesen, 1985; Nichols et al, 1981), but alpha actinin is involved (Carroll & Cox, 1983). Calmodulin may regulate tubulin assembly and coordinate actomyosin and microtubule systems.

In the resting platelet only 25-50% of actin is in the filamentous or F-actin form, the rest is globular or G-actin, probably complexed with profilin (profilactin), and very little is assembled as a contractile skeleton (Markey et al, 1981; Fox & Phillips, 1983; Carroll et al, 1982). On platelet activation the influence of profilin is removed and actin polymerisation occurs forming thin filaments in a process facilitated by actin binding protein and alpha actinin (Scollmeyer et al, 1978; Phillips et al, 1979). Bipolar filaments of myosin form so that globular myosin heads occur at the ends of filaments and can bind to actin filaments. These myosin heads are responsible for myosin ATPase activity which generates contractile force.

Regulation of the actin-myosin contraction in platelets results from changes in the phosphorylation of the light chain of myosin (Scholey et al, 1980; Adelstein & Conti, 1975). Light chain phosphorylation is caused by myosin light chain kinase, a calcium/calmodulin dependent enzyme. Activation of the latter is triggered by a rise in cytosolic calcium ion concentration, this leads to myosin filament formation and its interaction with actin and ATP producing contractile force (Adelstein & Pollard, 1978; Dabrowska & Hartshorne, 1978; Hathaway & Adelstein, 1979). The energy is provided by a magnesium ion dependent ATPase present in

myosin and stimulated by actin. Filaments of the latter are attached to the platelet membrane by alpha-actinin which binds to the GpIIb/IIIa complex, and actin binding protein which when phosphorylated, cross-links actin filaments (Zhuang et al, 1984) and appears to bind to GPIb (Solum & Olsen, 1984). Contraction occurs by actin filaments and myosin rods sliding over one another. Myosin light chain phosphatase may switch off myosin (Barylko et al, 1977). In the absence of calcium ions, tropomyosin inhibits the interaction of myosin with actin, and this may be an additional regulatory role of calcium in platelets.

1.1.10 BIOCHEMICAL MESSENGERS IN PLATELET ACTIVATION

Two important messengers are involved in the stimulus response coupling following agonist binding at the platelet membrane, calcium ions and diglyceride. An increase in cytoplasmic free calcium ion concentration leads to the phosphorylation of myosin light chain with subsequent contraction and granule centralisation as well as several other calcium mediated events associated with platelet activation (Gerrard et al, 1981). Diglyceride is able to activate protein kinase C (even at baseline cytosolic calcium ion concentrations) (Kaibuchi et al, 1983; Rink et al, 1983), and this enzyme phosphorylates a 40-47 Kd protein which appears to be associated with granule labilisation and calcium ion secretion. Calcium flux and diglyceride production result from the breakdown of phospholipids of the phosphoinositide class (Fig. 1.9), themselves produced when thrombin and other stimulants activate platelets.

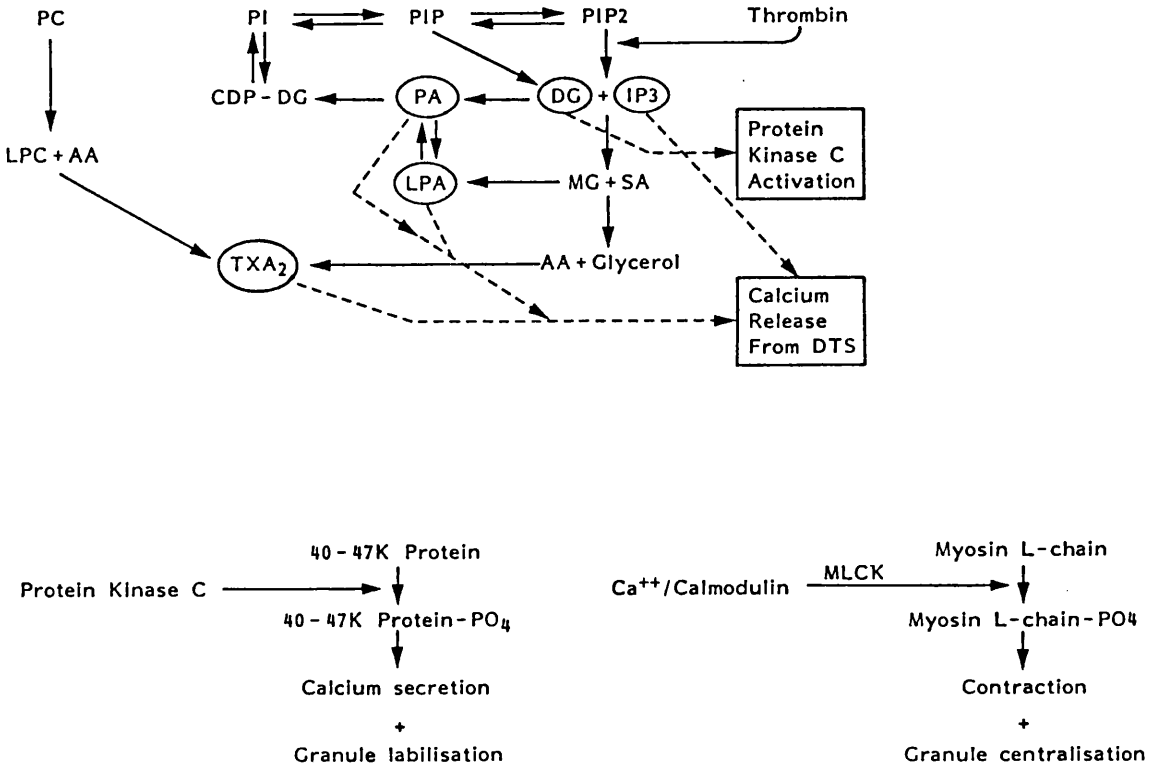

Fig. 1.9. The PI cycle.
Upper panel - production of active messengers; lower panel - end effects. key:- 1=phospholipase C; 2=phospholipase A_2; 3=diglyceride lipase; PC=phosphatidyl choline; LPC=lysophosphatidyl choline; MLCK=myosin light chain kinase; AA=arachidonic acid; TXA_2=thromboxane A_2; PI=phosphatidyl inositol; PIP=phosphatidyl inositol-4'-phosphate; DG=dyglyceride; PIP_2=phosphatidyl inositol-4',5'-bisphosphate; PA=phosphatidic acid; CDP-DG=cytidine diphosphate diglyceride; IP_3=inositol triphosphate; LPA=lysophosphatidic acid; MG=monoglyceride; SA=stearic acid.

Low concentrations of thrombin capable of inducing platelet shape change activate phospholipase C, which initially cleaves phosphatidylinositol 4',5'-bisphosphate (PIP2) and later phosphatidylinositol (PI), as well as phosphatidylinositol 4'-phosphate (PIP) (Agranoff et al, 1983; Rittenhouse, 1983), to yield diglyceride and inositol trisphosphate (IP3). The latter has been implicated as releasing calcium from intracellular storage sites in other cell types and could function similarly in relation to the platelet DTS. The cleavage of PIP2 itself may release calcium (Vickers et al, 1982) and increase platelet activation by other mechanisms.

There are PG endoperoxide/TXA_2 dependent and independent pathways of Ca^{++} flux (Gerrard et al, 1981a; Hallam et al, 1983), but the mechanisms are unclear. PIP2 hydrolysis may be involved in calcium mobilization by TXA_2, while IP3 or lysophosphatidic acid (LPA) could be responsible for calcium flux through the other pathway. LPA also promotes phosphorylation of both 40-47Kd protein and myosin light chain.

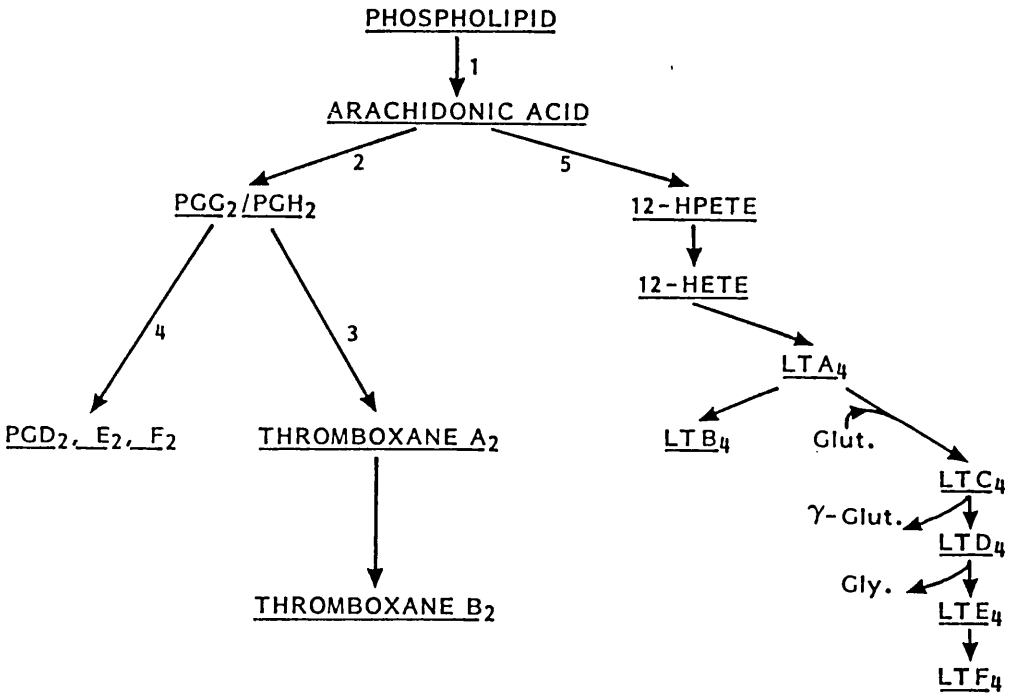

Fig. 1.10. Arachidonate metabolism. key:- 1=phospholipases; 2=cyclo-oxygenase; 3=thromboxane synthetase; 4=isomerase; 5=lipoxygenase; Glut=glutathione; G-Glut=G-glutamine; Gly=glycine.

The metabolism of arachidonate provides a series of compounds which influence platelet function. The rate limiting step in prostaglandin and thromboxane synthesis is the liberation of arachidonate from the membrane phospholipids. This is brought about by the action of phospholipase C and diglyceride lipase (Bell et al, 1979) on phosphatidyl inositides; and calcium activated phospholipase A_2 on phosphatidyl choline (McKean et al, 1981). Once liberated, arachidonate may be converted to a variety of possible products (Fig. 1.10) by the cyclo-oxygenase and lipoxygenase pathways (ratio approximately 70:30%). In the DTS, arachidonate is converted by 12-lipoxygenase to 12-hydroperoxy 5,8,10,14-eicosatetraenoic acid (12-HPETE) and its 12-hydroxy form (HETE) (Hamberg & Samuelsson, 1974), which may be further modified by neutrophils to give a series of potent bioactive substances known as leukotrienes.

The rest of the arachidonate is converted by cyclo-oxygenase to the prostaglandin endoperoxides, PGG_2 and PGH_2, in the DTS (Gerrard et al, 1976). A small percentage of these are converted to PGF_2 , PGE_2 and PGD_2, but their main fate in platelets is rapid conversion into TXA_2 by thromboxane synthetase. TXA_2 is very unstable and only exists in aqueous solution for about 30 seconds before hydrolysing to the stable TXB_2. TXA_2 is an exceptionally potent constrictor of vascular smooth muscle and a strong platelet aggregating agent (Hamberg et al, 1975).

Although TXA_2 may cause aggregation directly, and release ADP, it is not essential for aggregation. It does lower the activation threshold of other agents and has

various effects on calcium ion flux, liberating calcium from intracellular stores, possibly promoting the influx of extracellular calcium, and along with calcium and calmodulin activates the platelet contractile proteins. It is thought to cause some of these effects by acting as a calcium ionophore, but TXA_2 along with the PG endoperoxides (which have potent platelet activating effects in their own right) is also known to phosphorylate myosin light chain and 40-47kd protein (via protein kinase C) (Gerrard & Caroll, 1981). Platelets contain a high concentration of calcium, about 60% is present in dense granules and can be secreted into plasma (Holmsen & Karpatkin, 1983), but the cytoplasmic concentration is very low (Rink et al, 1982). A small amount of calcium is associated with the plasma membrane and most of the rest is located in the DTS, and may be released by TXA_2 dependent (Gerrard et al, 1978a) and independent pathways. Calcium is actively taken up by the DTS in a calcium stimulated ATPase driven mechanism (Kaser-Glanzmann et al, 1977; Cutler et al, 1978). Calcium re-uptake is stimulated by cyclic AMP and a cyclic AMP-dependent kinase which phosphorylates a 22Kd protein (Kaser-Glanzmann et al, 1979). This protein is believed to be analogous to a cardiac muscle protein which activates calcium ATPase, increasing calcium uptake.

Platelet calmodulin regulates Ca^{++} transport and appears to control the use and availability of Ca^{++} in the cell. Ca^{++} forms complexes with this protein, and most of the reactions involving Ca^{++} are probably controlled by or mediated through calmodulin.

ENZYME/PROCESS	CALCIUM	CYCLIC AMP
Myosin Light Chain Kinase (a,b)	(+)	(-)
Microtubule Polymerization (c,d)	(-)	(+)
Phospholipase C	(+)	(-)
Diglyceride Lipase	(+)	(?)
Phospholipase A_2	(+)	(-)
Cyclo-oxygenase	(?)	(-)

Table 1.4 - Some Functions of Calcium and Cyclic AMP. (+) = Stimulation; (-) = Inhibition. a - Hathaway & Adelstein, 1979; b - Hathaway et al, 1981; c - Ikeda et al, 1981; d - Steiner, 1978.

The major mechanism for down regulation of platelet function is the stimulation of adenylate cyclase which increases the cAMP concentration. Adenylate cyclase is mainly localised in the DTS and SCS (Cutler et al, 1978), and is stimulated by adenosine, PGI_2, PGE_1, PGD_2, and 6-keto-PGE_1 (Jakobs et al, 1979; Vigdahl et al, 1969; Gorman et al, 1978). These actions of PGE_1 and PGI_2 can be inhibited by TXA_2 and PG endoperoxides (Miller et al, 1977; Gorman et al, 1978). Adenylate cyclase is inhibited by Ca^{++} (Rodan & Feinstein, 1976), alpha-adrenergic agents (Jakobs et al, 1978), and ADP (Cooper & Rodbell, 1979). Cyclic AMP is broken down by phosphodiesterase which is stimulated by calcium- calmodulin (Wang & Sharma, 1980). Cyclic AMP inhibits platelet aggregation, fibrinogen binding, secretion and adhesion to the vessel wall. These effects are probably exerted by inhibiting calcium flux and/or promoting calcium re-uptake (Kaser-Glanzmann, 1977). Other effects of Cyclic AMP are shown in Table 1.4 above.

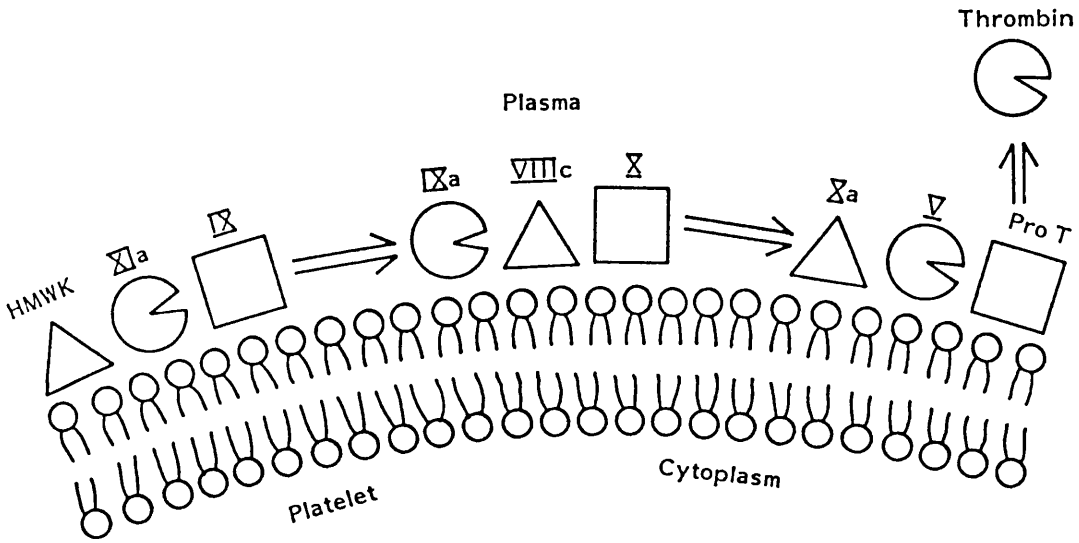

Fig. 1.11. Platelet coagulant activity. Scheme of reactions taking place on the platelet membrane. HMWK=high molecular weight kininogen; Prot=prothrombin.

1.1.11 PLATELET COAGULANT ACTIVITY

Platelets contribute to almost every stage of the coagulation system. They release calcium ions, factors V, XIII, and fibrinogen, as well as fibrinolytic substances and antithrombins. The platelet membrane provides a phospholipid surface on which reactions can occur, and has specific receptors for certain proteins, while others are non-specifically adsorbed to their surface, which results in the acceleration of coagulation reactions.

Platelet membranes have an asymmetric distribution of phospholipids between the two halves of the plasma membrane. Following platelet activation, negatively charged phospholipids such as phosphatidyl serine (PS) and phosphatidyl inositol (PI) are translocated to the outer half of the membrane, while phosphatidylcholine (PC) moves to the inner half, in a phenomenon known as a "flip-flop" reaction (Zwaal & Hemker, 1982). The exposed PS and other negatively charged phospholipids account for the activity traditionally known as Platelet Factor 3, by acting as a binding surface for the Factor X and Prothrombin activation complexes (Fig. 1.11). The vitamin K dependent coagulation zymogens bind to the phospholipid surface by virtue of their gamma-carboxy glutamic acid residues. Thus the Michaelis constant (Km) of the reaction is lowered and the overall rate of proteolysis significantly increased. This action of platelets can to some extent be mimicked by phospholipid vesicles, but the reaction rate is much lower, suggesting that platelets contribute by additional mechanisms.

Platelets have receptors for a number of clotting

factors, including Factors XI, IX, VIII, X, and V. Stimulated platelets activate Factor XII and Factor XI in the absence of calcium (Walsh, 1972a). Activated platelets bind factor XI to their membranes in the presence of high molecular weight kininogen (Greengard & Griffin, 1983), and it may then be activated by Factor XII dependent and independent mechanisms (Walsh, 1972b). Factor Xa binds to the platelet surface in a specific, and saturable manner (Miletich et al, 1977), which is not dependent on a free active site; the zymogen form does not bind. Factor Xa binding to washed platelets is dependent on the release reaction, and the receptor is proteolysed by thrombin (Miletich et al, 1977). The binding of Factor Xa appears to be dependent on Factor Va (Miletich et al, 1978a, 1978b, Kane et al, 1980), the receptor site for the latter remains unknown, but may involve a cytoskeletal protein (Tuszynski, et al, 1982).

As well as promoting fibrin formation, platelets possess anticoagulant activities and inhibitors of coagulation and fibrinolytic reactions. Platelets release Protein S (Schwarz et al, 1985) which acts as an enzymic cofactor for Protein C, and promotes the binding of this protein complex to cell membrane surfaces, thus localizing the reaction (Harris & Esmon, 1985). Activated Protein C binds to the platelet surface and degrades activated factor V (Comp & Esmon, 1979), therefore decreasing factor Xa binding to its receptor. Factor VIIIa is inactivated by activated Protein C in a similar way to factor Va (Fulcher et al, 1984). Apart from being a substrate for Protein C,

factor Va causes a fifty-fold enhancement of the rate of protein C activation by thrombin (Salem et al, 1983).

Antithrombin III has recently been immunochemically detected in supernatants following arachidonate stimulation of washed platelets, or after lysis with Triton or by freeze/thawing (Leon Alhenc-Gelas et al, 1985). Platelets contain large amounts of plasminogen activator inhibitor (PAI-1), although it does not appear to function as well as the form circulating in plasma, and may require activation.

During aggregation, platelets produce a calcium dependent protease which degrades a number of proteins involved in the cytoskeleton and contractile processes (Fox et al, 1983). A similar protease cleaves GpIb to liberate glycocalicin with the result that VWF can no longer bind (Solum et al, 1980). This same protease may degrade, and reduce the molecular weight of von Willebrand factor (Gralnick et al, 1985). The latter action may be of importance in limiting platelet adhesion, if it is true that only the higher multimers of VWF possess adhesive activity.

1.1.12 INHIBITORS OF PLATELET FUNCTION

A number of pharmacological and iv vitro agents inhibit various aspects of platelet function. The commonest ones may be broadly divided into membrane stabilisers, cAMP modulators, and antagonists of arachidonate metabolism. The first group include the thiazide diuretics and B-adrenoreceptor blockers, which are thought to bind to the platelet membrane and influence ion passage across the membrane. Increasing platelet cAMP levels down regulates

platelet function. This may be achieved by drugs which stimulate adenylate cyclase, such as PGI_2 and PGE_1, and compounds which inhibit the enzyme responsible for cAMP degradation, phosphodiesterase, and examples of the latter are adenosine and xanthines. Arachidonate metabolism may be inhibited at various levels. Acetyl salicylic acid (ASA) irreversibly inhibits cyclo-oxygenase, so that prostaglandins and thromboxanes cannot be formed during the lifespan of that platelet. Drugs like dazoxiben (Pfizer) inhibit thromboxane synthetase, but have no direct effect on prostaglandin synthesis. Alternatively, thromboxane receptor blockers such as the compound AH23848 (Glaxo Ltd) may be used. In addition, there are ADP scavengers such as the potato enzyme apyrase and the creatine phosphate/creatine phosphokinase (CP/CPK) combination, which are useful in vitro agents for limiting ADP dependent platelet aggregation.

1.2 METHODS AVAILABLE FOR STUDYING PLATELET AGGREGATION

In order to study the behaviour of platelets in whole blood, suitable technology had to be selected. This section of the thesis therefore assesses the available techniques and their particular problems.

1.2.1 OPTICAL METHODS

Platelet aggregation has been studied for many years by optical, turbidometric methods using platelet rich plasma (Born, 1962; O'Brien, 1962). These systems depend on light scattering by a suspension of platelets and the measurement

of light transmitted through the solution with a photomultiplier tube. Although of proven diagnostic value, these systems have certain limitations. Centrifugation is required during sample preparation so that red cells, white cells, and cell fragments are removed from the final platelet suspension to be tested. Platelets are heterogeneous in size, density and metabolic activity (Frojmovic and Milton, 1982; Karpatkin and Charmartz, 1969), and the heavier platelets are said to be more reactive (O'Brien, 1971). A subpopulation of platelets is almost certainly removed by centrifugation and platelet rich plasma therefore gives a false model of the aggregability of blood, making it difficult to equate in vitro results with the true clinical situation in the circulation.

1.2.2 INFLUENCE OF LEUCOCYTES

In vivo platelet function may be modulated by other formed elements present in blood. Leucocytes can synthesise PGI_2 (Blackwell et al, 1978); although there is little evidence that prostacyclin is a significant cyclo-oxygenase product in any leucocyte preparation so far studied, it may be important in certain pathologic states. Leucocytes also release other prostaglandins, as well as thromboxanes, and lipoxygenase products, which may have regulatory effects on platelets (Higgs, 1982). Production of these substances is usually enhanced by activating stimuli such as endotoxin and immunoglobulin fragments for monocytes, phagocytosis in neutrophils, and immune stimulation in eosinophils. The predominant stable prostaglandin produced by leucocytes is

PGE_2, but PGE_1 and PGD_2 as well as PGF_2, have also been detected (Higgs, 1982). PGE_2 potentiates platelet aggregation at low concentrations by inhibiting adenylate cyclase (Bonne et al, 1981), but at high levels it may exert inhibitory effects by combining with the PGI_2/PGE_1 receptor. PGE_1 and PGD_2 are both potent platelet function inhibitors (Whittle et al, 1978). However, the major cyclo-oxygenase metabolite in neutrophils, monocytes, macrophages, and lymphocytes (Morley et al, 1979; Davidson et al, 1978; Murota et al, 1978) is thromboxane B_2. Cyclo-oxygenase activity is greater in macrophages than in monocytes, neutrophils, eosinophils, lymphocytes, or platelets (Morley et al, 1979). Lymphocytes possess both cyclo-oxygenase and lipoxygenase, and thromboxane B_2 is a major metabolite, but they release much lower amounts of the cyclo-oxygenase products than neutrophils or monocytes, and their significance in haemostasis is unknown. In sheep blood, lymphocytes release thromboxane and aggregate in response to zymosan, and release more thromboxane than neutrophils in response to complement activation. However, sheep lymphocytes neither release thromboxane, nor aggregate in response to zymosan (Tahamont et al, 1984).

An alternative system for arachidonate metabolism exists in leucocytes, the lipoxygenase pathway which gives rise to a further series of substances known as leukotrienes (Borgeat et al, 1976), with potent pharmacological activity on platelets, leucocytes, and smooth muscle. 5-Lipoxygenase activity has been reported in human peripheral blood neutrophils (Goetzl & Sun, 1979) and

lymphocytes (Parker et al, 1979), whereas platelets possess 12-lipoxygenase (Hamberg & Samuelsson, 1974; Nugteren, 1975). It appears form cross labelling studies that 12-hydroxy products from platelets can be released and taken up by neutrophils to act as a further substrate for production of 5,12-hydroxy products and leukotrienes. Although leukotrienes (LT) B_4, C_4, D_4, and E_4 do not induce platelet aggregation or thromboxane B_2 production, LTC_4, D_4, and E_4 potentiate platelet aggregation induced by adrenaline or thrombin, probably by modulating thromboxane A_2 production (Mehta et al, 1986). In contrast, LTB_4, C_4, D_4, and E_4 have no effect on human platelet aggregation induced by PAF-acether (Filep & Foldes-Filep, 1987). PAF-acether (1-O-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine) (Benveniste et al, 1972; Benveniste et al, 1981), is formed by a variety of cell types, including neutrophils (Lynch et al, 1979; Jouvin-Marche et al, 1984), and is a potent stimulator of platelet aggregation. Platelets produce lyso-PAF, which can be converted to PAF-acether by neutrophils. PAF-acether may initiate platelet aggregation through a different pathway to ADP, adrenaline, and collagen (Chignard et al, 1979), but this remains controversial (Marcus et al, 1981; Chesney et al, 1982).

1.2.3 INFLUENCE OF ERYTHROCYTES

The presence of red cells may enhance platelet adhesion and aggregation by chemical and mechanical means (Hellem et al, 1961; Schmid-Schonbein et al, 1981; Turitto and

Baumgartner, 1975). Erythrocytes may preferentially bind prostacyclin, thus preventing its inhibitory action on platelets (Willems et al, 1983), and if cell damage occurs during centrifugation and separation, may release ADP and potentiate platelet aggregation (Gaarder et al, 1961). Red cells also have an active uptake system for adenosine, a compound capable of increasing platelet cAMP levels (Roos & Pfleger, 1972; Gresele et al, 1983), and thus tending to depress aggregation. Removal of the various formed elements of blood also influences its rheological properties, which may determine the distribution of platelets among the other cells, as well as influencing function.

1.2.4 MECHANICAL EFFECTS

Centrifugation itself induces biochemical changes, with alterations in platelet cyclic AMP levels, induction of prostaglandin synthesis (Salzman et al, 1976), and release of adenosine and ADP from red cells, due to mechanical activation occurring during the separation process. The exposure of platelets to these compounds could lead to minimal activation, without aggregation occurring. This is undesirable, since the primed platelets may subsequently be hyper responsive, or alternatively, refractory to ADP and other in-vitro agonists. Some workers have suggested that aggregation experiments should not be performed within one hour of centrifugation, to allow the platelets to recover. Labile substances that regulate platelet function such as prostacyclin (Moncada et al, 1976), thromboxane A_2

(Hamberg et al, 1975) and cyclic AMP may become biologically inactive during sample preparation for optical aggregation.

The measurement of platelet aggregation in whole blood is impossible by currently available optical methods, due to the optical density of blood and poor light transmission. Two basic techniques have been developed to overcome these problems, electrical impedance (Cardinal & Flower, 1980), and sequential platelet counting (Lumley & Humphrey, 1981). In these systems, blood may be analysed immediately after sampling without centrifugation, and aggregation is investigated in the presence of all blood elements. It is also possible to study certain antiplatelet drugs such as dipyridamole, which are only active in whole blood (Gresele et al, 1983), requiring erythrocytes for their function. The only factors lacking from the natural situation, are extracellular calcium ions because citrated samples are used, endothelial cells, and certain rheological factors.

1.2.5 WHOLE BLOOD AGGREGATION - PLATELET COUNTING METHODS

The platelet counting method (Lumley & Humphrey, 1981) involves the incubation of whole blood with aggregation reagents in a shaking water bath at 37°C, and the sequential removal and dilution of aliquots for platelet counting on instruments such as the Ultraflo (Clay Adams Corp.). This method is tedious and operator intensive, it requires a high level of technical ability, and is not suited to busy clinical laboratories as a general method. The counting apparatus used is large and heavy, unsuitable for transporting to the clinic or bedside. Other workers have

used a variety of types of cell counter, some of which do not require predilution. However the cycle time of the instrument is often relatively long in terms of platelet aggregation (>30 seconds) and therefore limits both sensitivity and accuracy. The results obtained by counting methods in some ways differ from those obtained with the turbidometric and impedance methods. This probably reflects the way in which the methods detect aggregates, since counting methods detect aggregation by the fall in free platelet numbers, which starts quite rapidly after the addition of aggregating reagent. In contrast, impedance methods involve the accretion of platelets to an adherent platelet monolayer on the electrode. Traditional optical turbidometric methods are insensitive to small aggregates and require large aggregate formation to influence light transmission.

1.2.6 IMPEDANCE AGGREGOMETRY

The impedance aggregometer (Fig. 1.12) consists of a cuvette holder, thermostatically controlled to keep the blood at 37°C, a magnetic stirrer to mix the cuvette contents, an electrode assembly, and electronic circuitry to monitor changes in electrical impedance (Fig. 1.13). The electrode consists of 2 fine platinum wires (0.25mm diameter, 1.5cm long) separated by a 1mm gap, and situated midway between the centre of the cell and the rim (Fig. 1.14).

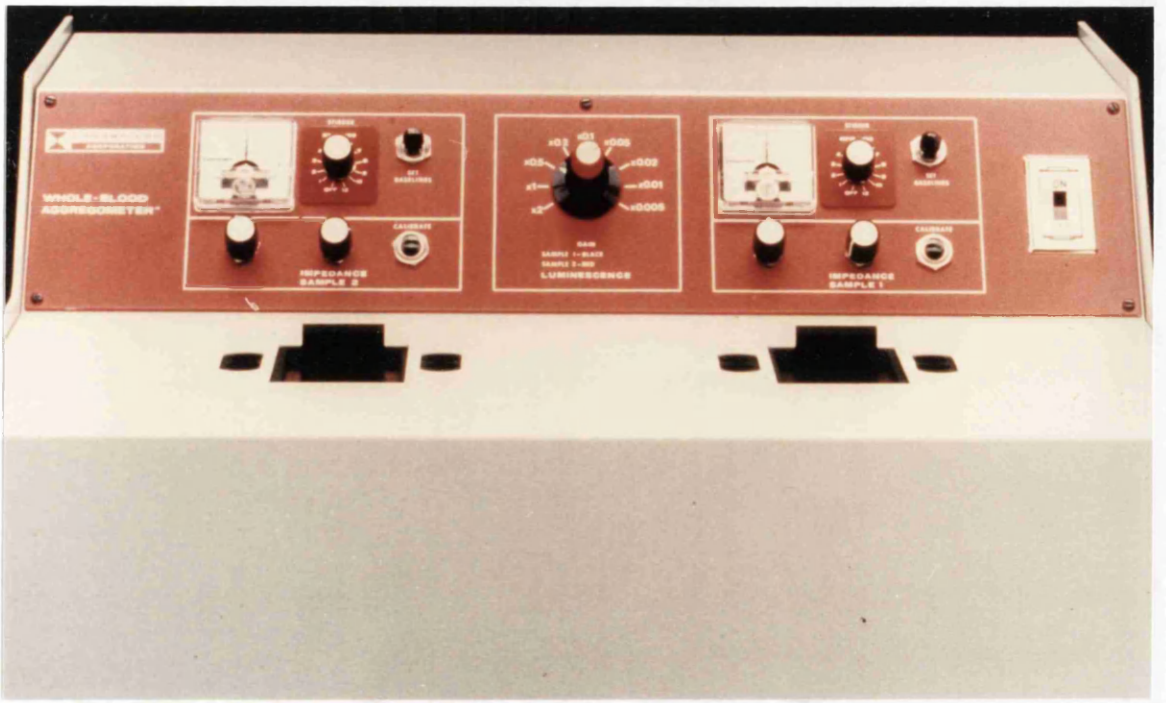

Fig. 1.12. The Chronolog Model 540 dual channel whole blood aggregometer.

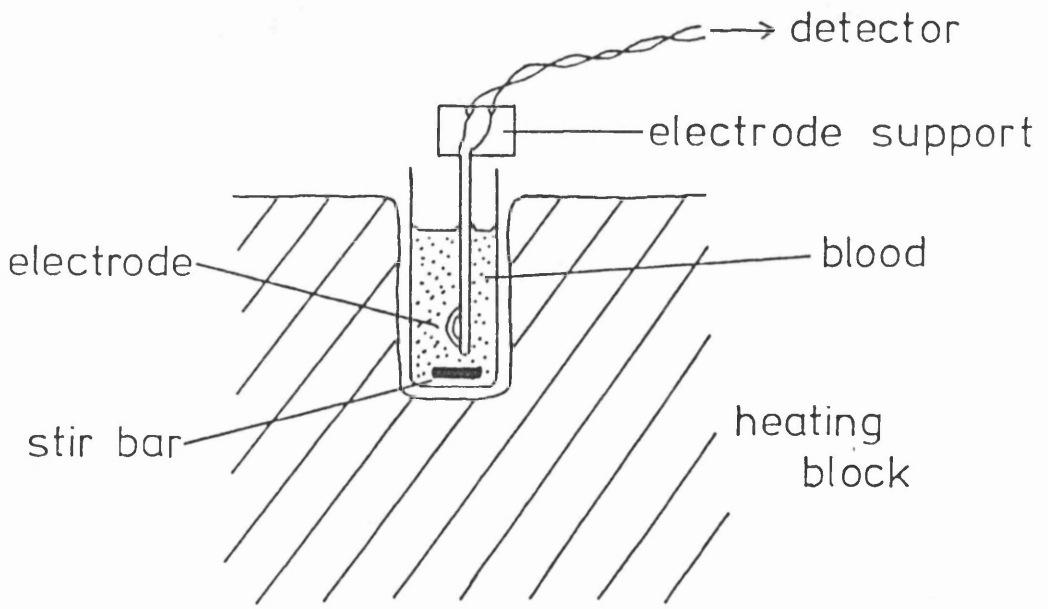

Fig. 1.13. Diagramatic representation of the impedance aggregometer.

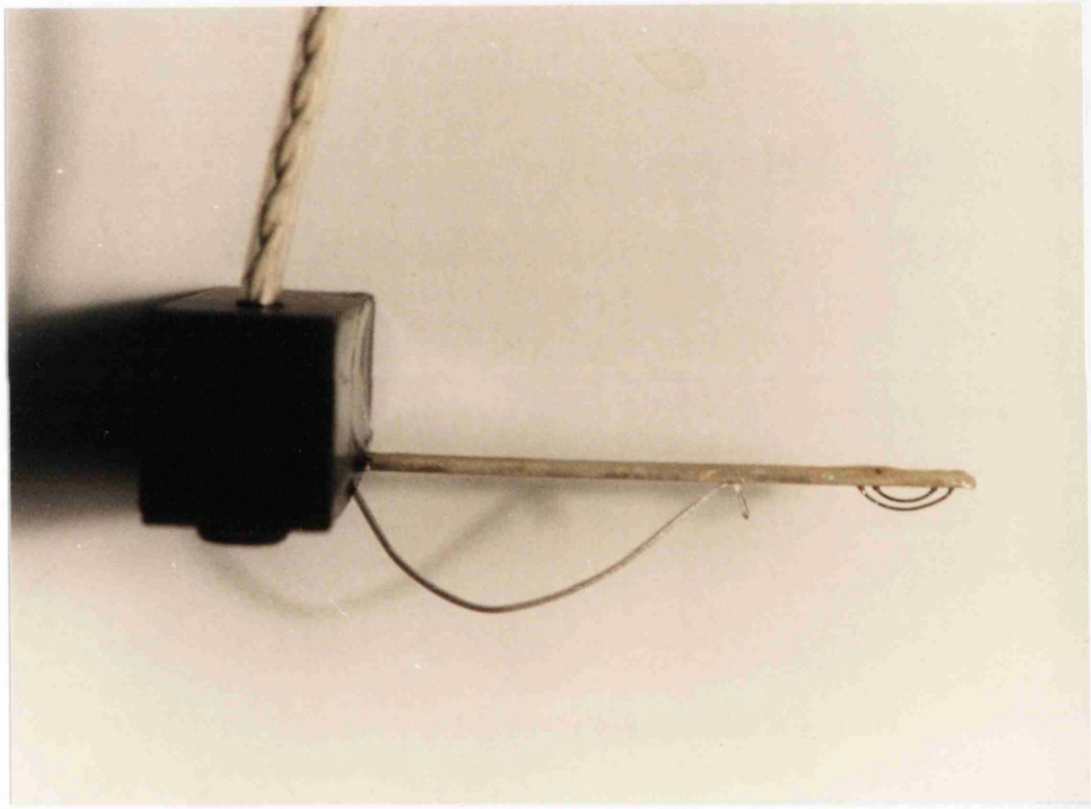

Fig. 1.14. The impedance electrode. The two platinum electrode wires can be seen at the right hand side of the electrode assembly.

A sine wave signal is passed across the electrodes with blood acting as the conductor, and after amplification and demodulation, is fed to a chart recorder. Since DC current causes polarization of one electrode, an AC current is used, and electrical impedance is therefore measured rather than resistance.

On immersion in blood the electrodes immediately become covered with a monolayer of platelets (Cardinal & Flower, 1980), but no further interactions occur, and a constant conductance is obtained. When an agonist is added, platelets aggregate to the monolayer and there is a gradual accretion of platelets (Challen et al, 1982). Conductance is thus impaired, and an increase in impedance is seen on the chart recorder. Red cells and leucocytes may be observed (by electron microscopy) trapped in the platelet aggregates, but their presence or absence has no obvious effect on the aggregation response. A standard impedance of 5 ohms may be added to the resting impedance of the electrode circuit to enable calibration of the chart recording.

The first studies of whole blood platelet aggregation by the impedance method were on blood from laboratory animals (Cardinal & Flower, 1980; Bult et al, 1981). Platelet numbers and behaviour vary considerably from species to species, and the influence of other formed elements and plasma proteins may well be different. Some of the early studies used heparin as an anticoagulant; this is undesirable since heparin can initiate and potentiate platelet aggregation after both in vitro addition, and in vivo infusion (Zucker, 1975; Thomson et al, 1973).

In order to use electrical impedance for clinical laboratory studies of platelets in whole blood, a detailed study of the technique was necessary to establish a working method and normal ranges with various aggregation reagents. The variables involved in whole blood impedance aggregation have been investigated and conditions optimised; this work is presented in Chapters 3 and 4 of this thesis.

1.3 THE PROTHROMBOTIC STATE

In discussions about the prothrombotic state, semantic as well as theoretical arguments are involved, since some authors prefer the terms "hypercoagulable state" or "prethrombotic state". These terms are often used interchangeably but really indicate subtly different conditions. The term prethrombotic implies the state of blood just before a thrombotic event occurs, and is difficult to investigate. The term hypercoagulable implies an increased ability to form a fibrin clot, but may not necessarily lead to thrombus formation. The observed alterations in haemostasis can be divided in various ways, according to the system that is affected: thrombin generation, natural anticoagulants, fibrinolysis, platelet activation; or into congenital and acquired conditions. Furthermore, prothrombotic states can arise from both physiological and pathological conditions. In situations like stress and pregnancy it is desirable for the body to prepare itself against the consequences of damage; a transient hypercoagulable state would allow the rapid cessation of bleeding at wound sites. Pathological

conditions which lead to this situation either involve a congenital deficiency of a protein involved in balancing thrombin generation, or the continuous and excessive activation of platelets or coagulation, swamping normal control mechanisms.

1.3.1 PHYSIOLOGICAL CONDITIONS

A number of physiological variations alter the balance between the procoagulant, inhibitor, and fibrinolytic systems of haemostasis towards a prothrombotic state. Often this is in response to challenge and may be advantageous, preparing the body for 'flight or fight' situations. Under conditions of chronic stress these changes may become more permanent and may cause an imbalance and vascular damage.

Stress and vigorous exercise result in a sharp rise in factor VIII, von Willebrand factor, and plasminogen activator, with increased fibrinolytic potential. Infusion of adrenaline (Gader et al, 1973 and Gader et al, 1975) or vasopressin analogues eg. DDAVP, mimic these changes (Mannucci et al, 1977; Ludlam et al, 1980; Cash, 1978; Prowse et al, 1984). In humans, the duration of the rise in FVIII and vWF exceeds that of plasminogen activator, which is readily exhausted by repeated stimulation. The adrenaline response can be inhibited with certain B-adrenoreceptor blockers, (Ingram et al, 1977). Thus adrenaline from the adrenal medulla and vasopressin may be mediators of stress responses. Adrenaline has also been shown to act in synergy with a variety of platelet activators, and lowers the threshold for aggregation and release. Venous occlusion has

similar effects, but probably acts by local neurohumoural mechanisms with vasodilatation and consequent release of endothelial cell proteins. Thus two key substances which modulate thrombin generation and platelet adhesion are produced. Since plasminogen activator inhibitor (PAI-1) exists in plasma in molar excess over tissue plasminogen activator (tPA), fibrinolysis only occurs under favourable conditions when there is a fibrin clot to protect released tPA from inactivation by PAI-1. The release of extra tPA due to stress or occlusion presumably favours clot dissolution when fibrin is deposited.

Some haematological variables are influenced by ambient air temperature and hypothermia. In particular, levels of factor VII and antithrombin III (AT-III) fall, while fibrinolytic activity increases in individuals exposed to low temperatures (Bull et al, 1978). In vitro experiments have shown that C_1-Inhibitor loses activity as temperature is decreased. These decreases in AT-III and C^1-Inhibitor activity may have clinical relevance since myocardial infarction and cerebrovascular episodes occur more frequently during cold weather (Bull, 1973).

From the beginning of the second trimester of pregnancy, major alterations in haemostatic parameters and blood rheology occur, and combine to give an increased risk of venous thrombosis. Factors VII, VIII, IX, X and fibrinogen increase, but AT-III falls, and despite an increase in plasminogen, the euglobulin clot lysis time is prolonged, apparently due to reduced plasminogen activator activity and increased PAI-1 (Bonnar 1973; Wiman et al,

1984; Astedt et al, 1984; Walker et al, 1983). The fibrinolytic response to venous occlusion and exercise is said to be abolished (Astedt, 1972; Woodfield et al, 1968). At parturition, fibrinolysis increases and there may be a further rise in coagulation factors, particularly FV and FVIII (Bonnar, 1973). Post partum, these changes gradually reverse to normal within six weeks. Platelets may also become hyperaggregable 2-5 days post partum, and coupled with the increased blood viscosity due to raised fibrinogen, and slower venous flow, this may account for the observed higher risk of thromboembolism.

Epidemiological studies have shown that women taking combined oral contraceptives (COC's) have an increased risk of venous thromboembolism (Sagar, 1976; Realini & Goldzieher, 1985). A number of alterations in the haemostatic system have been noted and they roughly parallel the changes seen during pregnancy. These haemostatic changes are not only related to the ethinyl oestradiol content of the COC, but also to the progestogen dose and type. The introduction and widespread use of low oestrogen content COC's has been linked with a reduction in morbidity and mortality in cardiovascular disease (Bottinger 1980). Levels of factors II, VII, IX, X, XII and fibrinogen generally increase, while ATIII decreases, and platelets are unchanged or become hyperaggregable (Mammen, 1982; Poller, 1978; Cohen et al, 1988; Robinson et al, 1990; Bonnar, 1987). These changes are offset by increased fibrinolysis, with raised levels of FXII and plasminogen, and decreased C1-inhibitor (Gordon et al, 1980; Meade et al, 1976). There

is a concurrent rise in cholesterol, high density lipoproteins, and blood viscosity.

Ageing causes an increase in plasma levels of factors V, VII, VIII, IX, and fibrinogen, while fibrinolytic activity and ATIII decrease (Meade & North, 1977; Chakrabarti et al, 1975; Dodds et al, 1975). The platelet count in elderly patients is generally slightly lower, but platelet adhesion in glass bead columns is increased. Vessel wall prostacyclin synthesis and release may also decline, favouring platelet hyperactivity. The combination of these changes, along with lesser physical mobility, probably contributes to the marked increase in thromboembolic risk in this group.

1.3.2 HYPERCOAGULABILITY IN DISEASE STATES

A number of risk factors have been noted for the occurrence of thrombosis, and there are certain clinical conditions which predispose to thromboembolism (Table 1.5).

RISK FACTORS	CLINICAL CONDITIONS
Surgery	Homocysteinuria
Trauma	Myeloproliferative Disorders
Age	Polycythaemia
Malignancy	Systemic Lupus Erythematosus
Immobilization	Antiphospholipid Syndrome
Heart Failure	Behcets Syndrome
Previous Venous Thrombosis	Paraproteinaemias
Myocardial Infarction	Nephrotic Syndrome
Paralysis of Lower Limbs	PNH
Varicose Veins	Congenital Deficiency of Haemostasis Inhibitors
Oestrogen Therapy	
Obesity	Acute Leukaemia
Pregnancy and the Peurperium	Diabetes Mellitus

Table 1.5. Risk Factors and Clinical Conditions Associated With an Increased Risk of Venous Thromboembolism (modified from Yardumian & Machin, 1985).

The acute and chronic phases of the inflammatory process following thermal or mechanical trauma, surgery, neoplastic growth, and infection, are associated with the increased synthesis of a number of plasma glycoproteins - the acute phase reactants (Table 1.6) (Koj, 1974; Egeberg, 1962; Holmberg & Nilsson, 1974; Denson, 1977). The magnitude of the increase in plasma proteins varies, but can be up to tenfold for fibrinogen. The period of acute phase reaction can persist for many months in the presence of chronic illness. The onset of protein elevation in the acute phase may be related to the plasma half life and availability of intracellular stores. Thus von Willebrand factor rises quickly due to endothelial release, whereas fibrinogen must be synthesised before an increase is seen. The changes in haemostatic proteins tip the balance towards hypercoagulability, particularly since blood viscosity is modulated by fibrinogen concentration (Dintenfass, 1971). In many clinical conditions, acute phase reaction is only the basic haemostatic change, and may be accompanied by specific pathogenetic changes, such as platelet activation, protease activation, and prostacyclin suppression.

HAEMOSTASIS PROTEINS	**OTHERS**
Factor VIII	Ceruloplasmin
von Willebrand Factor	C-Reactive Protein
Fibrinogen	Haptoglobin
Alpha-1-Antitrypsin	Serum Amyloid-A

Table 1.6. Examples of Acute Phase Reactants.

1.3.3 POST-OPERATIVE AND POST-TRAUMATIC CHANGES

The risk of thromboembolism following surgery is related to a number of features of the operation itself, such as site and duration of surgery, tissue involved, and amount of damage; as well as patient related variables, such as age, underlying disease, and other risk factors. 50% of patients having total hip replacement develop venous thrombosis within two weeks, usually in the vessels around the operation site (Hampson et al, 1974). This increased risk is probably due to a combination of localized blood vessel trauma, and exposure to thromboplastin from areas of damaged bone and muscle tissue in patients who are usually elderly. Hysterectomy and prostatectomy performed by the abdominal route rather than by vaginal or transurethral routes, carry a high risk (Gordon-Smith et al, 1972; Walsh et al, 1974). Prolonged immobility after surgery may cause stasis and is a further risk factor (Nicolaides & Irving, 1975).

Surgery and trauma stimulate acute phase reactions, so that fibrinogen and factor VIII rise and remain high for many days. Factor V levels fall and remain low for 2-3 days, and then rise temporarily above normal. Factor VII falls by about 50% soon after trauma, and then returns to normal 3-4 days later. Factor XII may remain low for a number of days. The fall in these factors is probably related to the degree of tissue damage with exposure of collagen and other activators of the contact system, as well as thromboplastin, which may cause dramatic local activation of extrinsic coagulation.

AT-III falls after surgery, perhaps due to increased consumption by activated clotting factors (Aberg et al, 1973; Korvald et al, 1974; Sagar et al, 1976).

Fibrinolysis is increased at the end of surgery, due to release of plasminogen activators, but a period of 'fibrinolytic shutdown' then follows, and lasts up to 10 days, with decreased plasminogen activators and plasminogen, and increased levels of alpha-2-antiplasmin (Chakrabarti et al, 1969; Innes & Sevitt, 1964; Ygge, 1970).

The platelet count usually shows no immediate change, although in extensive surgery, there may be some consumption, but within a few days the count rises as much as fourfold, and this may be sustained for about a month, particularly if there are post-operative complications. Platelet turnover is increased (Slichter et al, 1974), and B-Thromboglobulin levels may also increase, suggesting increased intravascular platelet activation (Pepper & Ludlam, 1977; Dawes et al, 1978).

1.3.4 VASCULAR DISEASE

The study of haemostatic variables in patients who already have arterial or venous thrombosis is complicated by the changes consequent of the vessel abnormality. Soon after an acute event, this differentiation is particularly difficult owing to the acute phase reaction and medication which the patient is receiving. Changes in fibrinogen and von Willebrand factor for example, are most likely to reflect the trauma associated with a thrombotic event, rather than to have been the cause.

Thrombin generation during acute thrombotic events is indicated by increased levels of fibrinopeptide A and soluble fibrin monomer complexes (Nossel et al, 1975; Fletcher & Alkjaersig, 1977). Fibrin degradation products may also be increased (Cooke et al, 1975; Ruckley et al, 1970), although fibrinolytic activity is often found to be depressed (Isaacson & Nilsson, 1972a, b, c). However, these changes are not specific to thrombosis, and may be found in other medical conditions, such as malignancy and infection. Platelet adhesion may be increased and platelet lifespan is shortened (Genton & Steele, 1977). Platelet aggregation studies have given conflicting results, but generally show an increase; while platelet release products (B-TG and PF4) are invariably raised (Pepper & Ludlam, 1977; Gjesdal, 1977), though the latter measurements are not necessarily specific.

1.3.5 ARTIFICIAL SURFACES AND PROSTHETIC VALVES

The clinical use of synthetic materials with prolonged exposure to flowing blood is increasing. The synthetic surface may be a dialysis membrane, cardiac bypass circuit, indwelling vascular catheter, Dacron vessel graft or prosthetic heart valve. There is no totally inert biocompatible material available, and all are thrombogenic to some extent. When exposed to flowing blood, platelets adhere to these materials, and aggregates form, accelerating local thrombin generation, and causing fibrin mesh formation. Red cells become trapped in the fibrin and proteins are deposited in this matrix. This results in the formation of an attached thrombus. Extracorporeal circuits

used in cardiac bypass, may cause extensive consumption of coagulation factors and platelets, with the generation of kinins and other biologically active substances, and sometimes the reinfusion of activated platelets, which may be deposited in the circulation.

1.3.6 MALIGNANCY

A number of tumour cell lines have been shown to express thromboplastin and sometimes coagulation factors such as factor VII at there surfaces, thus activating coagulation (Dvorak, 1987). In other conditions such as promyelocytic leukaemia, and pancreatic tumours, the malignant cell may be excessively granular, and cell lysis or activation may result in the liberation of large quantities of active proteolytic enzymes, which swamp the normal compensatory mechanisms in blood. In other leukaemias, there may be hyperviscosity syndromes due to the relative inflexibility and poor deformability of the malignant cells. Clinically, this can result in blockage of the small vessels, particularly in the cerebral and pulmonary circulations by the stacking of abnormal cells. The high paraprotein levels seen in IgM macroglobulinaemia and IgG or IgA myeloma may also predispose to thrombosis due the consequent hyperviscosity. Frequent thromboses also occur in the presence of solid tumours of the lung, stomach, pancreas and prostate, and may take the form of either localised thrombosis or disseminated intravascular coagulation. ATIII and fibrinolytic activity are frequently reduced, and the platelets may be hyperaggregable in these

patients (Dvorak, 1987).

1.3.7 SYSTEMIC LUPUS ERYTHEMATOSUS (SLE)

SLE and other related autoimmune collagen disorders have an increased frequency of arterial and venous thromboses as well as recurrent foetal loss. Many patients have been shown to have anti-phospholipid antibodies and tHere is a strong association between their presence (measured as lupus anticoagulant or anti-cardiolipin) and venous thromboembolism. Various attempts have been made to find a common pathogenetic mechanism, and defects in a number of different systems have been described, and it is likely that there is no single cause of the thrombotic events in all patients.

1.3.8 INHERITED THROMBOPHILIA

Congenital deficiency of AT-III, heparin cofactor II, protein C, or protein S may be associated with an increased risk of thrombosis (Egeberg, 1965; Sie et al, 1985; Tran et al, 1986; Horellou et al, 1984; Broekmans, 1985; Comp et al, 1984; Broekmans et al, 1985). The events may occur spontaneously, or in relation to other high risk factors, eg. pregnancy, peri-operatively, or while taking the oral contraceptive pill; acquired deficiencies may also occur.

The congenital dysfibrinogenaemias are a group of disorders which may be characterised by haemorrhage, thrombosis, or both. The mechanism of thrombosis in these patients remains unclear.

Homocysteinuria is another rare inherited disorder

associated with an increased incidence of both arterial and venous thromboses. This may result from direct damage by homocysteine and methionine to the vascular endothelium, or activation of the contact system.

1.3.9 MYELOPROLIFERATIVE DISORDERS

In the myeloproliferative disorders there is a high incidence of thrombotic episodes in certain subgroups of the disease, and quite gross changes in platelet function are seen. Abnormalities of platelet metabolism and function can be ascribed to the abnormal proliferation of a clone of cells in the bone marrow. The myeloproliferative disorders comprise four groups of diseases which frequently overlap and become indistinct: polycythaemia vera (PV), myelofibrosis (MF), essential thrombocythaemia (ET), and chronic granulocytic leukaemia (CGL). Paradoxically, both bleeding and thrombotic events, sometimes occur in the same patient, and are prominent complications in all of the myeloproliferative diseases (Wasserman & Gilbert, 1963, 1966; Ward & Block, 1971; Weinfeld et al, 1975). Thromboembolic complications occur in 14-63% of PV cases, and cause death in 10-40%, whereas bleeding occurs in 15-35%, and has a lower mortality (Rigby & Leavell, 1960; Wasserman & Gilbert, 1963, 1966; Berger et al, 1973). This tendency has been attributed to many different factors, including: hypervolaemia with an increased red cell mass, stasis of blood, capillary distension, abnormal clot formation, thrombocytosis, and qualitative platelet abnormalities (Gilbert, 1975). The haemorrheological

abnormalities found in PV are absent from ET, and yet both haemorrhagic and thrombotic complications, often associated with spontaneous platelet aggregation occur frequently in ET.

1.3.10 ADULT RESPIRATORY DISTRESS SYNDROME

Adult respiratory distress syndrome (ARDS) is a form of non-cardiogenic oedematous lung injury that can arise in patients with no obvious lung involvement, and was first described 20 years ago (Ashbaugh et al, 1967). It is a profound clinical syndrome which is frequently fatal with a mortality of 60-70%, where sepsis is the predominant underlying condition. ARDS arises as a complication of a variety of diverse disease processes, including: septicaemia, severe trauma, massive transfusion, and pancreatitis. The condition is characterised by hypoxia, pulmonary hypertension, increased capillary permeability, and widespread bilateral infiltrates. The increase in permeability of the pulmonary vasculature and tissue damage leads to alveolar flooding and haemorrhage; microthrombi may also occur.

The initiating mechanisms are unknown, but a variety of cellular elements and vasoactive substances have been implicated, including platelets, neutrophils, and there release products, as well as fibrin, complement, and kinins (Heffner et al, 1987). Platelets, neutrophils, and fibrin strands may be seen enmeshed in the pulmonary vascular bed at postmortem. Animal models suggest that these cells are involved, and their release products eg. prostaglandins,

leukotrienes, thromboxane, and growth factors, are able to simulate much of the pathophysiology of ARDS. There may also be direct effects of toxins such as oleic acid, fibrin, histamine, and bradykinin on the lungs (Hofman & Ehrhart, 1984; Gerdin & Saldeen, 1978; Brigham & Owen, 1975; Lewis, 1963).

During respiratory failure, neutrophils sequester in the pulmonary circulation at sites of endothelial injury (Redl & Schlag, 1980; Schlag et al, 1980), and there are increased numbers of degranulated neutrophils in bronchoalveolar lavage fluid (McGuire et al, 1982). In animals, endotoxin or phorbol myristate acetate infusion activates neutrophils, cause lung oedema and damage (Heflin & Brigham, 1981), but not if the animals are first rendered neutropenic (Shasby et al, 1982; Lloyd et al, 1983). Neutrophils release free radicals (shasby et al, 1982), eicosanoids (Bizios et al, 1983; Goldstein et al, 1977; Spagnuolo et al, 1980), and proteolytic enzymes (Janoff, 1970) which may damage the endothelium and alter permeability, increase vasoconstriction, recruit further neutrophils, and cause a zone of inflammation.

Platelets are heavily implicated in the pathogenesis, and may be an initiating factor of lung damage in early ARDS. A number of substances associated with conditions predisposing to ARDS are also potent activators of platelets. Endotoxin binds to specific membrane receptors on the platelet and could cause aggregation during sepsis related respiratory failure (Carvalho, 1985; Hawiger et al, 1975; Morrison & Ulevirch, 1978). Thrombin may be

generated in septicaemia, which frequently is associated with a consumptive coagulopathy. Infection may also involve the presence of viral bodies and immune complexes in the circulation, as well as PAF released from neutrophils and macrophages. In trauma, blood may be exposed to, tissue factor and procoagulants due to massive tissue damage. The reticuloendothelial system is frequently blocked by the massive demands on it and this may cause poor clearance of these foreign bodies, activated substances and effectors. Platelets release various substances once activated which could be implicated in the disease process. Platelets have the highest body concentration of serotonin outside the intestine, and it is the most potent pulmonary vasoconstrictor known in humans, affecting the precapillary pulmonary vessels the most (Malik, 1983). Platelet derived growth factor (PDGF) is a mitogen for mesenchymal cells such as fibroblasts and smooth muscle cells, is chemotactic for neutrophils and monocytes, stimulates neutrophil aggregation and release of free radicals and granules (Dueul et al, 1982; Dueul & Huang, 1984). Thromboxane A_2 is a potent pulmonary vasoconstrictor and 12-HETE or 12-HPETE increase airway mucous production, as well as promoting neutrophil activation and chemotaxis (Voelkel, 1985; Goetzl, 1980).

In ARDS, sequestered platelets may be detected by histological or radiochemical methods, in the pulmonary circulation (Carvalho, 1985; Pietra et al, 1981). Progressive thrombopenia occurs in 50% of non trauma ARDS cases, and the degree of thrombopenia parallels the worsening hypoxia. Half of the patients with early ARDS have

a pulmonary filling defect (Zapol et al, 1985; Greene et al, 1981), and biopsies and autopsies show pulmonary artery occlusions due to platelets, neutrophils, and fibrin (Hill et al, 1976; Pratt et al, 1979).

TXA_2 is a potent stimulator of platelet aggregation, but is also vasoactive, inducing pulmonary artery vasoconstriction (Bowers et al, 1979). Elevated plasma concentrations have been noted in patients dying of septic shock with ARDS (Reines et al, 1982). The source of the TXA_2 may not be entirely from platelets, since thrombocytopaenia only reduces TXA_2 production by 50% in sheep following endotoxin infusion, and has no significant effect on pulmonary hypertension (Wanders et al, 1981). Alveolar macrophages and neutrophils can synthesise and release TXA_2 (Cook et al, 1982; Goldstein et al, 1977), but not in the same quantities as released from platelets during aggregation. Endothelial cell slices from large branches of the pulmonary artery synthesise small amounts of TXA_2 compared to prostacyclin (McDonald et al, 1983). However in vivo evidence suggests that injured microvascular endothelial cells may synthesise more TXA_2 than prostacyclin (Ingerman et al, 1980; Serneri et al, 1983; Cooper et al, 1980). The exact contribution of platelets, leukocytes, alveolar macrophages, and endothelial cells to the final plasma concentration of TXA_2 in ARDS remains to be elucidated, as does its actual pathophysiological role. In addition, relatively little work has been performed in humans, and the results may be quite different to those obtained in animal models.

1.3.11 CIGARETTE SMOKING AND HYPERAGGREGABILITY

Extensive evidence that cigarette smoking is a major risk factor for cardiovascular diseases such as atherosclerosis, arterial thrombosis and coronary heart disease (Schwartz & Mcgill, 1980; Reid et al, 1976; Aronow, 1974; Murphy & Mustard, 1966), has accumulated. Epidemiological studies have shown a correlation between cigarette smoking and an increased frequency of arterial thrombosis (Murphy & Mustard, 1966), atherosclerosis (Strong & Richards, 1976; Hopkins & Williams, 1981), myocardial infarction (Aronow, 1976; Kannel, 1981), sudden death (Spain & Bradess, 1970), and cerebrovascular accident (Wolf et al, 1977). In arterial disease, cardiovascular morbidity and mortality rates are decreased when smoking is stopped (Wilhelmson et al, 1975). Cigarette smokers have a higher risk of occlusive peripheral arterial disease (Juergens et al, 1960) and persistent smoking after the development of the disease makes successful treatment more difficult (Wray, 1971). Even passive smoking has deleterious effects (Bocanegra & Espinoza, 1981; Meberg et al, 1979).

This relationship between smoking and cardiovascular disease has been attributed to nicotine, carbon monoxide, and other substances in cigarettes or generated after smoking them (Mansouri & Perry, 1982; Madsen & Dyerberg, 1984; Bierenbaum et al, 1978; Drummond et al, 1979). The pathophysiology of the effects of cigarette smoking has been investigated by a number of groups, and a range of biochemical, morphological, and functional changes have been noted. However, the smoking related changes seen in

different studies have not been consistent. Cigarette smoking causes shortened platelet survival (Mustard & Murphy, 1963; Fuster et al, 1981), increased platelet adhesiveness (Ambrus & Mink, 1964; Erikssen et al, 1977; Ashby et al, 1965; Birnstingl et al, 1971), and spontaneous in vivo aggregation (Bierenbaum et al, 1978; Wu & Hoak, 1975; Davis & Davis, 1979). In vitro aggregation (Grignani et al, 1977; Levine, 1973; Hawkins, 1972; Glynn et al, 1966) is also increased, However some studies have failed to show enhancement of adhesion (Murchison & Fyfe, 1966), or have found great variability in aggregation responses depending on the age of volunteers (Grignani et al, 1977; Hawkins, 1972). Chao et al (1982) found a shortened lag phase to collagen, but no change in the aggregation response to collagen (4ug/ml), ADP (1uM), or thrombin (0.3u/ml). In addition, Laszlo et al (1983) and Siess et al (1982) found no evidence of platelet activation after smoking, as measured by in vitro aggregation, or plasma BTG levels.

Most of these studies have been epidemiological in nature, comparing populations of smokers and non-smokers. Other investigations have looked at the effects of a smoking session on blood from regular smokers. The results obtained depend on the particular group selected, the smoking habits of the volunteers, and the type and composition of the tobacco.

Cigarette smoke causes subendothelial damage (Boutet et al, 1980), and Platelets from blood collected after smoking can adhere to intact endothelial surfaces, which does not occur with normal blood (Sieffert et al, 1981).

These changes in platelet behaviour are particularly relevant since platelets have a role in the initiation and growth of atherosclerotic plaques, and arterial thrombosis (Woolf & Wilson-Holt, 1981; Ross & Glomset, 1976; Fuster & Chesebro, 1981).

Chronic smoking causes an increase in platelet count, fibrinogen (Dintenfass, 1975; Meade et al, 1979), alpha-1-antitrypsin, orosomucoid, haptoglobin, properdin factor b, and whole blood viscosity (Dintenfass, 1975), without changes in antithrombin III, factor V, or factor X, which is indicative of an acute phase reaction, suggesting that there is an underlying inflammatory process (Chao et al, 1982). Variable fibrinolytic results have been recorded in smokers (Meade et al, 1979; Allen et al, 1981), but recent studies have shown increased fibrinogen and decreased plasminogen activator and plasminogen (Belch et al, 1984).

Patients with peripheral vascular disease (PVD) have similar abnormalities to those reported in some groups of smokers, ie. abnormal platelet function (Ward et al, 1978), raised plasma fibrinogen (Dormandy et al, 1973; Hamer et al, 1973; Harris et al, 1978), and decreased fibrinolysis (Browse et al, 1977), and yet smokers do not necessarily have PVD. This led Hurlow and colleagues (1981) to suggest that the changes in PVD may be a consequence of smoking rather than to endothelial cell damage; however, they were unable to demonstrate any differences in platelet function between smokers and non-smokers in both PVD and normal control groups, although the control smokers had an increased fibrinogen.

The object of this thesis was to investigate platelet function in whole blood, in an environment close to normal physiology. It was anticipated that such studies in whole blood would be more sensitive to in vivo changes than studies on isolated platelets, since all of the formed elements are present, there is less risk of in vitro activation, and labile substances should still be present. This approach was thought to be particularly applicable to platelet studies in blood from patients with prothrombotic states where the mechanism of thrombosis is unclear, and for examining anti-platelet drug efficacy. Existing methods were inadequate and it was first necessary to develop new techniques using electrical impedance.

CHAPTER 2 MATERIALS AND METHODS

2.1 GENERAL METHODS

2.1.1 SPECIMEN COLLECTION AND HANDLING

Venous blood was collected from the antecubital fossa with minimal stasis, using a 19 or 21 gauge needle, and plastic syringes. When large volumes of blood were drawn, a "butterfly" cannula (Abbott Laboratories Ltd) and several syringes were used. Blood was collected into either EDTA for full blood and platelet counts, 0.106M Tri-sodium citrate (1 volume to 9 volumes of blood) for citrated blood and plasma, or plain glass tubes for serum. Special anticoagulants were required for certain tests, and details are given in the appropriate methods. Plasma and serum samples were handled with plastic pipettes and tubes to avoid contact activation.

2.1.2 FULL BLOOD, DIFFERENTIAL, AND PLATELET COUNTS

These were performed using Coulter S+IV (Coulter Electronics Ltd., Luton, Beds), or Technicon H1 (Technicon Instruments Ltd, Basingstoke, Hants.) cell counters. In some cases (where there were atypical cell populations or characteristics) manual platelet counts were performed using a haemocytometer (Brecher & Cronkite, 1950), and manual differential counts with morphological examination were carried out on Romanowsky stained peripheral blood films.

2.1.3 STATISTICAL ANALYSIS

Statistical tests were performed as indicated in the text. Parameters with normal distributions were analysed by

2-tailed Student's "t" test and paired "t" tests as appropriate; data with non-Gaussian distribution was analysed by non-parametric tests; probability values of 0.05 or less were taken as being statistically significant. The coefficient of correlation was performed by the method of Pearson.

2.2 COAGULATION FACTOR ASSAYS

Unless otherwise specified, all general laboratory reagents were of 'Analar' or higher grade, and obtained from BDH Ltd (Dagenham, Essex) or Sigma Chemical Co. Ltd. (Poole, Dorset).

2.2.1 COAGULATION SCREENING TESTS

The thrombin clotting time (TT), prothrombin time (PT), and activated partial thromboplastin time (APTT) were performed by manual techniques or using a Coag-A-Mate X2 instrument (Organon-Teknika Ltd) (Machin & Mackie, 1989; Austen & Rhymes, 1975; Hardisty & Ingram, 1965). The following reagents were used: Diagen Bovine Thrombin, Diagen "Bell & Alton" Platelet Substitute (Diagnostic Reagents Ltd., Thame, Oxon.); Manchester Comparative Reagent (UK Reference Laboratory for anticoagulant control, Manchester), or in later experiments phenolysed rabbit brain thromboplastin (Diagnostic Reagents Ltd); Light Kaolin (BDH Ltd). All tests were controlled using pooled normal plasma (n=10). The TT and PT were considered normal if they were within 2 seconds of the control time, and the APTT within a range from 30-40 seconds. Samples with a TT greater than 20

seconds were repeated using protamine sulphate or toluidine blue instead of saline to identify heparin effects and dysfibrinogens.

2.2.2 FIBRINOGEN - CLAUSS ASSAY

Clottable fibrinogen was measured by a manual technique (von Clauss, 1957), using 100 u/ml bovine thrombin (Diagnostic Reagents Ltd) and standardised with Reference Plasma 100% (Immuno Ltd., Dunton Green, Kent).

When heparin contamination of the plasma was suspected, protamine sulphate was added to the diluting buffer to neutralise heparin. The reference range determined in 20 normal subjects was found to be 1.5 - 4.0 g/l.

2.2.3 FIBRINOGEN (GRAVIMETRIC) ASSAY (Biggs, 1976)

Reagents

0.025M Calcium chloride 10u/ml Bovine thrombin - Parke Davis (Warner Lambert), or Diagen (Diagnostic Reagents Ltd).

Method

1ml of plasma was placed in a 10ml glass tube and 1ml of calcium chloride was added along with 0.2ml thrombin. After mixing, 2 applicator sticks were inserted and the solution left to clot at 37°C for 30 minutes. The clot was then gently squeezed against the wall of the tube with the applicator sticks to express the serum. The clot was carefully removed from the sticks with a scalpel blade and placed in a fresh glass tube, before washing twice with saline for 10 minutes, once with distilled water for 10 minutes, and finally with acetone for 5 minutes. The ball of

fibrin was placed in a petri dish at 37°C overnight and then weighed on a sensitive chemical balance.

Calculation

The weight of the clot obtained from 1ml of plasma was corrected to g/l, and for dilution in citrate. The reference range determined in 20 normal subjects was found to be 1.50 - 4.00 g/L.

2.2.4 WHOLE BLOOD CLOTTING TIME

1ml of blood without anticoagulant was placed in a 75*12mm glass tube in a 37°C water bath immediately after collection. The tube was gently tilted at regular intervals until a clot was detected. In normal blood this usually occurred within 5 minutes of blood collection. In some experiments, for comparison with the clotting time obtained in non-anticoagulated whole blood aggregation, blood was diluted with prewarmed saline and collagen to give a final volume of 1ml, and the clotting time was then determined.

2.2.5 1-STAGE FACTOR VIIIC ASSAY (Biggs, 1976)

1-Stage FVIII:C assays were based on the APTT method, using triple point assays with factor VIII deficient substrate plasma from a local haemophiliac, and the British Standard for factor VIII (NIBSC). Results were obtained by parallel line bioassay procedures using a double logarithmic transform. The reference range was 0.5 - 2.0 IU/ml.

2.2.6 VON WILLEBRAND FACTOR IMMUNOLOGICAL (ELISA) ASSAY

von Willebrand factor antigen was measured by enzyme linked immunosorbent assay (ELISA) by a modification of the method of Short et al (1982). In brief, Immulon microtitre plates (Dynatech Laboratories Ltd, Billingshurst, Sussex) were coated with a 1/1000 dilution of rabbit anti-human von Willebrand factor serum (Dako Ltd, High Wycombe, Bucks), in 0.01M sodium phosphate, 0.145M saline buffer pH 7.2. Doubling dilutions (in the range 1/4-1/256) of British Standard plasma (NIBSC) or test samples were made in the above buffer modified by adding 20.75g NaCl and 1ml Tween 20 per Litre buffer. Horse radish peroxidase conjugated anti-human von Willebrand factor serum (Dako Ltd) was diluted 1/1000 and bound conjugate was detected using 1,2-phenylene-diamine dihydrochloride and an MR700 plate reader (Dynatech Laboratories Ltd).

A graph of log concentration against absorbance was plotted for the standard dilutions, and the test values were read from the standard curve and multiplied up for the dilution factor. If very high or low absorbance readings were obtained and were outside the linear range of the standard curve, the samples were assayed again at a higher or lower dilution as appropriate. If bad duplicates were obtained or non-parallel dilution curves were seen for test samples, the assay was repeated.

The reference range established in 20 apparently healthy normal subjects was 0.5 - 2.00 u/ml.

2.2.7 VON WILLEBRAND FACTOR ANTIGEN (IMMUNOELECTROPHORESIS)

Laurell rocket immunoelectrophoresis (Laurell, 1966) was carried out using 1% Agarose LE (ICN Biomedicals Ltd, High Wycombe, Bucks) prepared in Barbitone buffer pH 8.6, and containing rabbit antihuman factor VIII related antigen - Dako Ltd (High Wycombe, Bucks). Doubling dilutions of test and British Standard plasma (NIBSC) were made in buffer to give undiluted, 1/2, 1/4, 1/8, standard solutions, and test plasmas were diluted as appropriate for their VWF:Ag concentration. Dilutions were applied to 2.5mm wells in the agarose, and electrophoresis was carried out for 18 hours at 3 v/cm gel at 10°C. After staining with Page Blue 83 'Electran' (BDH Ltd), the height of each rocket was measured from the well to the tip of the rocket. A log/log graph of Rocket height against VWF:Ag concentration was plotted and the test values abstracted from the standard curve. This percentage of standard value was corrected to U/ml by multiplying the percentage by the standard potency and dividing by 100. The correct volume of antiserum to use was determined for each batch, but was usually in the region of 100ul per 10ml of agarose. Reference range 0.50 - 2.00 u/ml

2.2.8 RISTOCETIN COFACTOR ASSAY (vWF:RiCof)

(Modified from Weiss et al, 1983)

Preparation of Reagents

Washed Platelets were prepared by diluting platelet rich plasma 1/2 with calcium free Tyrode's buffer containing 10ng/ml Iloprost and 10mM EDTA at room temperature. The diluted platelets were placed in a plastic conical bottom

tube and centrifuged at 850g for 10 minutes. The supernatant was discarded using a plastic pasteur pipette and the pellet slowly and gently resuspended in buffer containing inhibitors. When the platelets were completely resuspended, they were again centrifuged. This process was repeated twice more. The supernatant was discarded, and the platelets were resuspended in Tyrode's Buffer containing 10ng/ml Iloprost and 1/4 PPF, to a platelet count of approximately $200*10^9/l$.

Serial doubling dilutions of standard were made from 1/2 to 1/64 using sample buffer. Test plasmas were initially diluted 1/8 to 1/32, but other dilutions were prepared as required. All dilutions were prepared just before use.

Method

0.175ml of platelet suspension was placed in each of a series of glass aggregometer cuvettes and stir bars were added. 0.3ml of buffer/PPF was placed in one cuvette without stir bar to act as a blank. The platelet aggregometer (Payton 300BD, Rotronics Ltd, Croydon) was calibrated using the blank to set the recorder to 95%, and a cuvette containing platelets plus 0.05ml undiluted standard plasma to set 10%.

0.05ml of 6mg/ml ristocetin sulphate (Lundbeck Ltd.) was added to the cuvettes containing washed platelets, and 2 of these were warmed in an aggregometer. 0.05ml of standard or test plasma dilution was added, and the wave of agglutination was recorded. This process was continued for the remaining dilutions of each sample. Suitable dilutions of test plasma were used so that at least 3 responses (where possible) were obtained in the range of the linear part of

the standard curve.

The slope of the initial linear part of each response was measured and a graph of slope against log dilution (concentration) was plotted for the standard dilutions. The concentrations of test sample results were abstracted from the curve, and the mean concentration determined and corrected according to the potency of the batch of standard.

A buffer blank was tested for agglutination and aggregation with each batch of tests, to ensure adequate platelet washing and absence of background aggregation.

2.2.9 ANTITHROMBIN III AMIDOLYTIC ASSAY

Antithrombin III (ATIII) was measured by a modification of the amidolytic method of Odegaard et al (1975), using a microtitre technique (Machin & Mackie, 1989), with bovine thrombin (Diagnostic Reagents Ltd) and the chromogenic substrate S-2238 (KabiVitrum Diagnostica Ltd, Sweden). Assays were standardised with the International Standard preparation (NIBSC). The reference range in 20 apparently healthy normal subjects was 0.80 - 1.20 IU/ml.

2.3 PLATELET FUNCTION TESTS

2.3.1 PLATELET AGGREGATION - TURBIDOMETRIC METHOD

Test samples

Citrated blood was centrifuged at 170g at ambient temperature, for 10 minutes to prepare platelet rich plasma (PRP). This was removed and stored at room temperature in a capped tube. All handling was kept to a minimum and was with plastic pipettes and tubes. The residual blood was then centrifuged at 2700g for 15 minutes and the resulting platelet poor plasma (PPP) collected into a separate, capped plastic tube. PRP was diluted with autologous PPP to give a final platelet count of $200*10^9$/L for aggregation studies.

Reagents

ADP (Grade III, Sigma Chemical Co. Ltd.) - 10mM stock solution in saline, stored in aliquots at -20°C. Working dilutions of 100, 50, 25, 10, and 5uM ADP were prepared in saline and kept on ice, for use within 4 hours.

Collagen (Hormon-Chemie, Munchen, W.Germany) - 1mg/ml equine tendon fibrils stored at 4°C. The stock solution was diluted with the kit buffer to give 40 and 10ug/ml which were stored in ice for one working day.

Adrenaline (Sigma) - 1mg/ml (5.5mM) stored in aliquots at -20°C. Before use, 100, 50, and 10uM solutions were prepared with saline as diluent and kept in ice.

Arachidonate (Sigma) - 20mM solution in distilled water, stored in aliquots at -20°C. For use, the stock solution was diluted with an equal volume of isotonic saline and kept on ice, to be used within 4 hours.

Thrombin (Parke-Davis, Bovine) - 50 u/ml solution in isotonic saline stored in aliquots at -20°C. Thrombin was thawed and diluted with saline to give 1 and 5 U/ml solutions when required.

Endoperoxide Analogue U46619 (a kind gift from Upjohn Ltd, Kalamazoo, Michigan, USA) - The vial contents were dissolved in a small quantity of ethanol, and diluted to 25ug/ml (0.071mM) with saline, and stored at -20°C in aliquots.

Calcium Ionophore A23187, Ca^{++} + Mg^{++} salt (Sigma) - 500ug/ml (1mM) in ethanol, stored in aliquots at -20°C. Calcium Ionophore was diluted in saline to give 5 and 10ug/ml solutions, stored in ice for 1 working day.

Ristocetin (Lundbeck) - 12.5mg/ml in saline, stored in aliquots at -20°C. The stock aliquots were thawed when required and kept on ice before diluting as necessary in saline.

Method

The heating block of a Payton 300BD dual channel platelet aggregometer (Rotronics Ltd, Croydon) was allowed to warm to 37°C and the stirrer speed was set to 900 rpm. The chart recorder was set to 10mv and 3cm/min. Using

microcuvettes and appropriate holders, the aggregometer signal to the chart recorder was calibrated to 10% and 95% settings using PRP and PPP, and the output and zero controls respectively. 270ul of undiluted PRP was placed in a cuvette in the aggregometer, and stirred for 15 minutes to check for spontaneous aggregation (pen deflection >20% of chart). If spontaneity was present, the PRP was diluted in PPP and the test repeated, until a dilution of PRP was found where spontaneous aggregation had disappeared. If this point was found at or above a platelet count of $200*10^9/L$, aggregation tests with various reagents could be performed. 270ul of diluted PRP were placed in a cuvette and warmed until a steady baseline was obtained. 30ul of aggregating reagent was added rapidly to the PRP, and the response recorded.

Calculation

The length of the lag phase, aggregation rate, and amplitude of the aggregation wave were measured (Fig. 2.1). Alternatively, threshold concentrations were determined by starting with a very low dose of an aggregating agent and progressively adding higher concentrations of the agonist until a response was obtained. EC_{50} (50% of effective concentration) values were obtained by drawing a dose/response curve for a number of concentrations of agonist and finding the dose of aggregating reagent which caused 50% of the maximum response.

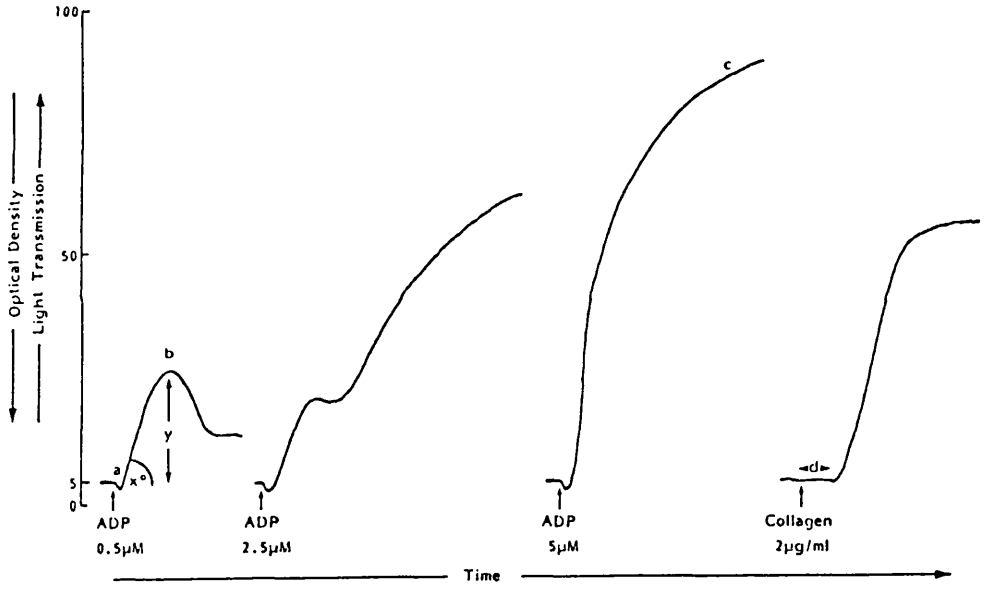

Fig. 2.1. The measurement of platelet aggregation responses in platelet rich plasma. a) initial rate (slope) of aggregation; b) extent (amplitude) of primary wave aggregation; c) extent of secondary (maximal) aggregation; d) lag period; x°) angle of initial slope.

Comments

Care was taken to exclude red cells and granulocytes from the PRP, as they interfere with the light transmittance. In patients with conditions like thallasaemia, there were red cell fragments and membranes in the PRP; these were removed where necessary by further centrifugation of PRP at 1500g for 2 minutes, or after allowing them to settle out.

Interpretation

Subjective assessment of aggregation responses by a trained eye is usually sufficient for clinical interpretations, but normal values for the lag phase, aggregation rate and amplitude of response may be established, and are indicated at relevant places in this thesis. ADP usually gives a secondary wave at 2.5uM or above, with no disaggregation, but only a primary, reversible wave at less than 1uM. Both 1 and 4ug/ml collagen give a lag phase of 1 minute or less followed by a steep response, spanning the chart paper width. Arachidonate normally gives a response similar to the secondary wave of ADP. Ristocetin gives a strong response with a steep slope at 1.25mg/ml, a more protracted response at 1.00mg/ml, and may give no response at 0.75mg/ml.

2.3.2 ADP AGGREGATION THRESHOLD

Platelet aggregation was performed with a range of doses of ADP as described in the section on aggregation. The ADP dose which just induced a secondary wave of aggregation was taken as the threshold dose. This dose was generally

between 0.5 and 2.5M ADP.

2.3.3 SPONTANEOUS AGGREGATION

Undiluted PRP was stirred in a cuvette in an aggregometer at 37°C for 15 minutes, and transmission changes monitored on a chart recorder. Spontaneous platelet aggregation was defined as sharp deflection of the pen recorder similar to that seen for collagen aggregation, with an amplitude greater than 20% of the chart width. Where positive responses were obtained, PPP was monitored at 37°C as cryoglobulins and cold agglutinins can mimic spontaneous aggregation responses, when plasma is warmed to 37°C. When spontaneous aggregation was detected, the PRP was diluted 1/2 in autologous PPP, and the test repeated. If it was still present at a platelet count of $200*10^9/L$, or occurred in unstirred PRP at room temperature, Platelet aggregation to ADP and other reagents could not be tested.

Tests for spontaneous aggregation were also carried out in whole blood impedance aggregation methods, in a similar fashion, on undiluted and diluted citrated whole blood. Not all patients with spontaneous PRP aggregation have a spontaneous response in whole blood.

2.3.4 PLATELET AGGREGATE RATIO

The platelet aggregate ratio (PAR) was measured by a modification (Bowry et al, 1985) of the method of Wu & Hoak (1974). Blood was collected into either buffered EDTA or buffered EDTA with formalin. Platelet rich plasma was prepared, and platelets counted using a Coulter S+IV

instrument. Results were expressed as a ratio of the counts from formalin EDTA and EDTA buffered tubes. The reference range quoted in the published method was used (0.82-1.1), and increased circulating platelet aggregates were indicated by a decreased platelet aggregate ratio.

2.3.5 PLATELET ADENINE NUCLEOTIDE MEASUREMENT

Principle

The firefly luciferase/luciferin system emits energy as luminescence if ATP is added. If standard ATP solutions are used, a luminometer may be calibrated and unknown test samples assayed (Machin & Mackie, 1989, modified from Bangelmaier & Holmsen, 1983). ADP may also be measured after conversion to ATP via the PEP/Pyruvate kinase reaction.

Reagents

200ug/ml Collagen (Hormon-Chemie, Munchen).

100mM Na_2EDTA - 3.71g in 100ml distilled water Absolute Alcohol Tri-Chloroacetic Acid (TCA) - 20% w/v in distilled water.

TCA/EDTA Solution - Equal Volumes of TCA and 8mM EDTA.

Tris Acetate EDTA Buffer - 12.1g Tris and 0.744g Na_2EDTA in distilled water, adjusted to pH 7.75 with 50% acetic acid, and made to 1L with distilled water.

Luciferase/luciferin - ATP Monitoring Reagent (Pharmacia Ltd), reconstituted in 10ml sterile water, and stored in aliquots at -20°C.

0.1M Tris Acetate Buffer - 12.1g Tris dissolved in distilled water, pH adjusted to 7.75 with 50% acetic acid, volume made to 1L with distilled water.

0.4M $MgSO_4$ and 1.3M KCl soln. - 9.86g $MgSO_4.7H_2O$ and 9.69g KCl in 100ml distilled water.

100mM Phosphoenol Pyruvate (PEP) (Sigma Chemical Co.) - 0.234g in 10ml sterile distilled water, stored in aliquots at -20°C. Pyruvate Kinase (PK) (Sigma Type II) $(NH_4)_2SO_4$ Suspension.

BSA Buffer - 10mg Bovine Serum Albumin, Fraction V (Sigma) in 10ml Tris Acetate buffer, stored at 4°C.

Preparation of test samples

For the measurement of "Total Nucleotide Content" :- 100ul of PRP were pipetted into a plastic microcentrifuge tube, and 100ul of TCA/EDTA solution were added. The tube was vortex mixed and stored on ice for 10 minutes, before centrifugation for 3 minutes in a microcentrifuge. 10ul of the supernatant was diluted in 490ul of Tris Acetate EDTA buffer in a fresh plastic microcentrifuge tube, and the sample was assayed or frozen below -20°C for later use.

For the measurement of "Release Nucleotides" :- 225ul of PRP was stirred in an aggregometer cuvette at 37°C, and 25ul of 200ug/ml Collagen was added. After 4 minutes 25ul of 100mM EDTA was added, and then the stirbar was removed. The cuvette was placed in a centrifuge and the platelets pelleted at 1500g for 5 minutes. 100ul of the supernatant was mixed with 100ul of absolute ethanol in a plastic microcentrifuge tube, on a vortex mixer. The precipitate was then separated by spinning the tube in a microcentrifuge for 3 minutes. 10ul of the supernatant was diluted in 490ul Tris Acetate EDTA buffer, and the samples were stored on ice for immediate assay, or at -20°C for

subsequent assay at a later date.

Preparation of working reagents and standards

"Active Buffer" was prepared by mixing 90ul BSA Buffer with 10ul PK, 84ul $MgSO_4$/KCl solution and 16ul PEP in a micro- centrifuge tube and storing on ice. 20ul of this solution was added to 330ul Tris acetate buffer in a cuvette.

"Inactive Buffer" was prepared by mixing 20.34ml Tris acetate buffer, 504ul $MgSO_4$/KCl solution, 60ul PK and 96ul PEP in a loosely capped glass universal tube, and boiling in a waterbath for 30 minutes. After cooling to ambient temperature, 350ul of this boiled mixture was placed in a cuvette.

ATP standard (Pharmacia Ltd) was reconstituted with 10ml of sterile water to give a 10uM stock solution, and then further diluted to give 1.0, 0.5, 0.25, and 0.125 and 0.063uM solutions in saline. ADP standard was prepared from powdered ADP (Sigma Chemical Co) as a 0.5uM solution in saline. Stock solutions were stored frozen at -70°C and working solutions at 4°C.

Method

6 pairs of "Active" and "Inactive" cuvettes were prepared and 100ul of monitoring reagent was added to each. 50ul of test or standard sample was added to each pair of cuvettes. The "inactive" cuvette was mixed and placed in a Luminometer (Pharmacia Ltd), and a reading taken. The "active" cuvette was mixed and left to stand so that ADP conversion to ATP would occur. After the 6 "inactive"

cuvettes had been used for ATP measurement, the "active" ADP cuvettes were inserted into the luminometer and readings taken. An internal standard was used if abnormally low results were obtained, by adding 5ul of 10uM ATP to the same cuvette after measuring the response to test sample. ADP standard was also added to one of the "active" cuvettes to ensure that the pyruvate kinase reaction was occurring adequately.

Calculation

A graph of luminescence response against concentration of ATP standard was plotted on linear graph paper. The ATP and ADP concentrations of test samples were obtained by abstraction of their luminescence values from the graph. The actual concentration as $nM/10^9$ Platelets is obtained by the following formulae:-

$$\text{Released ATP} = \text{ATPl} * \frac{1000}{P} * df * 2 * \frac{275}{225}$$

$$\text{Total ATP} = \text{ATPl} * \frac{1000}{P} * df * 2$$

Where:-
ATPl = uMol ATP obtained from standard curve;
P = Platelet count ($*10^9/L$);
df = Dilution Factor (25 if a 1/25 dilution is made in sample preparation, 50 if a 1/50 dilution is made).

Released ADP and Total ADP are calculated in the same way, but the ATP figure must be subtracted from the final value.

Interpretation

Normal ranges for "Total Nucleotide Content" and "Release Nucleotides" (to 20ug/ml collagen, were determined in 20 healthy normal laboratory staff, who had not received any medication or patent medicines for at least 10 days prior to sample collection, and were expressed as $nM/10^9$

Platelets, as follows:-

	TOTAL	RELEASE
ATP	41-61	8-20
ADP	19-38	18-28
RATIO	1.24-2.56	0.43-0.79
SUM	60-99	22-50

35-55 % of Total Nucleotides are Released.

2.3.6 PLATELET AGGREGATION IN WHOLE BLOOD - IMPEDANCE METHOD

Principle

The measurement of platelet aggregation by electrical impedance (Cardinal & Flower, 1980) allows the analysis of blood samples immediately after sampling and aggregation is investigated in the presence of all blood elements. A cuvette of blood is stirred at 37°C, and an electrode consisting of 2 fine platinum wires set a fixed distance apart is inserted. The electrode immediately becomes covered with a monolayer of platelets and after the addition of a suitable agonist, formed aggregates adhere to the monolayer. The mass of platelets on the electrode influences the electrical impedance between the two wires, and this can be visualised using a chart recorder. A standard resistance of 5 ohms may be applied to the system to enable calibration of the chart recording. The method described is the final optimised method determined by the work presented in Chapter 3.

Reagents

Citrated Blood Isotonic saline Collagen (Hormon-Chemie, Munchen, W.Germany) - diluted in SKF buffer (provided by manufacturer) to give 200, 100, and 40ug/ml solutions.
ADP (Grade III, Sigma Chemical Co Ltd). Dissolved in saline to give 10mM stock solution stored at -20°C. The stock solution was diluted to 1mM before use, and further dilutions were made as necessary in saline.
Arachidonic Acid (Sigma Chemical Co. Ltd.) - 10mg dissolved in 200ul absolute ethanol, which had been purged of air with nitrogen. Stored under nitrogen at -70°C. Just Before use, 20ul of the stock solution was added to 80ul of normal (or patient) platelet poor plasma to obtain the working reagent (40mM). In initial experiments, sodium arachidonate was used, prepared as indicated in the turbidometric aggregation method, further details are given in chapter 3.

Equipment

Chronolog Whole Blood Impedance Aggregometer Model 540 (Chronolog Corp., Philadelphia) R302 Chart Recorder (Rikadenki-Mitsui, Chessington, Surrey) Sacriston, Co. Durham) Teflon coated stir bars (supplied with aggregometer) Glass and polystyrene 9*44mm cuvettes.

Preparation of test samples

The haematocrit was determined and the citrated blood diluted to a haematocrit of 0.31/l (PCV 30%) with isotonic saline. The diluted blood was warmed to 37°C in a polystyrene cuvette in the aggregometer heating block. When the haematocrit was unknown, and aggregation was to be

performed as soon as possible after blood collection, 722ul of citrated blood was diluted in 253ul of saline, as this dilution reduced the haematocrit of most (non-anaemia) blood samples to below 0.3l/l without having any deleterious effect on the platelet count.

Method

The aggregometer heating block was allowed to warm to 37°C by switching the instrument on about 30 minutes before required. 975ul of diluted citrated blood was placed in a 9*44mm cuvette and allowed to warm to 37°C in the machine heating block. A teflon coated stirbar was added, and the cuvette placed in the hole marked "PRP or Whole Blood" in the aggregometer. The electrode was then carefully positioned in the blood, and the stirrer speed set to 600 rpm. The "Set Aggregation Baseline" button was depressed and held, while the chart recorder zero control was adjusted to give a pen reading of 20% of the chart width. The aggregometer "zero" control was then adjusted to give a pen reading of 20% chart width. The "Calibrate" button was depressed and held, while the gain control was adjusted to calibrate 10 small divisions of the chart scale to equal 5 Ohms; this procedure was repeated several times to check the calibration. A steady baseline was obtained and then 25ul of aggregating reagent was rapidly added directly into the blood. The impedance changes were recorded at a chart speed of 2cm/min.

Calculation

Three measurements were made:-

The lag period between addition of reagent and beginning of aggregation wave (Fig. 2.2a).

The rate, which was measured by drawing a tangent to the initial exponential part of the response, and measuring the angle between this line and the horizontal (Fig. 2.2b).

The maximum impedance change was determined by measuring the vertical height between the minimum point of the trace after addition of reagent and the maximum height, in chart paper units. This figure could then be converted into a resistance (Ohms) by referring to the calibration response (Fig. 2.2c).

Comments

Checks were made for spontaneous aggregation by stirring blood at 37°C for 15 minutes without the addition of any reagents. Samples were considered to show spontaneous aggregation if a response of 10 Ohms or more was obtained. Undiluted blood was tested, and if spontaneous aggregation was obtained, the process was repeated with various dilutions of blood in saline or plasma as appropriate.

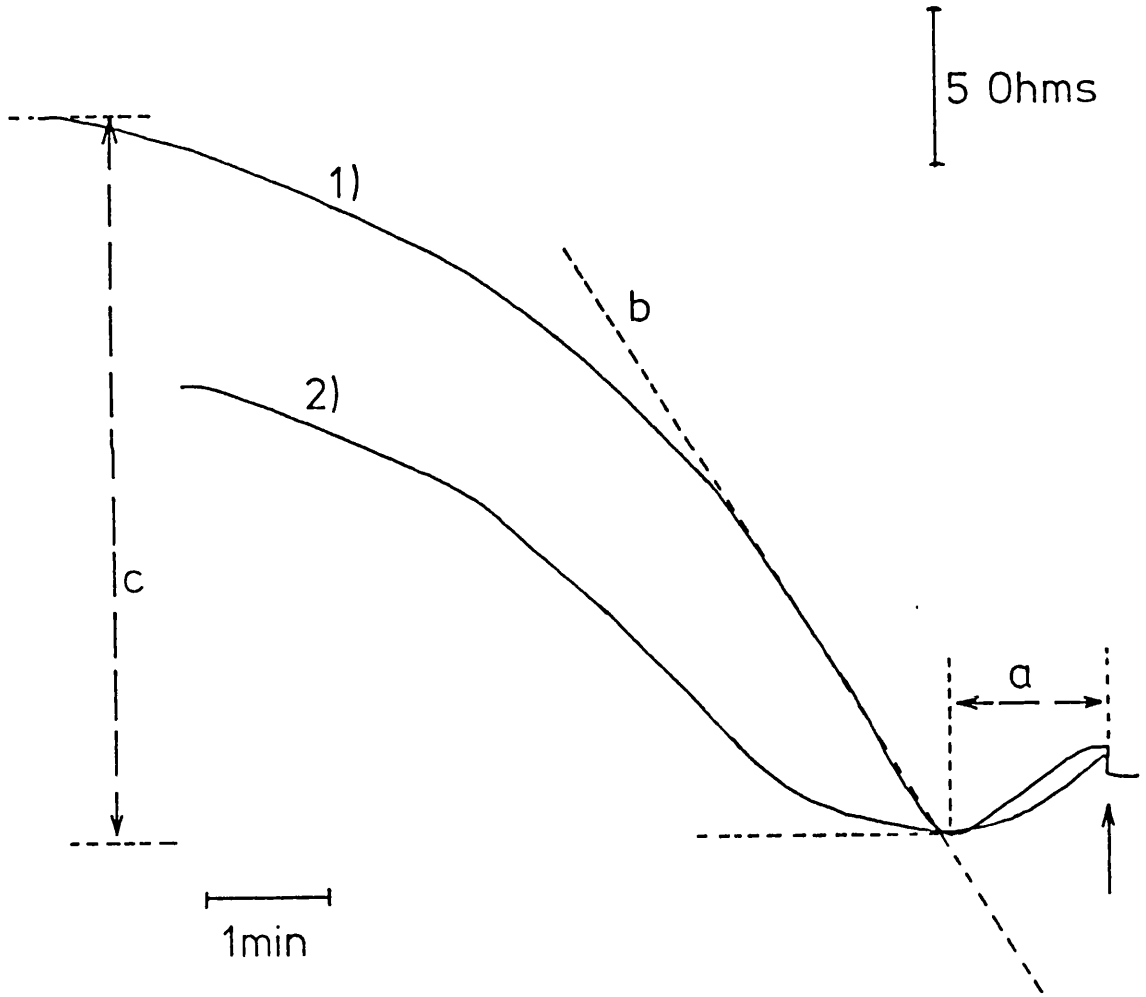

Fig. 2.2. - The measurement of platelet impedance aggregation responses in citrated whole blood. a) lag period; b) angle of initial slope; c) maximum impedance or amplitude; 1) 5ug/ml collagen; 2) 25uM ADP.

Samples with a high sedimentation rate would sometimes mimic the effects of spontaneous aggregation, particularly in undiluted blood, but this could be checked by mixing the blood manually and replacing the cuvette to see whether the impedance value had returned to baseline.

The instrument was recalibrated for each new blood sample or if there was any change in the dilution of blood used.

If the response was weak, the gain was sometimes increased prior to the aggregation response to give better accuracy (eg. 5 Ohms set to 20 chart divisions). When the gain was altered, the results had to be corrected appropriately, to be able to compare them with results at other gain settings. For the extent of aggregation, this was straightforward, but for the angle of initial slope measurement, the expression: Tan A = a/c was used, where A = angle of response slope, a = gain setting, and c = length of base (adjacent side of triangle). Thus for the measured response, "c" can be determined mathematically, and inserted into the equation: $c = a'/\text{Tan } A'$, where a' is the normal gain setting, and A' is the corrected angle to be determined.

After completion of aggregation, the electrodes were cleaned scrupulously with isotonic saline and a camel hair brush, to remove all aggregated cells.

2.3.7 SIMULTANEOUS MEASUREMENT OF AGGREGATION AND ATP RELEASE

Reagents

Chronolume (Chronolog Corporation, Philadelphia)

-Reconstituted with 1.25ml distilled water and kept on ice.

ATP Standard- 100uM, stored on ice, aliquoted at -70°C.

Equipment

Model 560 whole blood Lumiaggregometer (Chronolog Corp.)

Method for PRP

The aggregometer was set up for microvolume samples, and the heating block allowed to reach 37°C. A cuvette containing 350ul of platelet poor plasma was placed in the PPP blank hole. 250ul of platelet rich plasma (diluted in autologous platelet poor plasma to give a platelet count of $200-400*10^9/l$) was delivered into a series of aggregometer cuvettes containing stirbars. The cuvettes were placed in the aggregometer heating block, and 20ul of Chronolume reagent was added just before use. After bringing the cuvette to 37°C, and establishing a steady baseline on the chart recorder, 30ul of aggregating reagent was added, and the aggregation and luminescence responses were recorded. The method was standardised using a fresh cuvette of PRP, and adding Chronolume followed by 5ul of ATP standard. The luminescence gain control was initially set at x0.1, but was adjusted according to the nucleotide content and release, as well as the activity of the Chronolume reagent.

Method for Whole Blood

The aggregometer was prepared for use with large (9*44mm) cuvettes, and warmed to 37°C. 100ul of Chronolume reagent was added to the citrated whole blood at the time of dilution with saline, to give a final haematocrit of 0.3 l/l. The aggregometer controls were adjusted for calibration (section 2.3.6 above). The luminescence gain control was initially set at x0.1, but adjusted according to the observed luminescence. 25ul of ATP standard (100uM) was added to a fresh cuvette of blood and Chronolume, and the luminescence change recorded, in order to calibrate the system.

2.3.8 PLATELET WASHING METHOD

Reagents

Histopaque-1077 (Sigma Chemical Co. Ltd.).

ACD Anticoagulant - 25g Tri-sodium citrate Dihydrate, 15g Citric Acid, 20g D-Glucose, in 1L distilled water, adjust pH to 4.5. Store at 4°C.

Calcium Free Tyrode's Buffer - 8.0g NaCl, 0.2g KCl, 0.065g $NaH_2.PO_4.2H_2O$, 0.415g $MgCl_2.6H_2O$, 1.0g $NaHCO_3$, 1.0g D-Glucose, dissolved in 900ml distilled water, pH to 6.5 make to 1L with distilled water.

Modified Calcium Free Tyrode's - 0.2g Bovine Serum Albumin (Fraction V, essentially fatty acid free) and 10mg Apyrase (Sigma Chemical Co. Ltd) added to 100ml buffer.

Sample Buffer - 50mg Apyrase in 10ml Calcium free Tyrode's Buffer.

Sample collection

Blood was into ACD (1.7 ml ACD : 8.3 ml blood) at room temperature (18-20°C), and PRP was prepared by centrifugation at 170g for 10 minutes. 200ul of sample buffer was mixed with 10ml of PRP.

Method

A column of Sepharose 2B was equilibrated with Calcium free Tyrode's buffer containing glucose/BSA/Apyrase at room temperature. 5-10ml of PRP in sample buffer was carefully applied to the surface of the column, and the PRP was washed down the column with modified Calcium free Tyrode's buffer. 1-2ml fractions were collected into 75*13mm plastic tubes, until the platelet peak had eluted (visualised by a white 'milky' colouration of the column effluent).

Platelet counts were performed on the fractions with the highest density, and the fractions with counts exceeding $200*10^9/L$ (or peak counts if lower than this range) were pooled. The column was thoroughly washed in buffer without additives (at least 3 column volumes), so that the yellow (albumin) colour had eluted, and stored at 4°C until next use. If the platelet count was too low a concentration step was used as follows:- 2ml of Histopaque was placed into the bottom of a plastic 12ml centrifuge tube, and 10ml of gel filtered platelets or PRP were gently layered on top, before centrifugation 2000g for 10 minutes. The supernatant was carefully removed, avoiding the band of platelets at the interface of the Histopaque and buffer. The platelet band was then separately removed with a plastic fine-tipped

pipette and placed in a clean plastic tube. The platelets were counted, and diluted as appropriate in buffer (composition will vary depending on use of the washed platelets).

2.3.9 BETA THROMBOGLOBULIN (B-TG)

Principle

This radioimmunoassay technique is based on the competition between unlabelled B-TG and known amounts of ^{125}I-labelled B-TG for binding sites on B-TG specific antibody. The amount of bound ^{125}I-labelled B-TG is proportional to the amount of B-TG in the plasma samples.

Test samples

Blood was collected by venepuncture with minimal stasis, and the first 5ml discarded, or used for other test samples. 2.5ml of blood was rapidly added to a chilled sample collection tube in crushed ice, and gently mixed by inverting 2-3 times, and replaced in crushed ice.

Reagents

Sample collection tubes - 75*12mm polystyrene tubes containing:- 140ul 0.336M dipotassium EDTA (135mg/ml in sterile water), 36ul Theophylline (12.5mg/ml in absolute ethanol), 10ul Prostaglandin E_1 (25ug/ml in saline).

All other reagents were obtained in a kit from Amersham Ltd (The sample collection tubes provided with the kit were not used, as they were considered unsuitable due to inadequate platelet inhibitors in the anticoagulant). The kit contained sufficient reagents for the determination of

B-TG in 19 test samples in duplicate. The following reagents were reconstituted just before use:- ^{125}I-labelled B-TG anti-B-TG serum, Ammonium Sulphate solution, Reference Standards: 10, 20, 50, 100, and 225 ng/ml.

Preparation of test plasmas and reagents

The blood sample was left on ice for at least 15 minutes, but not longer than 2 hours, before centrifugation at 2000g and 4°C for 30 minutes, to obtain platelet poor plasma. The top third of the plasma was removed, and the next third pipetted into a clean polypropylene microcentrifuge tube, and frozen at -20°C until the time of assay.

Each standard was reconstituted with 500ul distilled water, and the antiserum and labelled B-TG with 10ml distilled water each.

Method

Starting with the lowest concentration, 50ul of each standard in duplicate, was pipetted into the first 10 microcentrifuge tubes. 50ul of each test sample in duplicate were pipetted into subsequent microcentrifuge tubes. 200ul ^{125}I-labelled B-TG was added to each tube, followed by 200ul of anti-B-TG serum. All tubes were mixed well on a vortex mixer, and incubated at room temperature for 1 hour. 500ul of ammonium sulphate solution was then added to each tube, and they were vortex mixed within 8 minutes, and then centrifuged within the next 10 minutes, in a microcentrifuge at 1500g, for 15 minutes. Each tube was inverted over a tissue, and tapped gently to remove any fluid, and then

allowed to drain onto the tissue paper for 5 minutes. Any remaining fluid was removed from the rim of the tube, and all tubes were placed in a gamma counter and counted for 60 seconds, or until 10000 counts were achieved.

A graph of gamma count against the B-TG concentration of the standards was plotted and a smooth curve drawn. Test sample values were abstracted from the curve using their count values. Reference Values were determined in 20 normal subjects and found to be <50 ng/ml.

2.3.10 PLATELET FACTOR 4 (PF4)

Principle

This radioimmunoassay technique is based on the competition between unlabelled PF4 and known amounts of ^{125}I-labelled PF4 for binding sites on PF4 specific antibody. The amount of bound ^{125}I-labelled PF4 is proportional to the amount of PF4 in the plasma samples.

Test samples

Blood was collected by venepuncture with minimal stasis, and the first 5ml discarded, or used for other test samples. 2.5ml blood was rapidly added to a chilled sample collection tube in crushed ice, and gently mixed by inverting 2-3 times, and replaced in crushed ice.

Reagents

Sample collection tubes - 75*12mm polystyrene tubes containing:- 140ul dipotassium EDTA 0.336M (135mg/ml in sterile water), 36ul Theophylline (12.5mg/ml in absolute

ethanol), 10ul Prostaglandin E_1 (25ug/ml in saline). Platelet Factor 4 Radioimmunoassay Kit (Abbot Laboratories Ltd, Wokingham, Berks). The kit was sufficient for 50 test samples in duplicate, and the following reagents were included:- ^{125}I-labelled PF4, PF4 Antiserum, PF4 Standards: 10, 30, 50, and 100 ng/ml, Dilution Buffer, 73% Saturated Ammonium Sulphate.

Preparation of test plasmas and reagents

Blood samples were left on ice for at least 15 minutes, but not longer than 2 hours, before centrifugation at 2000g and 4°C for 30 minutes. The top third of the plasma was removed, and the next third pipetted into a clean polypropylene microcentrifuge tube; and stored at -20°C until the assay. All reagents were brought to room temperature.

Method

The tubes provided in the kit were numbered, and tubes 1-3 were used for total counts (TC), 4-5 for non specific binding (NSB), 6-15 for the standards in duplicate, and 16-n for test samples. 50ul of dilution buffer was added to tubes 4 and 5, 50ul of each standard in duplicate to tubes 6-15, using dilution buffer as the 0ng/ml standard, and 50ul of each test sample in duplicate to the appropriate remaining tubes.

250ul of ^{125}I-labelled PF4 was added to every tube, and tubes 1-3 were then capped and placed to one side. 250ul of dilution buffer was added to tubes 4 and 5, and 250ul anti-PF4 serum to tubes 6-n. The tubes were vortex mixed, except those for TC, and all of the tubes were then

incubated at room temperature for 2 hours. 1ml of ammonium sulphate solution was added to each tube except the TC tubes, and they were Mixed, and allowed to stand for at least 10 minutes (but not longer than 60 minutes). All of the tubes except those for TC were centrifuged at 1500g for 20 minutes, and the supernatant was carefully decanted from each tube and the rim of each tube blotted with a tissue.

Gamma counts were performed on all tubes, by counting for 60 seconds or 10000 counts.

Calculation

The percentage binding was calculated for each tube as follows:-

$$\text{Percentage Binding} = \frac{\text{cpm test or standard}}{\text{cpm of TC tube}} * 100$$

The percentage binding for each standard was plotted against its concentration to give a smooth curve. The PF4 values for the test samples were abstracted from this curve using their percentage binding values. Reference values were established in 20 normal subjects, and found to be <10 ng/ml.

2.3.11 THROMBOXANE B_2 ASSAY

Principle

The assay of thromboxane B_2 (TXB_2) is performed by a radio-immunoassay technique. The method depends on competition between TXB_2 (from the sample) and a ^3H-labelled TXB_2 reagent for a limited number of binding sites on a TXB_2 specific antibody. The TXB_2:antibody complexes are separated, and the radioactivity in the supernatant

measured. The amount of radioactivity remaining in the supernatant is dependent on the amount of ^3H-TXB_2 displaced from the antibody by unlabelled TXB_2 in the test sample. The measurement of free, unbound ^3H-TXB_2 in the presence of a series of TXB_2 standards allows the concentration of TXB_2 in test samples to be interpolated from a standard curve.

Test samples

Serum Thromboxane B_2 - 1ml of fresh blood was collected into a 75*10mm glass tube and incubated at 37°C for 1 hour. Serum was collected after centrifugation at 1500g for 5 minutes, and stored at -20°C until required. Thromboxane B_2 Release - 270ul of platelet rich plasma (platelets $200*10^9$/L) were stirred in an aggregometer cuvette at 37°C and 30ul of either 200ug/ml collagen or 1.5mM sodium arachidonate were added. Aggregation was allowed to occur for 4 minutes, and then 30ul of 100mM EDTA was added. The platelets were pelleted by centrifugation at 1500g for 10 minutes, and the supernatant stored at -20°C until assayed.

Reagents

PBS pH 7.3 - 0.42g $NaH_2PO_4.2H_2O$, 1.75g Na_2HPO_4 (anhydrous), 0.9g NaCl in 200 ml distilled water.

TXB_2 Antisera (Universal Biologicals Ltd, London).

^3H-TXB_2 Tracer 1uCi vials (New England Nuclear, Boston).

TXB_2 Standard (Universal Biologicals Ltd, or New England Nuclear).

Optiphase 'safe' Scintillant (LKB Ltd, Croydon, Surrey).

Dextran T2000 (Pharmacia Ltd, Milton Keynes, Bucks).

Activated Charcoal (BDH Ltd, Dagenham, Essex).

Bovine Serum Albumin (Sigma Chem Co, Poole, Dorset).

Preparation of reagents and samples

The samples were normally diluted 1/4 and 1/8 in PBS; and standards were diluted to give 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, and 0.039ng/ml solutions.
Charcoal mix - the following reagents were mixed immediately before use:- 120mg Activated Charcoal, 10mg Dextran T2000, 10mg Bovine Serum Albumin, and Add 20ml of PBS and keep on ice.

Method

A series of polystyrene tubes were labelled 1-n, and 250ul PBS was added to tubes 1,2 for the top count (TC), 100ul PBS to tubes 3,4 for non specific binding (NSB), and 50ul PBS to tubes 5,6 for blank (BL). 50ul amounts of each standard concentration were added in duplicate to further numbered pairs of tubes, and 50ul of each test sample dilution in duplicate was added to subsequent pairs of tubes.

50ul of ^{3}H-labelled TXB_2 tracer was then added to each tube, followed by 50ul TXB_2 Antibody to all tubes except tubes 1 - 4. The contents of each tube were mixed and incubated overnight at 4°C.

150ul of Charcoal Mix were added to all tubes, excluding tubes 1 and 2 (TC). The contents were mixed well, and centrifuged at 2000g and 4°C for 15 minutes. 200ul of supernatant was removed from each tube and added to 5ml of Optiphase scintillation fluid. The samples were then counted for 2 minutes or 10000 counts on a Beta counter, and cpm obtained.

Calculation

Percentage binding was calculated from the following equation:-

$$\text{Percentage Binding} = \frac{\text{sample cpm} * \text{TC cpm}}{\text{NSB}}$$

A graph of percentage binding against dilution of standard was then plotted and the test sample values abstracted.

The blank count was used as a measure of maximum binding of the antibody to the tracer in the absence of any other antigen, and sensitivity was confirmed if this was between 35% and 45%. Test samples which gave values outside the linear portion of the standard curve were repeated after appropriate adjustment of the sample dilution.

Normal Values

Normal values obtained in healthy normal laboratory staff were as follows:- serum thromboxane B_2 215-544ng/ml (0.89-1.87ng/10^6 Platelets); Thromboxane B_2 release to collagen in PRP 362-722ng/ml (1.60-2.68ng/10^6/L).

2.3.12 PLATELET ADHESION STUDIES

(Turitto & Baumgartner, 1983)

Rabbits were killed by intravenous injection of sodium pentobarbitone (0.5 ml/kg). The aorta was dissected free from below the renal arteries to the bifurcation, transected at these points, and immediately washed in oxygenated Krebs's ringer solution within which it was subsequently maintained. A narrow gauge catheter was then passed gently through the lumen of the vessel, and where its tip emerged a strong tie was placed around the vessel and catheter. The

free end of the vessel was gently gripped by the operator with forceps and the catheter withdrawn thus everting the vessel. The vessel was then cut into 3 uniform pieces, and stored in oxygenated Krebs's ringer.

With forceps, a piece of aorta was carefully mounted onto the central spear of a Baumgartner chamber. The endothelial surface was at all times kept exposed to oxygenated Krebs's ringer solution. Blood was equilibrated for 10 minutes at 37°C prior to being circulated over the vessel at 150ml/min for 10 minutes, with oxygenation during the circulation. Experiments were repeated with further pieces of the aorta.

The aortic samples were briefly rinsed after each run in Krebs's ringer solution, and then fixed for 12 hours in 2.5% (w/v) glutaraldehyde in cacodylate buffer at pH 7.2. The samples were then washed in buffer and post fixed in 1% (w/v) osmium tetroxide (buffered as before) for 30 minutes. Following a water wash the specimens were dehydrated through a graded series of alcohol into tri-chloro, tri-fluoroethane from which they were critically point dried. After mounting on stubs and coating with gold-palladium, the specimens were examined in a Jeol JSM 35 scanning electron microscope.

2.4 OTHER METHODS

2.4.1 C-REACTIVE PROTEIN ASSAY (ELISA)

Assays for C-Reactive protein (CRP) were carried out by the ELISA technique described above for von Willebrand factor, but using anti-human CRP serum and peroxidase conjugate (Dako Ltd, High Wycombe, Bucks). Aliquots of a plasma calibrated against CRP standard serum (Behring Ltd, Hounslow, Middlesex) were used as a routine standard, at dilutions from 1/8-1/512, and test samples were diluted in the range 1/16-1/64). Coating antisera was diluted 1/2000, and conjugate 1/250, in buffer.

2.4.2 NEUTROPHIL WASHING

Reagents

ACD Anticoagulant - 25g Tri-sodium citrate Dihydrate, 15g Citric Acid, 20g D-Glucose, 1L distilled water, pH 4.5, stored at 4°C.

10% Dextran T500 (Pharmacia) in isotonic saline.

Ficol-Histopaque (Sigma Chemical Co. Ltd).

Mono-Poly Resolving Medium (Flow Laboratories Ltd)

Calcium Free Tyrode's Buffer - 8.0g NaCl, 0.2g KCl, 0.065g $NaH_2.PO_4.2H_2O$, 0.415g $MgCl_2.6H_2O$, 1.0g $NaHCO_3$, 1.0g D-Glucose, dissolved in 900ml distilled water, pH adjusted to 6.5, and made to 1L with distilled water.

Modified Calcium Free Tyrode's - 0.2g Bovine Serum Albumin (Fraction V, essentially fatty acid free), and 10mg Apyrase (Sigma Chemical Co. Ltd) added to 100ml buffer.

Sample collection

Blood was collected into ACD (1.7 ml ACD : 8.3 ml blood) at room temperature (18-20°C).

Method

Blood was twice centrifuged at 170g for 10 minutes and each lot of PRP removed. The remaining blood was diluted 1/2 with buffer, and 1ml of Dextran was added to every 9ml of blood. The tubes were then mixed and left to stand undisturbed for 1 minute for every 1ml of solution, at room temperature. The supernatant leucocyte rich plasma was then collected into separate 20ml plastic universal tubes. A density centrifugation gradient was then prepared by layering 2ml of mono-poly resolving medium followed by 1ml Ficol-Histopaque and up to 10ml leucocyte rich plasma into a plastic centrifuge tube, taking care not to mix the layers. The tubes were then centrifuged at 800g for 10 minutes at room temperature. and the supernatant (which contained plasma and platelets) was discarded. The next band (upper) containing mononuclear cells was removed,as was the clear medium separating the two bands. The lower band (polymorphonuclear cells) was then collected and saved, and the remaining red cells left pelleted at the bottom of the tube and discarded. The polymorph band was collected directly into 8-10ml of modified Calcium free Tyrode's buffer and centrifuged at 1000g for 10 minutes at ambient temperature, and the supernatant removed. The remaining red cells were removed by adding 1ml of distilled water to the cell pellet and resuspending for 20 seconds, before adding 2x concentrated buffer. The cells were centrifuged

immediately for 10 minutes at 1000g. The supernatant was discarded, and the pellet of white cells resuspended in buffer. The cells were counted and diluted to working concentrations (typically $10*10^9$/L for aggregation).

2.4.3 NEUTROPHIL AGGREGATION (TURBIDOMETRIC TECHNIQUE)

Reagents

Calcium Free Tyrode's Buffer - 8.0g NaCl, 0.2g KCl, 0.065g $NaH_2.PO_4.2H_2O$, 0.415g $MgCl_2.6H_2O$, 1.0g $NaHCO_3$, 1.0g D-Glucose, dissolved in 900ml distilled water, pH adjusted to 6.5, and made to 1L with distilled water.

Modified Calcium Free Tyrode's - 0.2g Bovine Serum Albumin (Fraction V, essentially fatty acid free) and 10mg Apyrase (Sigma Chemical Co. Ltd) added to 100ml buffer.

10mM FMLP - Formyl-Met-Leu-Phe peptide (Sigma) 5mg dissolved in 1.143ml DMSO and stored at -20°C. For working solutions FMLP was further diluted in saline to give 1mM, 100uM, and 10uM solutions.

100ug/ml PMA - Phorbol Myristate Acetate (Sigma) 1mg dissolved in 1ml DMSO (100ug/ml), and stored at -20°C. Working concentrations of 10, 1, and 0.1ug/ml were prepared in saline. Calimycin - Calcium Ionophore A23187 (Sigma) 5mg dissolved in 10ml DMSO to give a 500ug/ml solution, and stored at -20°C. Working solutions were prepared by dilution in saline to give 100 and 50ug/ml.

Method

An aggregometer blank was prepared by mixing 60ul of washed neutrophils and 240ul buffer in an aggregometer cuvette with stirbar. The instrument was calibrated in a

similar way to PRP aggregation, using 270ul of washed neutrophils to set 10% transmission, and the blank to set 100%. When a stable baseline had been obtained, 3ul of 200mM Calcium chloride, was added, and equilibrated for 2 minutes, before adding 30ul of aggregating reagent (final concentration in the cuvette of: 1-100uM FMLP; 0.01-0.1ug/ml PMA; or 5-10 ug/ml Calimycin). The response was monitored until maximum aggregation was obtained (up to 10 minutes).

2.4.4 NEUTROPHIL ELASTASE:ALPHA-1-ANTITRYPSIN COMPLEX ASSAY PRINCIPLE

Microtitre plates are coated with antisera against Neutrophil Elastase (NE), and dilutions of standard and test plasmas are added. During the incubation period, NE and its complexes with alpha-1-antitrypsin (A-1-AT) bind to the coating antibody. The plates are washed in buffer, and a peroxidase conjugated antibody directed against A-1-AT is added. After a final wash, a substrate for peroxidase is added which generates a yellow colour on reaction. The greater the colour, the more NE:A-1-AT is bound to the plate and therefore the higher the plasma level of complex.

NE:A-1-AT is the main inhibitor complex formed by NE under normal conditions, and free elastase does not usually circulate in blood, however, in certain pathological conditions where the inhibitors of elastase are depleted, free neutrophil elastase may interfere in the assay. The technique may be used as an estimate of neutrophil release.

Reagents

Buffer A - PBS Coating Buffer pH 7.2 - 0.345g $NaH_2PO_4.H_2O$ (0.0025M), 2.680g $Na_2HPO_4.12H_2O$ (0.0075M), 8.474g NaCl (0.145M), in distilled water.

Buffer B - Washing and Dilution Buffer - 1L PBS (Buffer A), 20.75g NaCl, and 1ml Tween 20.

Blocking Buffer - 1% BSA in Buffer A.

Sheep antiserum to human Neutrophil Elastase (anti-NE) and sheep antiserum to human Alpha-1-Antitrypsin conjugated to Horse radish peroxidase (HRP:anti-A-1-AT) (Serotec Ltd, Kidlington, Oxford).

1,2-phenylenediamine dihydrochloride (orthophenylenediamine, OPD) (Sigma Chemical Co Ltd, Poole, Dorset).

0.1M Citrate Phosphate buffer pH 5.0 - 7.3g Citric Acid.H_2O (0.0347M), 23.87g $Na_2HPO_4.12H_2O$ (0.0667M) in 1L distilled water.

3% Hydrogen Peroxide

2M Sulphuric acid

Equipment

Microtitre Plate Reader - Dynatech MR700 (Dynatech Laboratories Ltd, Billingshurst, Sussex).

Microtitre plates (Gibco Ltd, Uxbridge, Middx).

Multichannel pipettes (Labsystems Ltd, Uxbridge, Middx.).

Standard

An 'in house' standard was prepared by adding purified neutrophil elastase (NE) to an excess of normal plasma so that NE:A-1-AT complexes were formed. This preparation was calibrated against a commercial standard (Merck, Frankfurt, FRG).

Preparation of standard and control samples

Doubling dilutions from 1/50 to 1/3200 were made of standard, and 1/10, 1/50, 1/100 and 1/200 dilutions of test samples in buffer B.

The substrate was prepared by mixing the following reagents just before use:- 8mg OPD, 12ml Citrate Phosphate Buffer, and 5ul 3% H_2O_2.

Method

180ul of 1/2000 anti-human NE (diluted in Buffer A) was placed in each well of a microtitre plate, which was covered and placed at 4°C overnight. The wells were then emptied, and the plate washed 3 times for 3 minutes each with 200ul buffer B. After emptying the wells, 180ul of Blocking buffer was added to each well and the plate incubated at 4°C for 1 hour. The wells were then washed once with 200ul of buffer B, and 180ul of sample or standard dilution were placed in duplicate wells, except for wells A1/B1 which received buffer B only, and acted as a blank. The plate was then covered and left at room temperature for 2 hours. The plate was emptied and washed 4 times with 200 ul buffer B, before adding 180ul of HRP:anti-A-1-AT (diluted 1/2000 in buffer B) to each well. The plate was covered and incubated for 2 hours at room temperature, before a further washing cycle of 3 times for 3 minutes each with 200 ul buffer B. 180ul of fresh substrate was added to the emptied wells, and the plate placed in the dark until optimum colour development had occurred. The peroxidase reaction was stopped by adding 50ul of sulphuric acid to each well, and the absorbance at 490nm was measured on a microtitre plate reader.

Calculation

A graph of log concentration against log absorbance was plotted for the standard dilutions, and the test values were read from the standard curve.

The dilutions of test samples were adjusted for samples with very high levels of complexes, or in the presence of high concentrations of free elastase.

A reference range was established in 20 healthy normal subjects, and was as follows:- 8.9 - 196.5ug/l. There was no difference between males and females, and specimens were stable for long periods at -70°C. The assay was insensitive to pancreatic elastase.

2.4.5 ERYTHROCYTE WASHING

Reagents

Calcium Free Tyrode's Buffer - 8.0g NaCl, 0.2g KCl, 0.065g $NaH_2.PO_4.2H_2O$, 0.415g $MgCl_2.6H_2O$, 1.0g $NaHCO_3$, 1.0g D-Glucose, dissolved in 900ml distilled water and adjusted to pH 6.5, then made to 1l with distilled water.

Method

Citrated blood was centrifuged at 2000g for 15 minutes, and the plasma was discarded. The buffy coat was then removed with a plastic pasteur pipette, at the expense of some red cells. The remaining blood was resuspended to the original volume with buffer, and centrifuged again. The supernatant and buffy coat were again removed, the cells resuspended in buffer, and the process repeated a further two times. After final resuspension (usually to about 75% of

the original volume) in buffer, a full blood count was performed to check the haematocrit and depletion of platelets and white cells.

2.4.6 NICOTINE AND COTININE ASSAYS

Serum nicotine and cotinine levels were measured by gas-liquid chromatography (Feyerabend & Russel, 1979; Feyerabend & Russel, 1980).

CHAPTER 3

PLATELET AGGREGATION IN NORMAL WHOLE BLOOD

3.1 INTRODUCTION

The most suitable technique for studying platelets in whole blood appeared to be impedance aggregation. This allowed tests to be performed on an independent instrument which was mobile and could be taken to the clinic or bedside, and gave a dynamic measurement without the need to subsample. The technology allows the various formed elements of blood and plasma factors to influence platelet function. The influence of a variety of agonists and antagonists could also be studied.

A great deal of the early work on platelets in whole blood was carried out using blood from laboratory animals. A thorough evaluation was therefore necessary along with the optimization and standardization of measurements, before the technique was suitable for clinical laboratory studies. The variables influencing impedance aggregation in citrated normal whole human blood were studied. The mechanism of detection of platelet aggregates by the impedance electrode was also investigated using a variety of agonists and antagonists, and comparisons were made with other methods of measuring platelet aggregation.

3.2 PHYSICAL VARIABLES

The maintenance of a constant temperature was essential in impedance aggregation, since impedance is a function of temperature. Failure to prewarm the cuvettes of blood resulted in a steady increase in impedance, which mimicked spontaneous

aggregation. Approximately 5 minutes were required to adequately warm the cuvettes and blood to 37°C, but this varied according to ambient temperature and the time since blood collection. Agonists were added after a stable baseline had been obtained. Separate aliquots of blood were tested for spontaneous aggregation by stirring at 37°C for up to 15 minutes; no spontaneous responses were detected in any healthy normal individual studied.

The influence of the stirring speed was studied by varying the control to give a range of speeds between 100 and 1200rpm, using the same blood donation and dose of aggregating reagent. In the absence of a stir bar, impedance changes were not observed, at low stirring speeds poor aggregation responses were obtained, while optimal aggregation occurred at about 600rpm, and higher speeds caused a gradual reduction in the extent of impedance change (Fig. 3.1).

The results obtained with different types of aggregometer cuvette were compared, and glass cuvettes were found to cause a steeper slope of aggregation, and higher maximum impedance value than polystyrene cuvettes (Table 3.1), although statistical significance was only achieved for aggregation slope. Similar results were obtained when ADP was used as agonist. The method showed better reproducibility in polystyrene cuvettes, giving a within run coefficient of variation of 9% with collagen, compared to 15% using glass cuvettes.

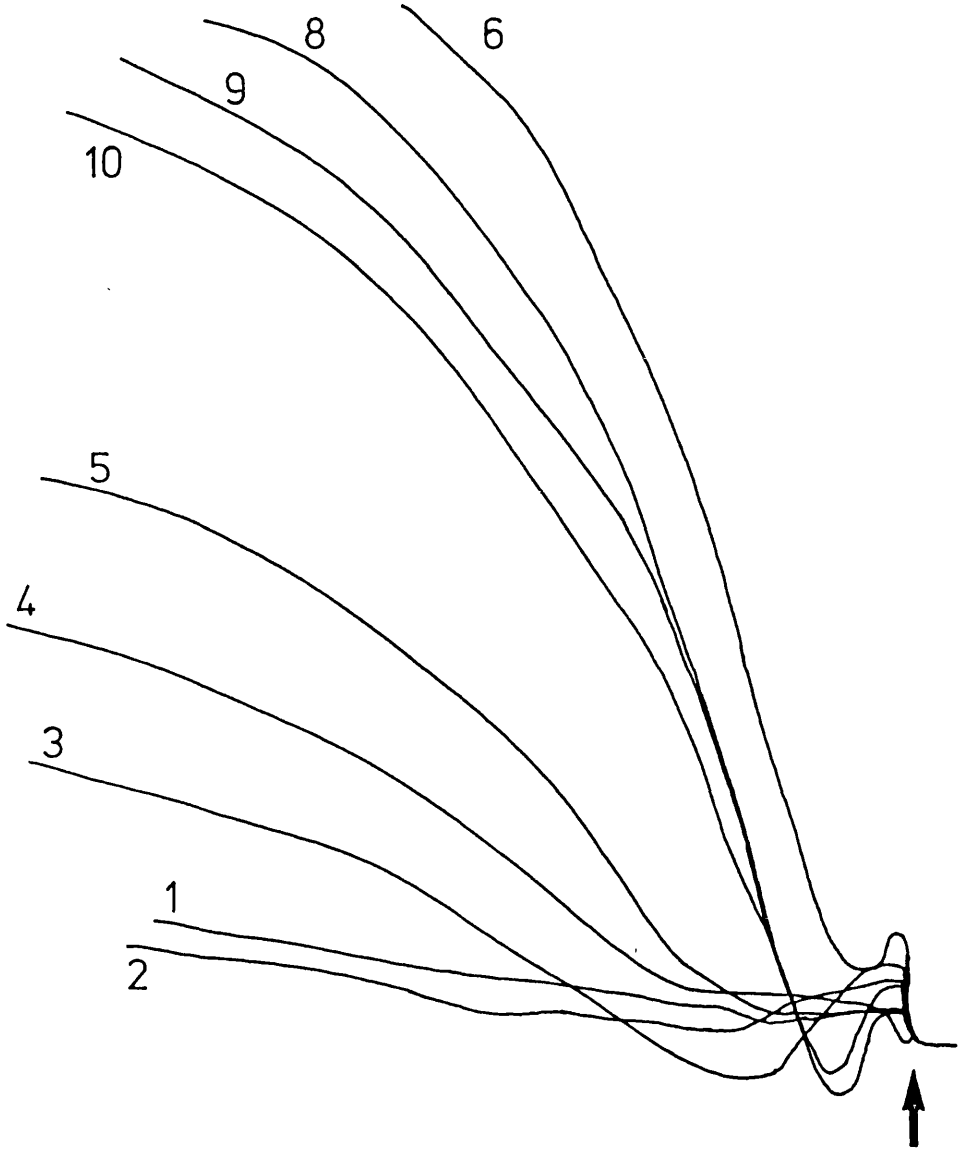

Fig. 3.1. The Influence of Stirrer Speed on Whole Blood Impedance Responses.
Tests were performed on blood with a Haematocrit of 0.3l/l and platelet count of $150*10^9$/l, using 5ug/ml collagen. The numbers indicate the stirrer speed x100 (rpm).

	P LAG	G LAG	P SLOPE	G SLOPE	P OHMS	G OHMS
Mean	1.09	1.09	50.3	58.4	14.8	16.7
SD	0.39	9.29	13.1	12.9	5.8	4.5
t-test	NS		$p<0.01$		$p<0.1$	

Table 3.1. Effect of Cuvette Type. Whole blood impedance aggregation to collagen was studied with normal blood in either polystyrene (P) or glass (G) cuvettes (n=11).

3.3 INFLUENCE OF HAEMATOCRIT

The effect of changes in the haematocrit was investigated in several ways:- a) Various amounts of whole citrated blood from normals and polycythaemic patients were diluted with platelet poor plasma or isotonic saline before aggregation. b) Platelet rich plasma was mixed with autologous washed red cells, in varying proportions. c) Whole blood from a severe thrombocytopaenic patient (platelets $<5*10^9/l$) was mixed with normal platelet rich plasma.

Manipulation of the haematocrit influenced the aggregation response (Fig. 3.2) in all three means of study. High haematocrits caused erratic tracings which were difficult to quantify, and often very weak aggregation traces were obtained, resulting in poor precision. Large dilutions of the blood with saline to reduce the haematocrit also caused an excessive reduction in platelet count which decreased the aggregation response. Dilution to a haematocrit of 0.3 l/l appeared to be optimal, giving a less erratic aggregation trace and reproducible responses, and causing a minimal change in the blood composition. Lower haematocrits showed no significant difference in aggregation response until the platelet count became limiting.

	HCt	Plats
a)	400	304
b)	350	266
c)	300	228
d)	250	190
e)	200	152
f)	100	76
g)	50	38

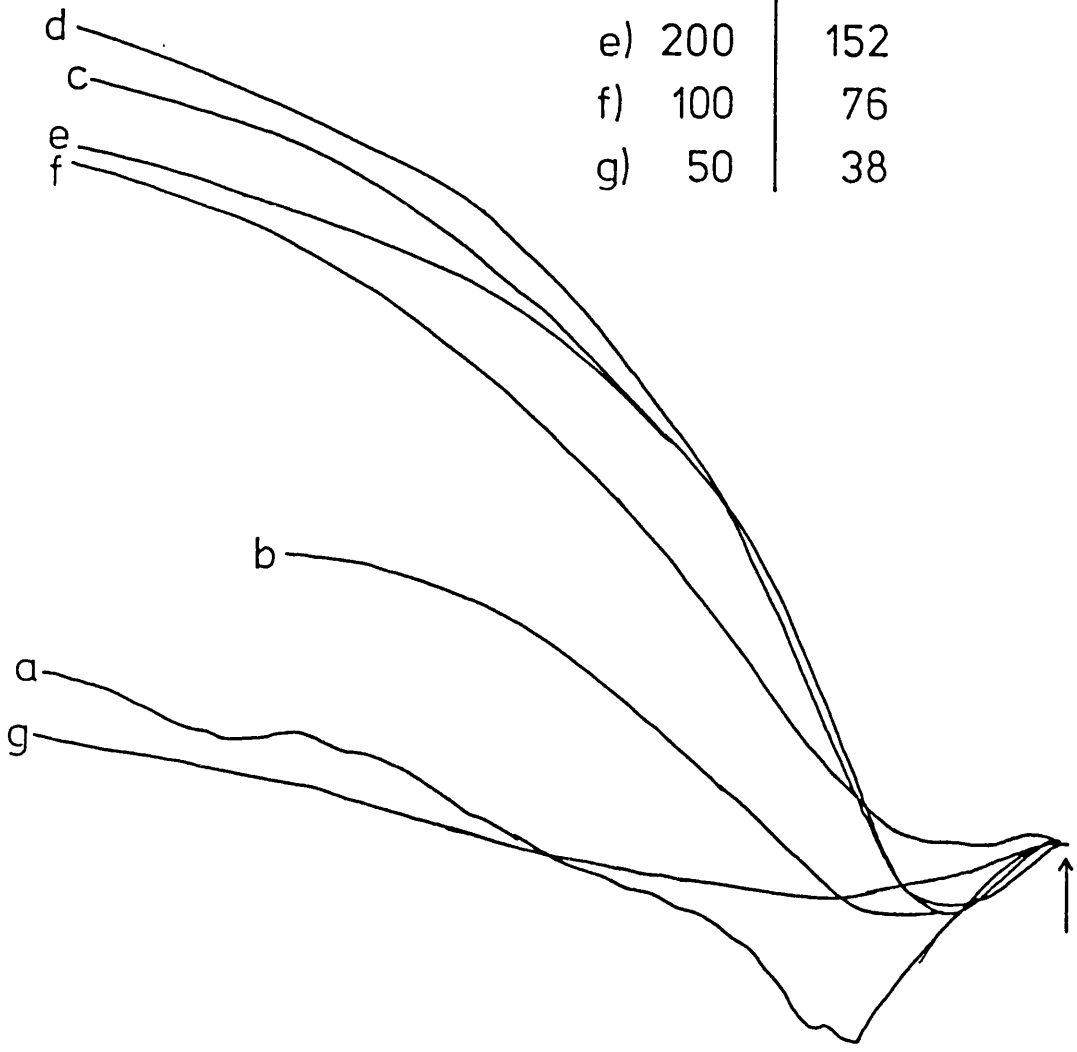

Fig. 3.2. The Influence of Haematocrit (and Platelet Count) on Whole Blood Impedance Aggregation. Citrated blood from a normal donor was diluted with isotonic saline and aggregation induced by 5ug/ml collagen.

Isotonic saline, calcium-free Tyrode's solution, or autologous plasma were all suitable diluents, but the former was usually used since it required no special preparation. Autologous plasma may be important if one is looking at plasma factors but it is undesirable to delay aggregation while the plasma is prepared by centrifugation. The use of plasma from other donors is also undesirable owing to the introduction of foreign proteins and potential immune reactions. The basal impedances of various solutions are shown in Table 3.3, saline and plasma caused a similar, small increase in impedance, and thus caused no significant loss of sensitivity.

SOLUTION	IMPEDANCE (Ohms)
Distilled Water	5
Isotonic Saline	28
PPP (normal)	21
PPP (severe anaemia)	20
PRP	20.5
Whole Blood	10.5
Blood + PPP 1:1	14.5
Blood + Saline 1:1	16.5

Table 3.3. Basal Impedance of Various Solutions.

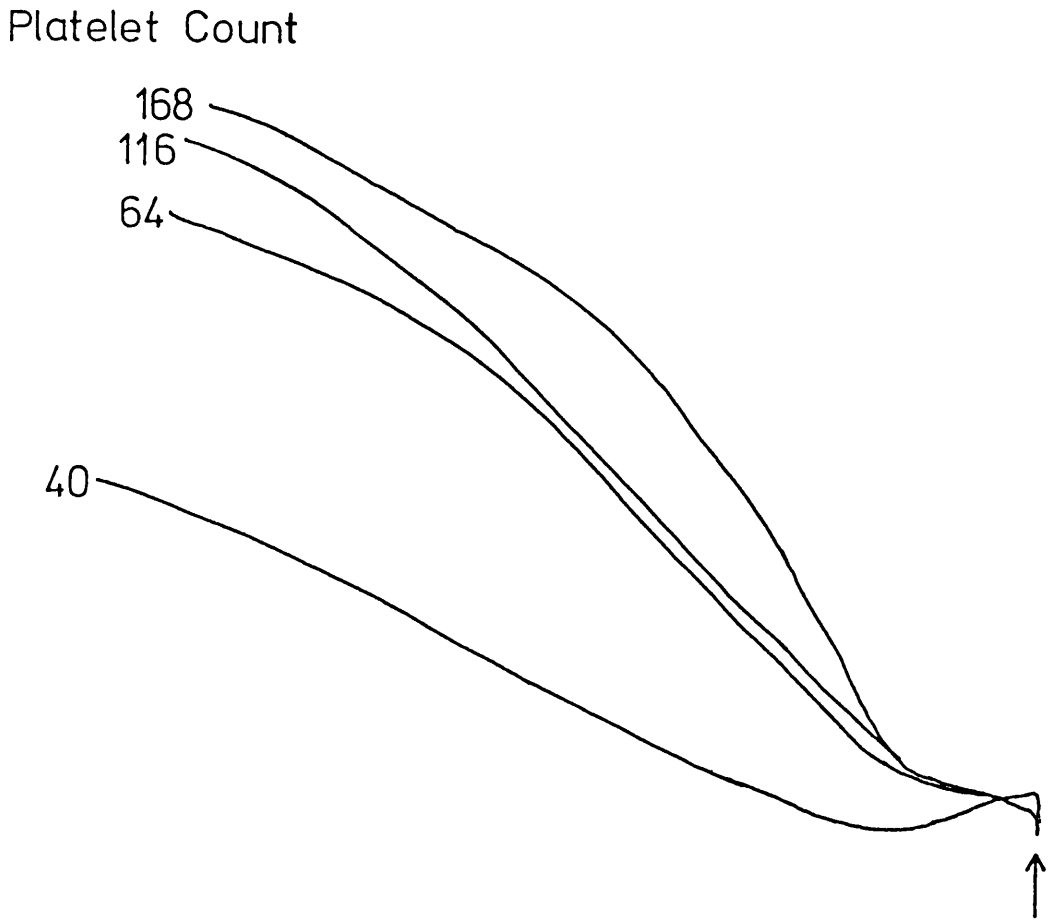

Fig. 3.3. The Influence of Platelet count on whole blood impedance aggregation. Normal PRP was mixed with citrated blood from a patient with thrombocytopaenia (platelets $<5x10^9/l$) to achieve a range of platelet counts ($*10^9/l$) at constant haematocrit. Aggregation was induced with 5ug/ml collagen.

3.4 PLATELET COUNT

The effect of platelet count was investigated by mixing platelet rich plasma with autologous washed red cells, and by mixing whole blood from a severe thrombocytopaenic patient with normal platelet rich plasma. In the latter, varying amounts of PRP from normal, ABO group compatible donors were diluted in the blood from two subjects with platelet counts less than $5*10^9/l$ (who had no detectable aggregation responses to ADP or collagen), and the final volume was corrected with PPP to a haematocrit of 0.3 l/l. Alternatively, 975ul of thrombocytopaenic blood was allowed to settle for 20 minutes and the PRP was removed and replaced by PRP from a normal donor.

Reduction of the platelet count had no significant effect on aggregation until the platelet count reached about $50*10^9/l$. Platelet counts below this value gave a progressive reduction in response (Fig. 3.3). If normal red cells were washed in buffer by centrifugation and repeated removal of the buffy coat, so that no platelets or leukocytes remained, no aggregation responses could be detected to collagen or ADP.

The influence of platelet count on the whole blood impedance response was also studied in 38 thrombocytopaenic patients (16 ITP, 11 AML, 8 other malignancy, 2 marrow aplasia, and 1 TTP). There was a good correlation between platelet count and the impedance response to collagen, in the group as a whole (platelet count $<150*10^9/l$) (Fig. 3.4, Table 3.4). A few individuals had completely absent responses, some had an excessively low platelet count ($<5*10^9/l$), but others had higher counts, and may therefore have had a coexistent

platelet functional defect. Patients with AML tended to have less aggregation at any given platelet count than the ITP group. The degree of response appeared to plateau later in the AML group than in others. When the regression and correlation data (Table 3.4) were studied at lower platelet counts, the whole group and ITP patients alone showed a good correlation and steeper slope of regression line, because the plateau effect had been removed. The regression line was much steeper for the ITP group than for the AML patients (Fig. 3.5). The data in thrombocytopaenia patients was therefore in close agreement with the in vitro experiments described above. Patients with platelet counts above $50*10^9/l$ gave aggregation responses within the reference range for healthy normal controls (with platelet counts $150-400*10^9/l$).

	ALL	**ITP**	**AML**
A)			
No.	38	16	11
SLOPE	0.35	0.24	0.42
INTERCEPT	7.55	20.5	2.2
r	0.77	0.70	0.91
p	<0.01	<0.01	<0.01
B)			
No.	33	12	10
SLOPE	0.43	0.55	0.30
INTERCEPT	5.35	10.63	1.45
r	0.67	0.72	0.70
p	<0.01	<0.01	<0.05
C)			
No.	23	9	7
SLOPE	0.68	0.53	0.34
INTERCEPT	-0.77	10.1	-0.38
r	0.72	0.65	0.53
p	<0.01	<0.05	NS

Table 3.4 Linear regression analysis and coefficient of correlation (r) between platelet count and impedance response in patients with thrombocytopaenia, at three different platelet count ranges: A) <150, B) <100, and C) <50 $*10^9/l$.

Thrombocytopaenic patients with populations of larger platelets generally had stronger aggregation responses, and the mean platelet volume correlated with the aggregation response ($r=0.6$, $p<0.01$) in ITP patients (Fig. 3.6). ITP patients had platelets with the largest volume (MPV 5.9-12.6), while AML patients showed a smaller range (MPV 6.2-8.4). When the thrombocytopaenic patients were studied by turbidometric aggregation in PRP, either no response could be detected, or the responses were all abnormal as compared with the normal reference ranges obtained at higher platelet counts, and were therefore difficult to interpret.

Patients with high platelet counts (not shown; $>400*10^9/l$) generally gave responses in the upper part of the normal reference range, although some essential thrombocythaemia patients had increased aggregation (see chapter 5).

3.5 STABILITY

Repeated testing of 3 normal blood samples by impedance aggregation at regular intervals with the same doses of agonists revealed no significant changes in responsiveness over a 2 hour period. The only exception to this was a progressive increase in aggregation over the first 30 minutes seen in one individual only. The aggregation response then remained stable for the next 90 minutes.

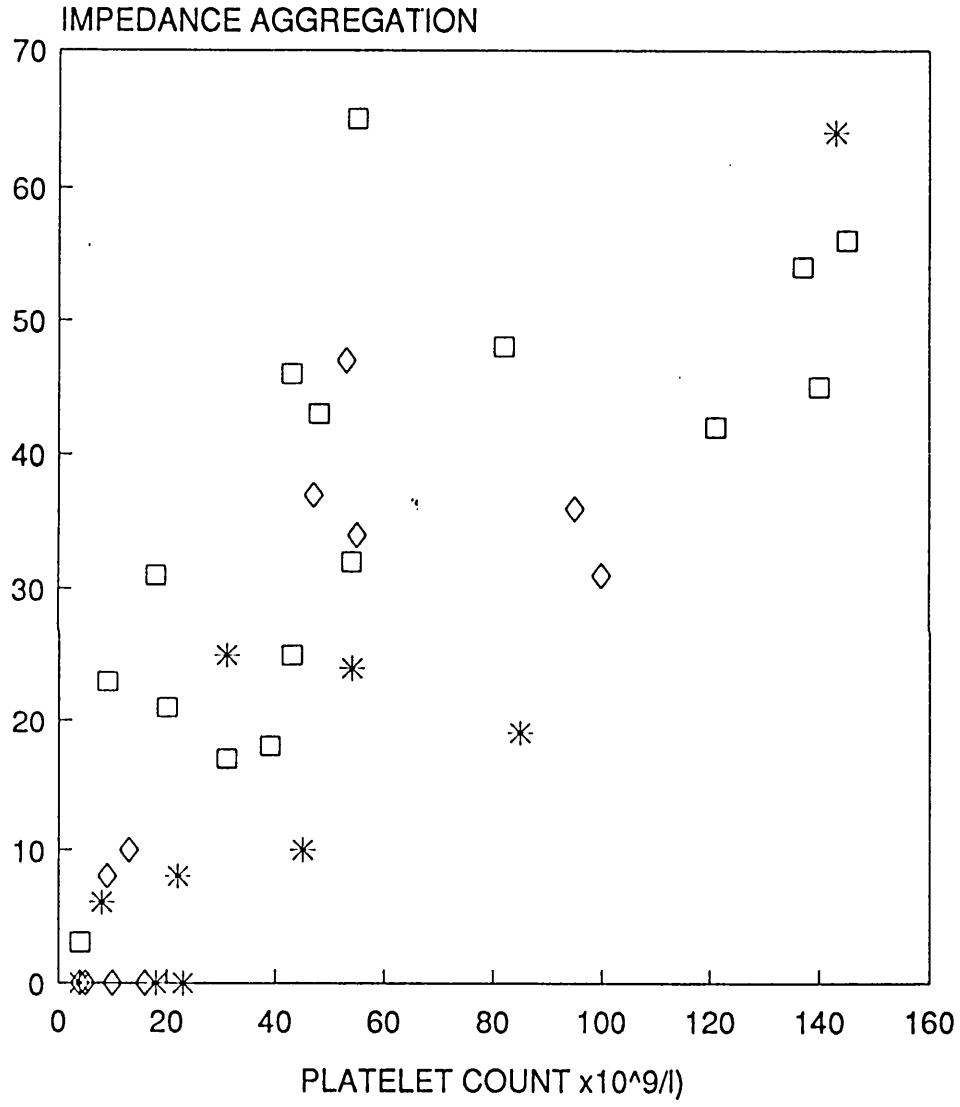

Fig. 3.4. The Relationship Between Impedance Aggregation and platelet count in Thrombocytopaenia. The slope of response was measured in 37 patients with thrombocytopaenia (platelets $<150x10^9/l$) due to ITP (Squares), AML (asterisks), or other causes (diamonds).

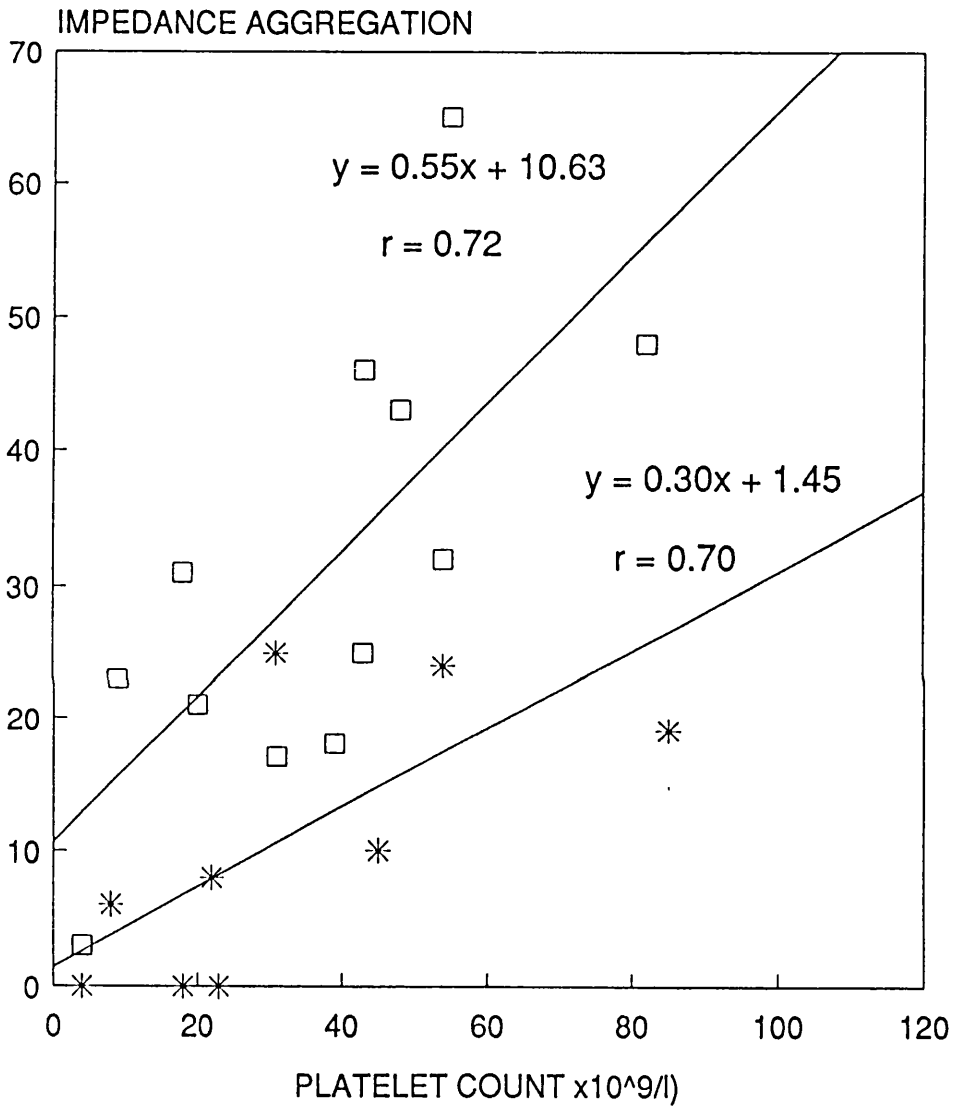

Fig. 3.5. Regression Analysis For Impedance Response Against Platelet Count in ITP and AML. Impedance was measured as the slope of response in patients with a platelet count less than $100x10^9/l$; squares = ITP, asterisks = AML.

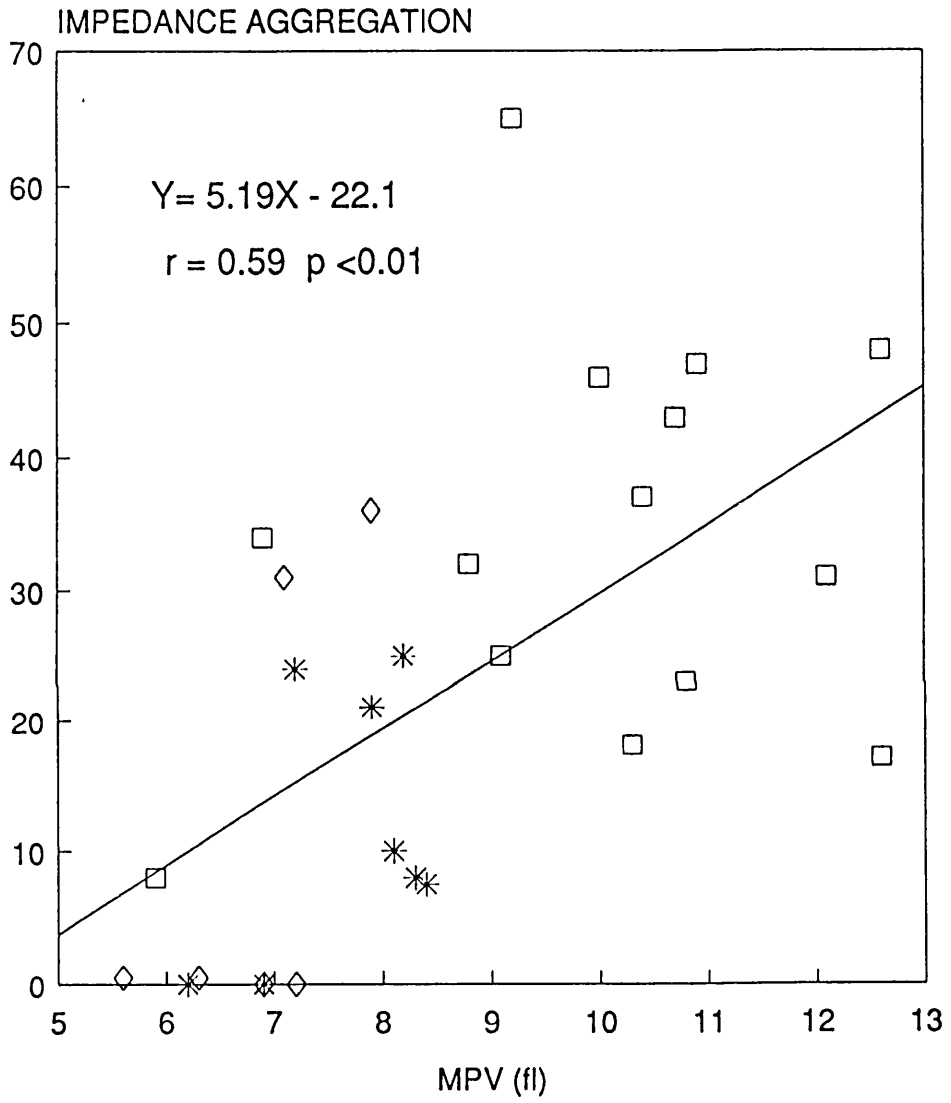

Fig. 3.6. The Correlation Between Mean Platelet Volume (MPV) and Impedance Response in Patients With Thrombocytopaenia. 28 patients with platelet counts less than $100x10^9/l$ were tested. Squares = ITP, asterisks = AML, diamonds = other diseases.

3.6 PLATELET AGGREGATION AGONISTS

Collagen caused dose dependent aggregation in citrated whole blood, and elicited strong aggregation responses at concentrations of 1-5ug/ml (Fig. 3.7). The dose of collagen causing 50% of the maximal response was determined in 6 volunteers, and was found to be 0.45ug/ml (range 0.10-0.90). The aggregation response was preceded by a lag period of about one minute in normal blood. During this lag phase, there was often a transient decrease in the impedance value, before an aggregation response became apparent.

Different collagen preparations influenced the results obtained; when soluble calf skin collagen (Bio/Data Corporation, Philadelphia, USA) was used, no aggregation response could be obtained, even when concentrations up to 200 fold higher than those shown above for equine tendon collagen (Hormon-Chemie) were used (Fig. 3.7). In PRP, by turbidometric aggregation, responses to skin collagen could be obtained at approximately 100 fold higher concentrations than for tendon collagen. Very long lag periods were observed, which increased with the dilution of the reagent, whereas the slope of the response remained relatively unchanged (Fig. 3.8). With tendon collagen, a clear dose response relationship is observed in PRP with regard to the lag period, slope, and amplitude of response (Fig. 3.9). Higher concentrations of skin collagen were not available to see whether there was a threshold at which aggregation would occur in whole blood. Results with tendon collagen are very similar between PRP and whole blood.

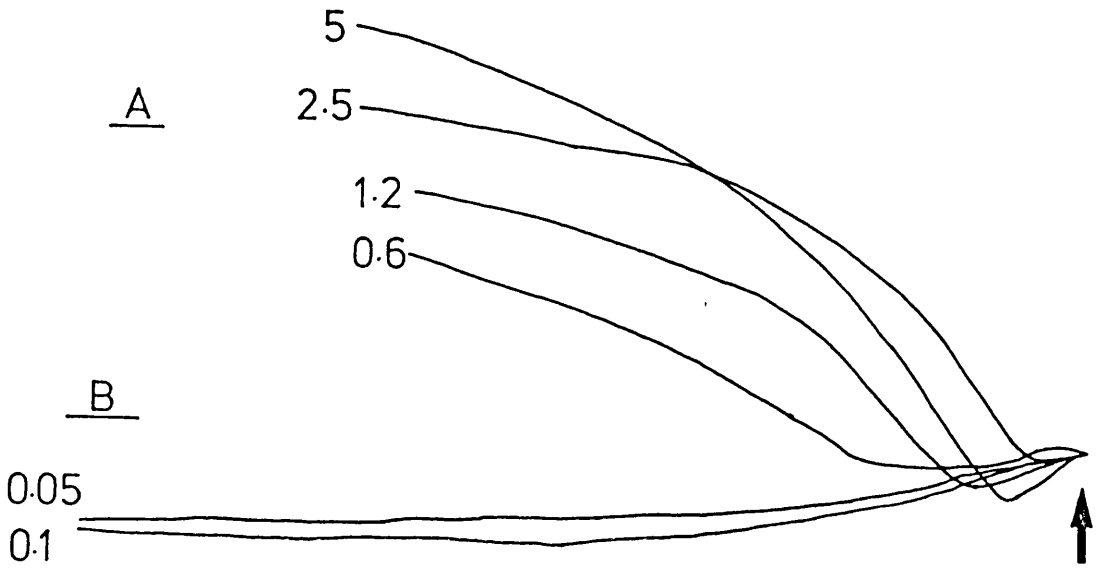

Fig. 3.7. Whole Blood Impedance Aggregation in Normal Blood With Different Collagen Types. Responses to various doses of tendon collagen (A, ug/ml) and skin collagen (B, mg/ml) are shown.

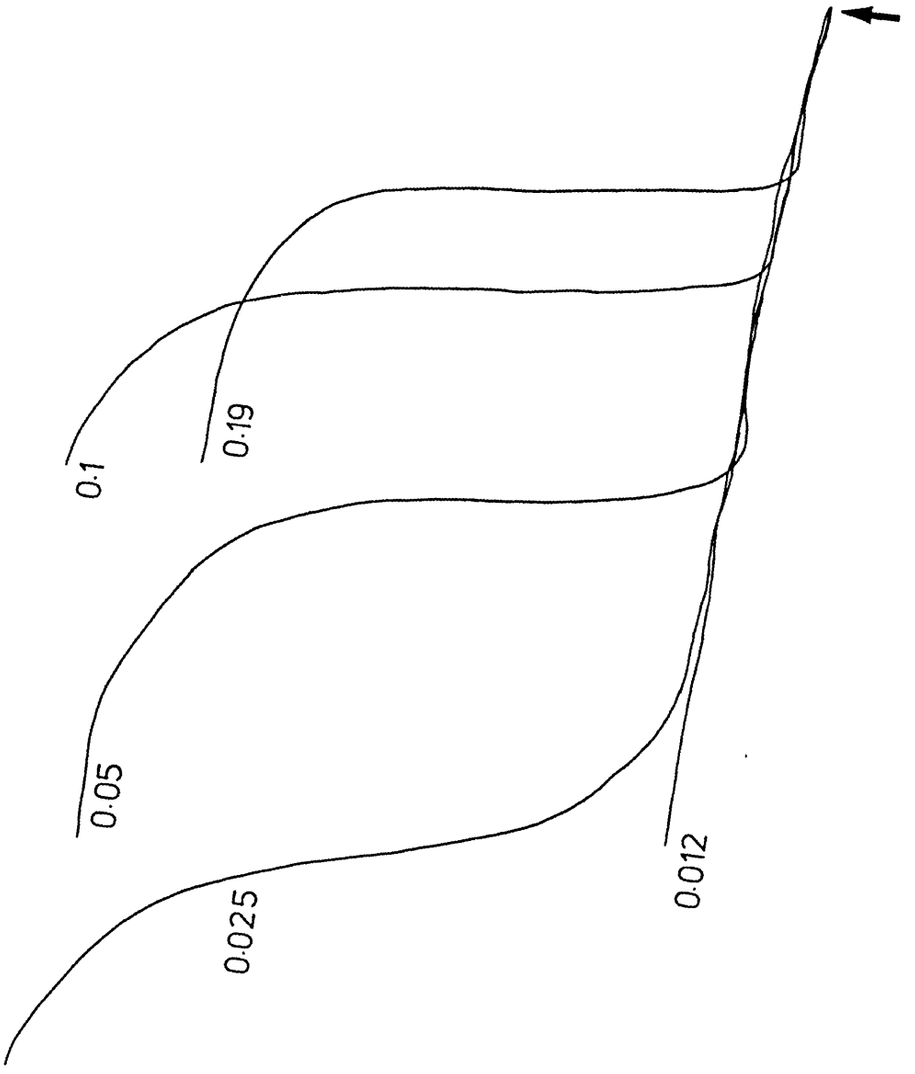

Fig. 3.8. Turbidometric Platelet Aggregation of Normal PRP Using Skin Collagen (mg/ml).

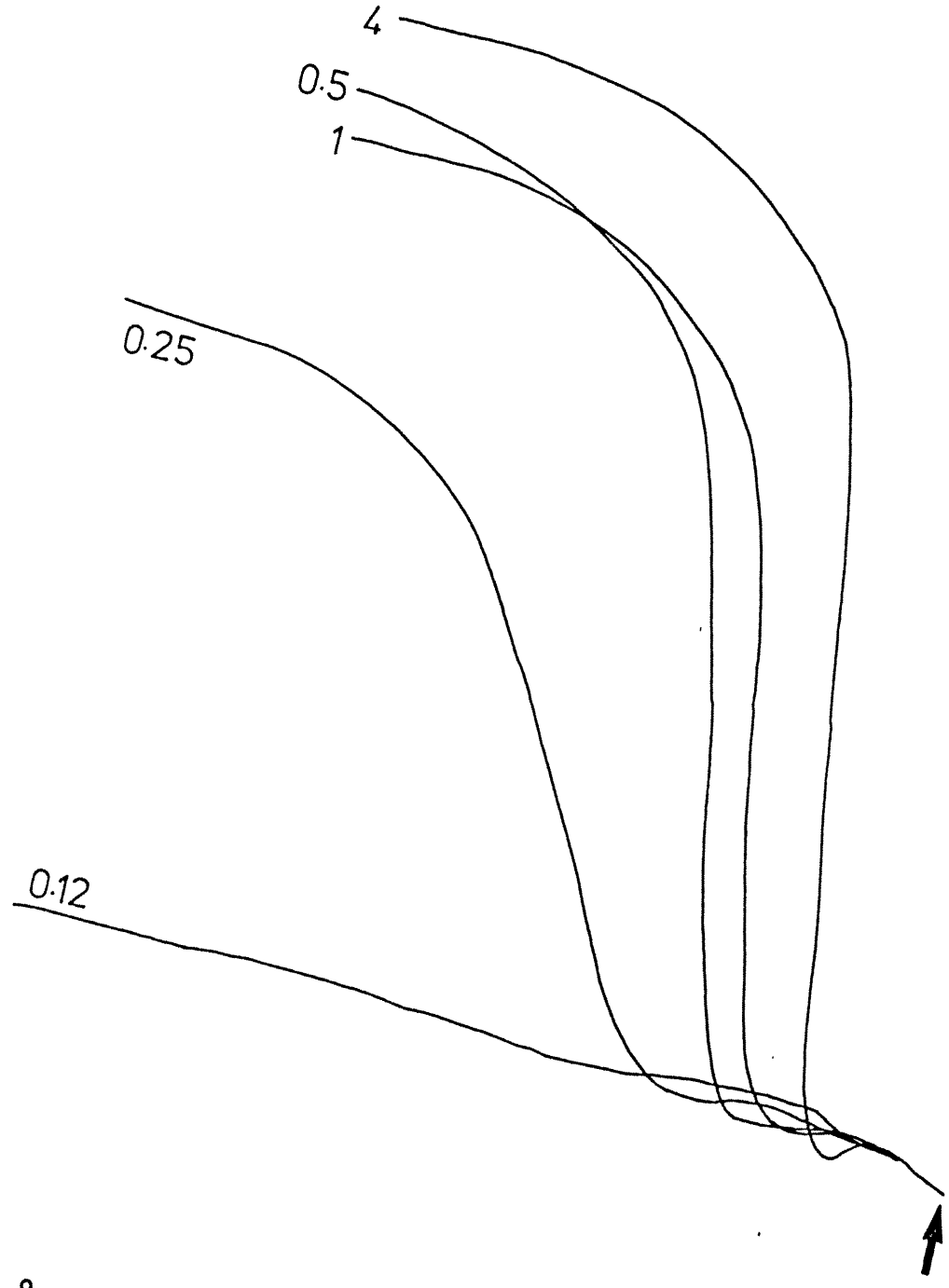

Fig. 3.9. Turbidometric Platelet Aggregation of Normal PRP Using Tendon Collagen (ug/ml).

ADP aggregation required doses about 10 fold higher than those used with PRP optical aggregation to give strong, reproducible responses (Fig. 3.10). Dose/response curves could be obtained to ADP giving an EC50 value of about 10uM, but only a single wave of aggregation was observed at all doses studied, unlike the results with turbidometric PRP methods (Fig. 3.11). An initial decrease in impedance was usually seen after adding the ADP, and this effect occurred independently of the haematocrit or platelet count.

When blood counts were performed (using a Coulter S+IV electronic counter) on normal samples before and after aggregation to 5ug/ml collagen or 25uM ADP, there was no significant change in red or white cell counts, but the platelet count fell by approximately 90%. The changes in platelet count were further evaluated by removing serial aliquots from the impedance cuvette, and diluting them 1/5 in a fixative (4% formaldehyde, 10mM Na_2EDTA, 0.145M NaCl). The changes in platelet count could be related to the change in impedance pattern (Fig. 3.12). An immediate fall in numbers of free platelets was seen on addition of ADP, followed by a slower decrease in numbers after about one minute. Most of the platelets aggregated before detection of increased impedance.

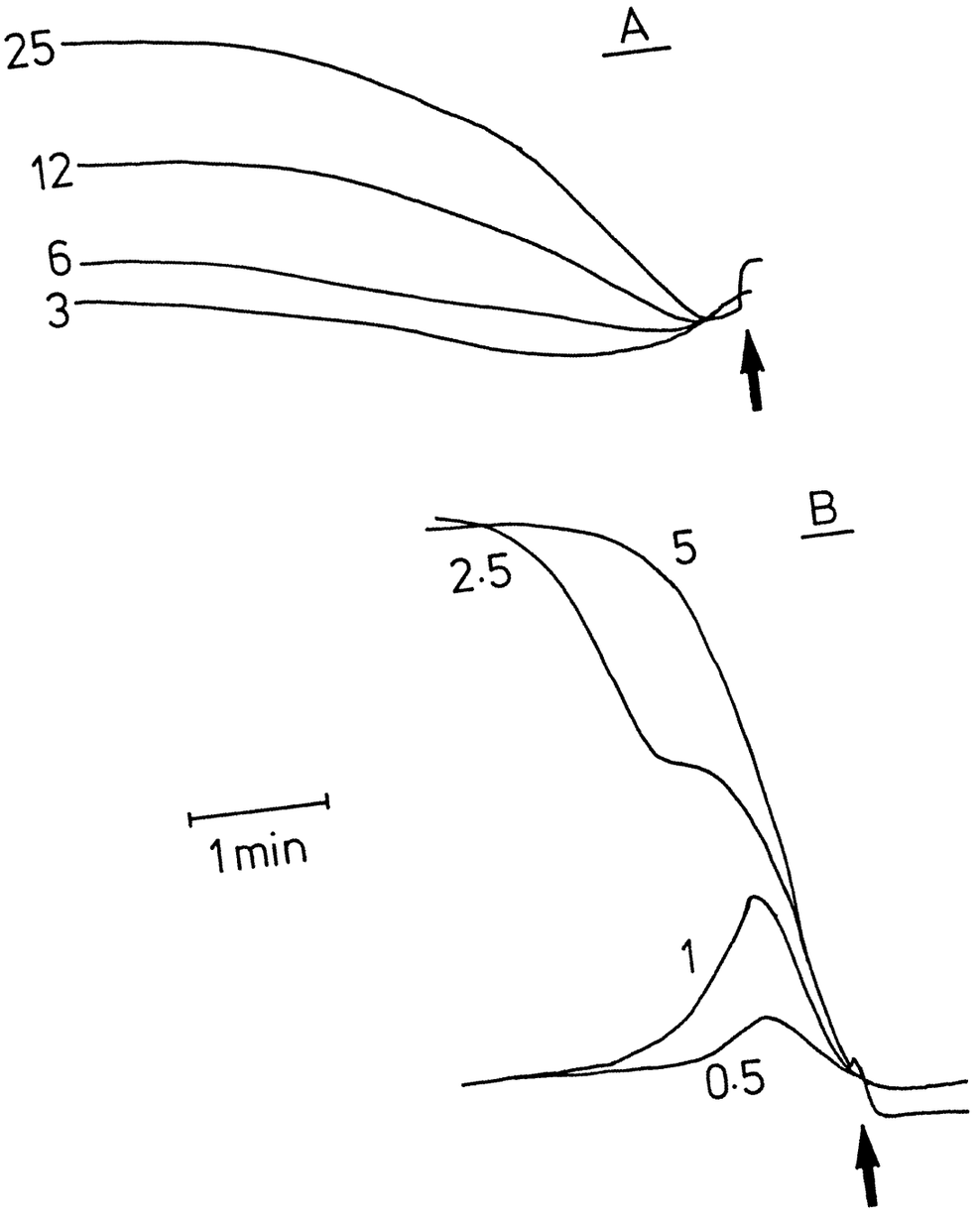

Fig. 3.10. ADP Aggregation. Impedance (A) and turbidometric (B) aggregation responses to various doses of ADP (uM).

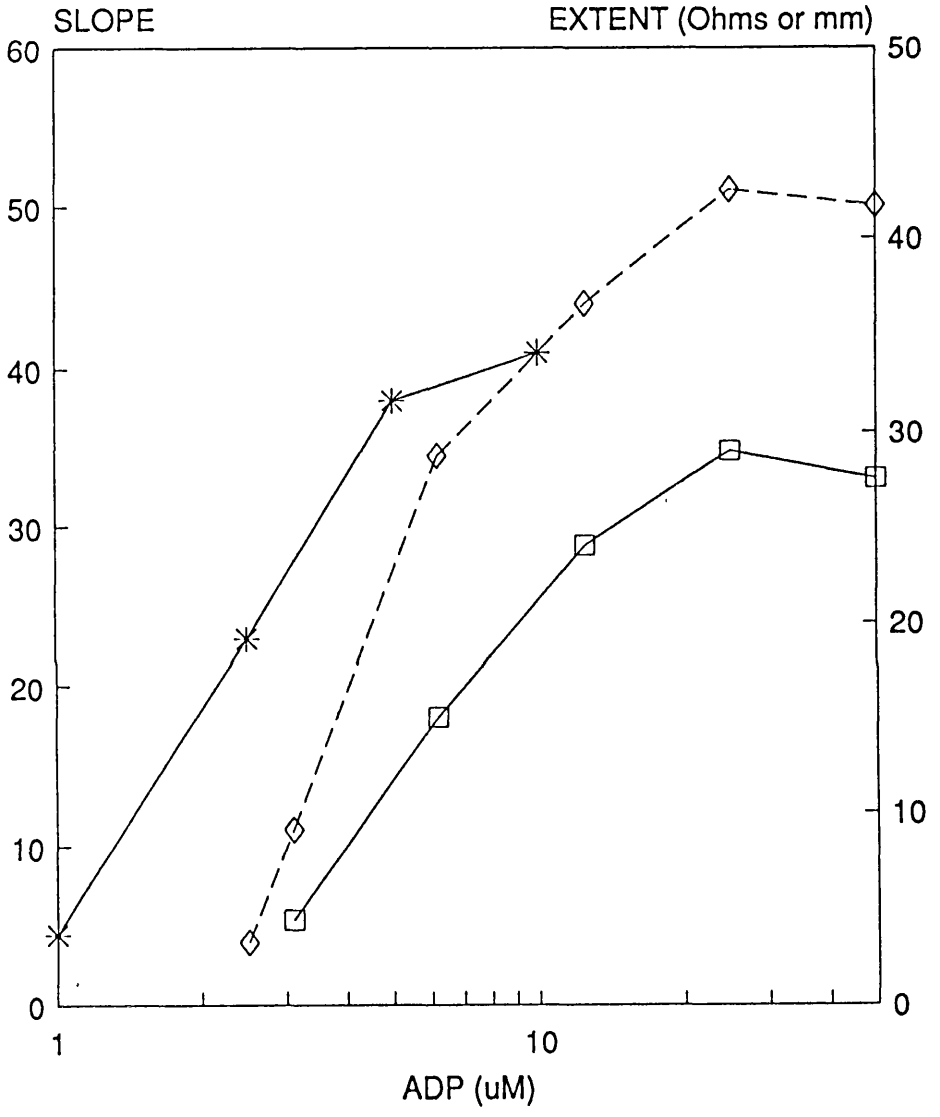

Fig. 3.11. Log Dose Response Curves For Aggregation to ADP. Aggregation in PRP was measured by the turbidometric method as amplitude of response (asterisks and solid line), and whole blood aggregation was measured as either slope (diamonds and broken line) or amplitude (ohms, open squares and solid line).

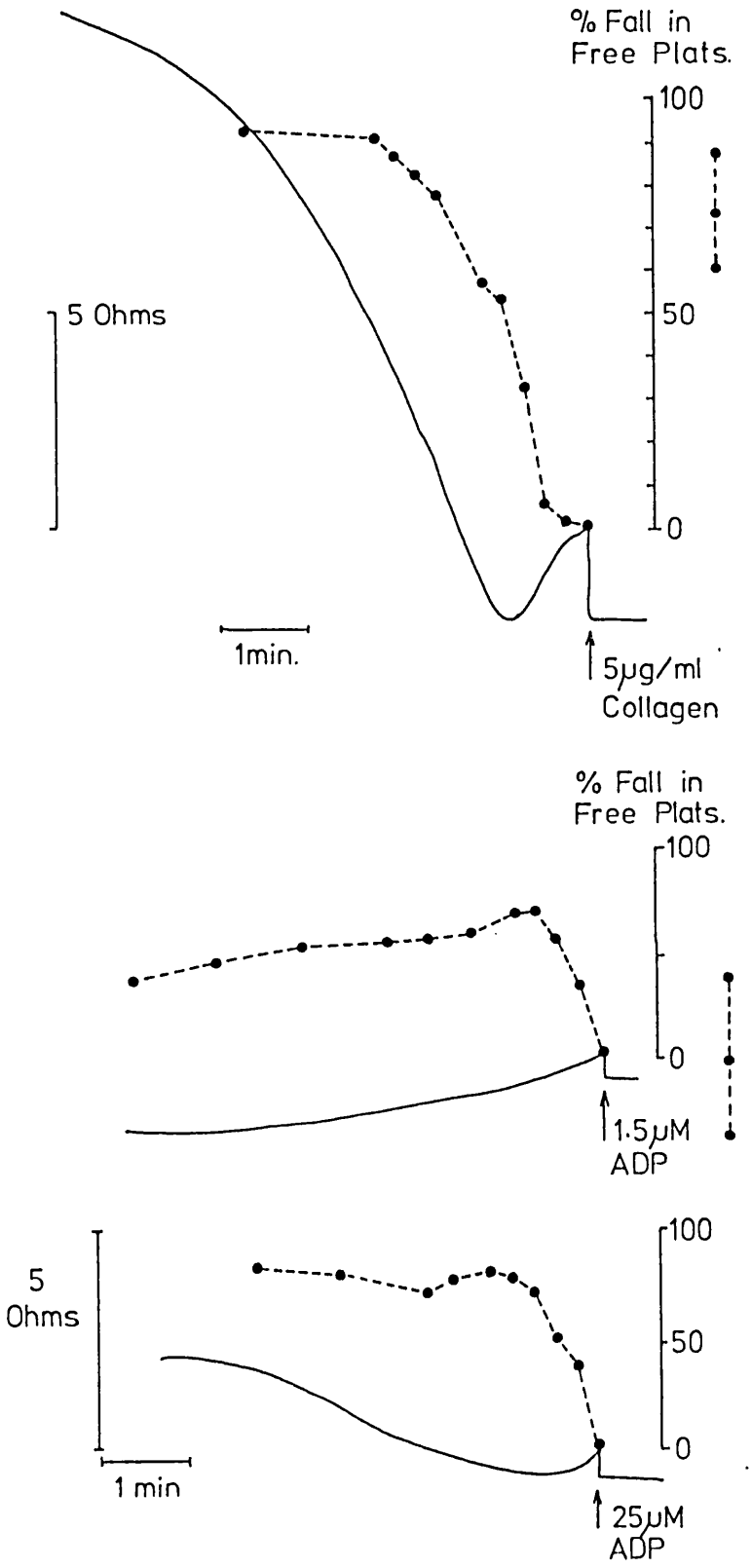

Fig. 3.12. Relationship between impedance tracings and the percentage fall in free platelet numbers, in response to ADP and collagen.

After the addition of collagen to whole blood, there was an initial slow fall in platelet count, followed by a rapid decrease, which just preceded the detection of an increase in impedance, but reached a plateau before 50% aggregation had been detected in the impedance aggregometer.

For adrenaline induced aggregation, normal individuals could be divided into responders and non-responders. The impedance responses were extremely weak even amongst the responders, usually requiring high threshold agonist doses (>10uM) for aggregation (Fig. 3.13). Sodium Arachidonate (Sigma Product No. A-8897) gave measurable responses (Table. 3.5), but its commercial replacement by a product with higher purity (Sigma product No. A-6523), caused excessive red cell lysis and interference in the aggregation responses, and was therefore unsuitable. Arachidonic acid gave suitable responses in whole blood, if prepared as detailed in chapter 2 , using plasma or 10% albumin as final diluents. The synthetic endoperoxide analogue U46619, gave biphasic dose response curves (Fig. 3.14), with an overall EC_{50} value of 0.92uM. The secondary aggregation was blocked by the addition of acetyl salicylic acid.

Normal ranges for collagen and ADP induced platelet aggregation are given in Tables 3.6 and 3.7, values for both polystyrene and glass cuvettes are presented, since the initial work was performed in glass cuvettes, before polystyrene ones were available, and the problem of glass activation became apparent. Appropriate normal data is given with each set of data in this and subsequent chapters of this thesis. The mean lag period before platelet aggregation

obtained with 5ug/ml collagen and plastic cuvettes was 1.29min (n=20, SD 0.40, range 0.7-2.0), which was comparable to that obtained with similar doses of collagen using PRP and a turbidometric method (n=14, mean 0.9min, SD 0.22).

	SLOPE	EXTENT
Mean	46.1	20.6
SD	11.8	12.0
RANGE	27-69	2-38

Table 3.5. Whole Blood Impedance Aggregation to Sodium Arachidonate.
A final concentration of 1mM arachidonate (Sigma Product No. A-8897) was used. Slope and Extent (impedance, Ohms) were measured in 20 healthy normal subjects, using glass cuvettes.

A)	**5ug/ml**	
	SLOPE	**EXTENT**
MEAN	52.4	16.1
SD	11.1	4.1
RANGE	28-70	7-26
MEAN +/- 2SD	30.2-74.6	7.9-24.3

B)	**1ug/ml**		**5ug/ml**	
	SLOPE	**EXTENT**	**SLOPE**	**EXTENT**
MEAN	45.1	13.3	61.4	22.6
SD	10.2	4.2	8.1	4.6
RANGE	27-62	6.5-20	45-73	13.5-31

Table 3.6 - Whole Blood Impedance Aggregation to Collagen in Blood from Healthy Normal Donors.
A) Polystyrene cuvettes; B) glass cuvettes (lag phase not measured); n = 20.

A)	**12.5uM**		**25uM**	
	SLOPE	**EXTENT**	**SLOPE**	**EXTENT**
MEAN	17.1	4.1	25.2	5.6
SD	8.2	3.3	11.3	4.1
RANGE	7-26	1.7-11	11-43	2.1-16
B)	**SLOPE**	**EXTENT**	**SLOPE**	**EXTENT**
MEAN	45.0	13.5	48.6	15.2
SD	18.1	7.6	10.9	6.2
RANGE	13-77	6.5-40	31-75	9-32

Table 3.7 - Whole Blood Impedance Aggregation to ADP in Blood From Healthy Normal Donors.
A) Polystyrene (n=10), and B) glass cuvettes (n=20).

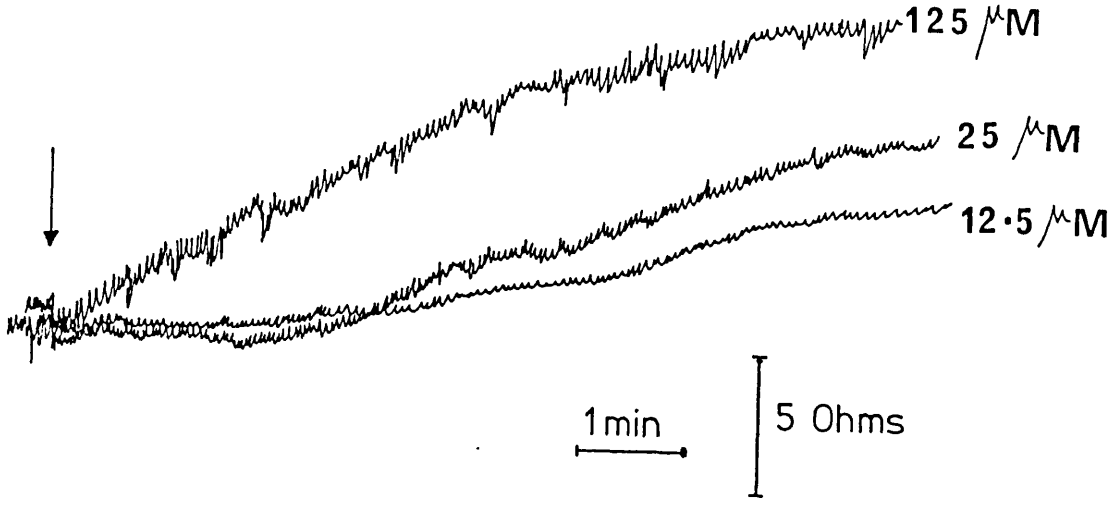

Fig. 3.13. Whole blood Aggregation Responses to Adrenaline.

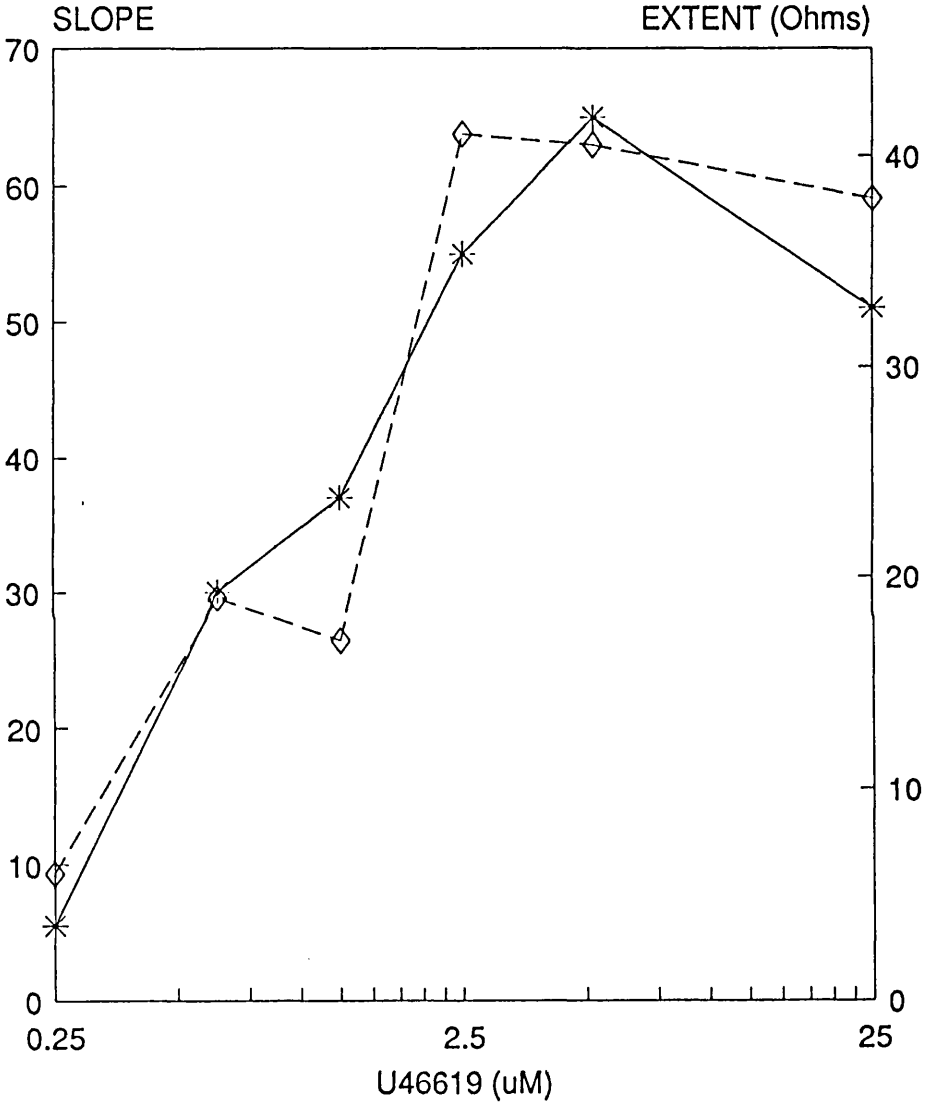

Fig. 3.14. Log Dose Response Curve For the Endoperoxide Analogue, U46619 by Whole Blood Impedance Aggregation. Asterisks indicate the slope of response, and squares indicate extent of aggregation.

3.7 PLATELET AGGREGATION ANTAGONISTS

3.7.1 INHIBITION BY ACETYL SALICYLIC ACID

Acetyl salicylic acid (ASA) caused parallel inhibition of collagen induced impedance aggregation when used in vitro (Table 3.8), or when studied ex vivo (24 hours after a single oral dose of 600mg). A further depression of aggregation could be achieved by adding ASA in vitro to the ex vivo samples (not shown). Stronger inhibition was obtained with weaker collagen concentrations (1ug/ml or less).

ADP induced impedance aggregation was more susceptible to ASA inhibition (Table 3.8), and even high doses (25uMol/l) could be completely inhibited by ASA in vitro. These results were in contrast to the effects of ASA on PRP aggregation to ADP, where the response remains unchanged, or only a small effect on the secondary wave is seen. In two individuals studied after oral ASA, impedance aggregation to ADP was increased rather than inhibited. Compared to the pre-ASA control response, 2mM ASA in vitro gave 93/95% and 45/88% of control, and oral ASA gave 150/203 and 116/147 for slope/amplitude in each individual respectively.

Partial inhibition of U46619 responses could be achieved with both in vitro and ex vivo ASA, but there was always a component of the aggregation response which was resistant to ASA inhibition. Adrenaline aggregation was more sensitive than ADP to inhibition by ASA both in vitro, and in ex vivo samples.

		Lag	Slope	Ohms
1ug/ml Collagen	**No.**	4	4	4
	Mean	23	29.5	39.6
	Range	84-139	23-43	14-24
5ug/ml Collagen	**No.**	10	10	10
	Mean	112.2	93.7	94.4
	SD	25.7	26.9	27.2
	Range	70-167	60-149	54-142
25uM ADP	**No.**	8	8	8
	Mean	238.0	29.0	39.6
	SD	75.8	34.2	43.1
	Range	115-300	1-93	1-95

Table 3.8 - Effect of 2mM Acetyl Salicylic Acid on Whole Blood Impedance Aggregation.
Results expressed as a percentage of the control response. For ADP aggregation, responses completely blocked by ASA were recorded as 300% for the lag period, or 1% for slope and amplitude.

3.7.2 THROMBOXANE RECEPTOR BLOCKADE AND ASA - EFFECTS ON DIFFERENT PLATELET AGGREGATION METHODS

The effects of an orally active thromboxane A_2 (TXA_2) receptor blocking drug (AH23848, Glaxo) and ASA on platelet aggregation responses were studied by whole blood impedance, whole blood platelet counting (using the Clay Adams Ultraflo 100), and by a turbidometric technique using platelet rich plasma (PRP). Blood samples were obtained from 6 healthy laboratory staff, before and 120 minutes after a single oral dose of 70mg AH23848. 2mM ASA was incorporated into the PRP or whole blood for U46619 and ADP aggregation to block secondary thromboxane generation. AH23848 was studied in vitro at a concentration of 1uM for ADP and collagen or 0.1uM for U46619 aggregation.

The dose causing 50% of the maximal response (EC_{50} value) was determined for each reagent by the three different techniques, and is shown in Tables 3.9-3.11. The sensitivity

of each method varied depending on the reagent used. There was a marked difference in the EC_{50} values for ADP by each method, the counting method being most sensitive, and the impedance method having relatively poor sensitivity. Responses to U46619 and collagen were similar between the three methods.

ASA had relatively little effect on U46619 aggregation compared to control data. Collagen aggregation was significantly reduced in the presence of ASA, but to a similar degree between methods. AH23848 both ex vivo and in vitro, inhibited aggregation induced by U46619 in all three methods (Table 3.9). The EC_{50} values were similar for in vitro AH23848 by each aggregation method, but paradoxically the impedance and turbidometric methods were much more sensitive in ex vivo studies of the drug, with inhibition at all doses of U46619.

There was inhibition of collagen induced aggregation with both in vitro and ex vivo AH23848 in all three methods, with dose response curves parallel to those obtained with ASA inhibition. There were however, some differences in sensitivity between the methods (Table 3.10, Fig. 3.15 - 3.17). AH23848 had no inhibitory effect on ADP aggregation by any of the three methods studied (Table 3.11).

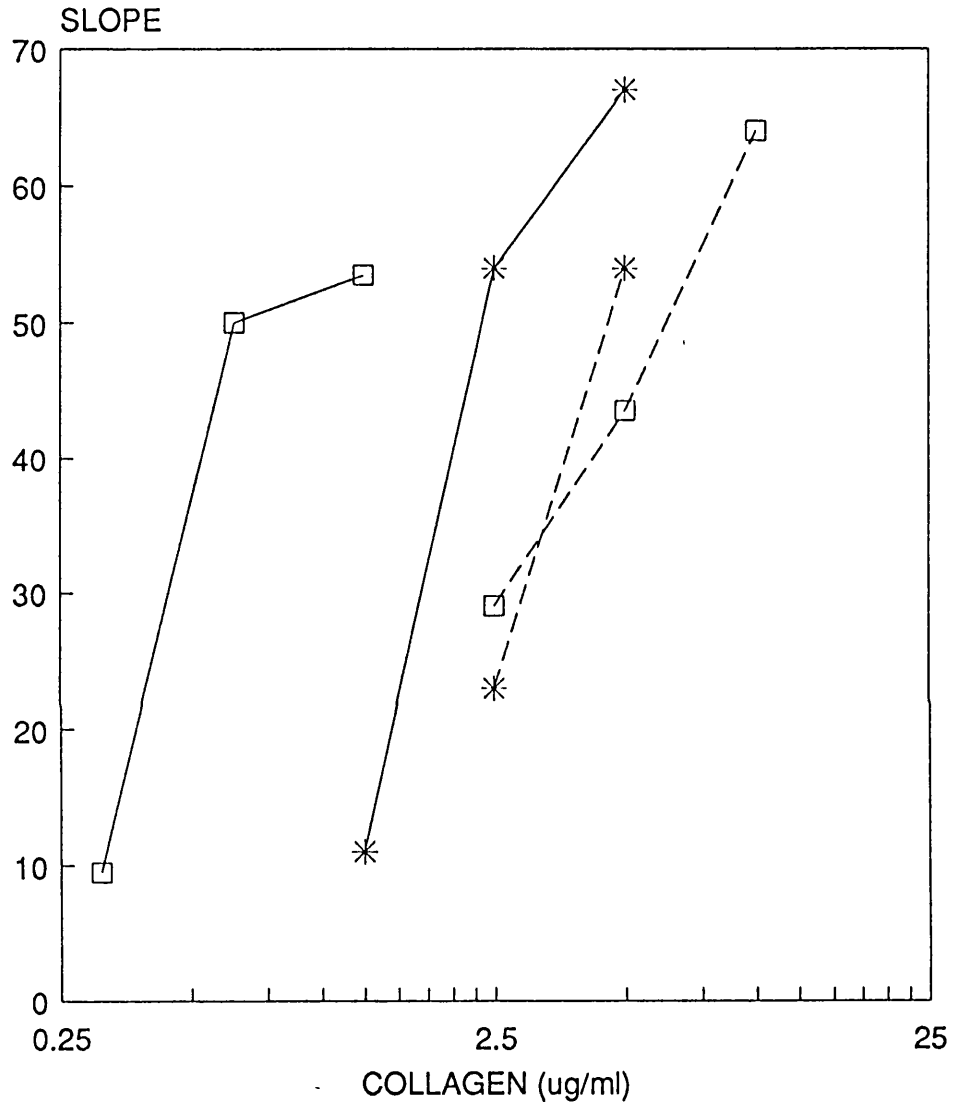

Fig. 3.15. The Effects of ASA and AH23848 on Collagen Dose Response Curves in Whole Blood Using the Impedance Method. Squares and solid line = Saline control; squares and broken line = ASA in vitro; asterisks and solid line = AH23848 ex vivo; asterisks and broken line = AH23848 in vitro.

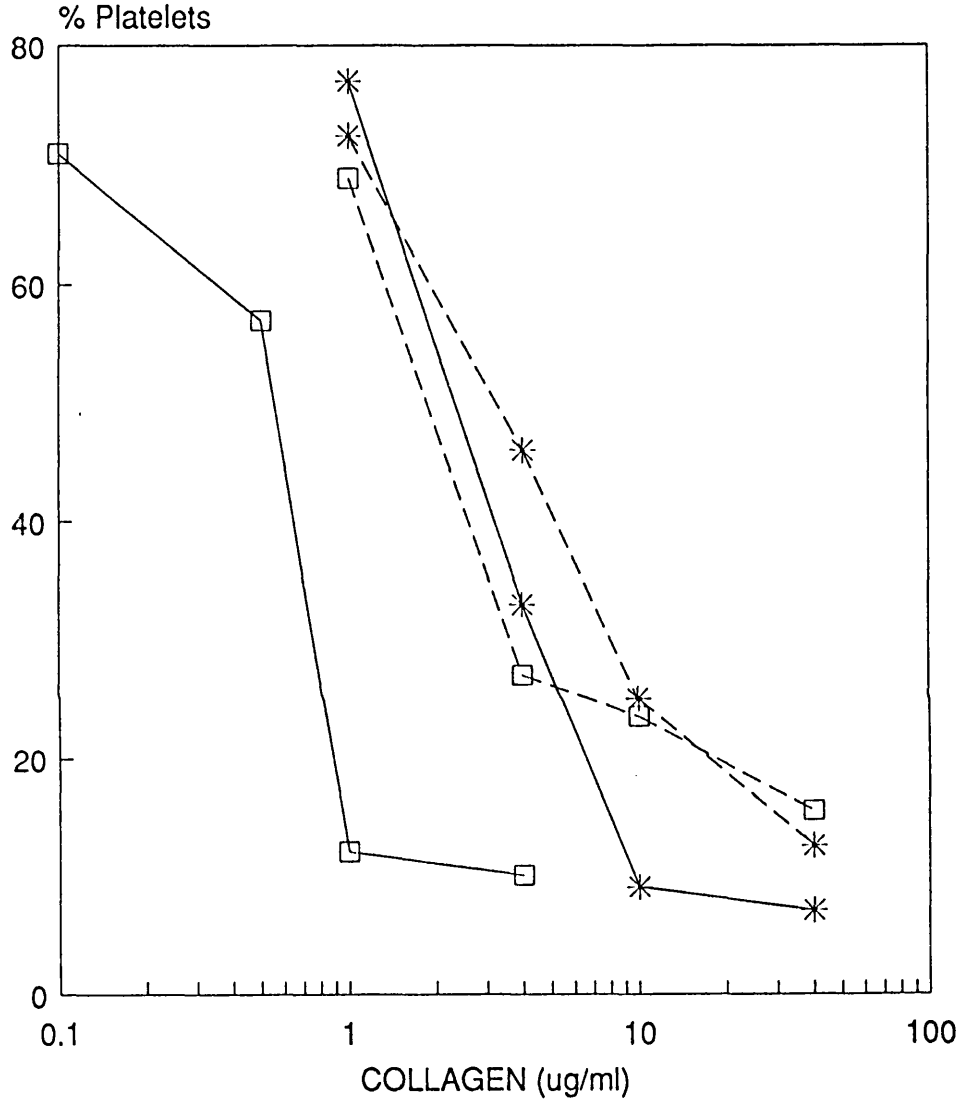

Fig. 3.16. The Effects of ASA and AH23848 on Collagen Dose Response Curves in Whole Blood Using the Platelet Counting Method. Results were expressed as a percentage of the initial platelet count. Legend as in Fig. 3.15.

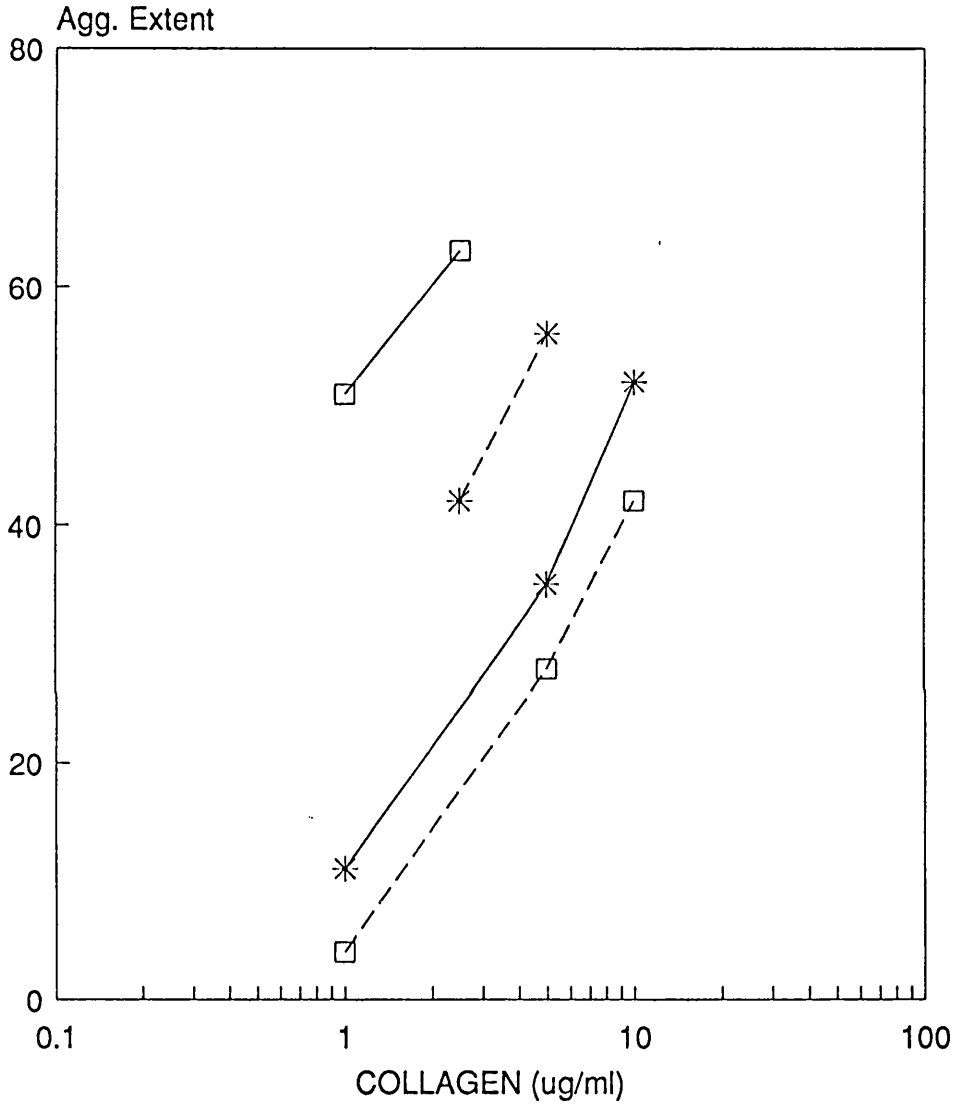

Fig. 3.17. The Effects of ASA and AH23848 on Collagen Dose Response Curves Using PRP and the turbidometric technique. Results are expressed as the extent of aggregation (%transmittance). Legend as in Fig. 3.15.

		WB UF-100	WB IMPEDANCE	PRP OPTICAL
CONTROL	**MEAN**	0.14	0.25	0.62
	RANGE	0.06-1.80	0.15-0.35	0.20-0.98
AH 23848	**MEAN**	1.87	0.95	1.59
IN VITRO	**RANGE**	0.73-4.30	0.49-2.60	0.74-3.30
AH 23848	**MEAN**	7.36	>250	>100
EX VIVO	**RANGE**	2.7-14.7	.....	.....

Table 3.9 Aggregation Responses in Three Different Methods With The Synthetic Endoperoxide Analogue U46619. Aggregation was performed in the presence of 2mM ASA to block secondary thromboxane generation (n=6); results are expressed as EC_{50} values (uM).

		WB UF-100	WB IMPEDANCE	PRP OPTICAL
CONTROL	**MEAN**	0.61	0.45	0.66
(+ Saline)	**RANGE**	0.25-1.0	0.1-0.9	0.17-1.05
CONTROL	**MEAN**	2.20	2.60	3.98
(+ ASA)	**RANGE**	1.43-4.25	1.60-4.10	1.50-6.00
AH 23848	**MEAN**	3.10	2.60	1.29
in vitro	**RANGE**	1.81-5.55	1.90-3.90	0.40-3.20
AH 23848	**MEAN**	2.50	1.80	3.81
ex vivo	**RANGE**	2.02-3.40	1.40-2.50	0.96-7.00

Table 3.10 - Aggregation Responses to Collagen in Three Different Methods. Results are expressed as EC_{50} values (ug/ml).

		WB UF-100	WB IMPEDANCE	PRP OPTICAL
CONTROL	**MEAN**	0.53	7.70	1.38
	RANGE	0.32-0.83	3.30-11.50	0.80-2.60
AH 23848	**MEAN**	0.58	--	1.60
IN VITRO	**RANGE**	0.29-0.82		0.66-2.55
AH 23848	**MEAN**	0.46	6.70	1.19
EX VIVO	**RANGE**	0.21-0.69	3.70-8.40	0.35-2.80

Table 3.11 - Aggregation Responses to ADP in Three Different Methods. Aggregation was performed in the presence of 2mM ASA, to block thromboxane generation. Results are expressed as EC_{50} values (uM). EC_{50} values for ADP could not be calculated in the impedance method with in vitro AH23848 in 4 of the subjects studied.

3.7.3 THE IN VITRO EFFECTS OF THE PROSTACYCLIN ANALOGUE ILOPROST

In vitro experiments showed that 2.5ng/ml Iloprost was required to reduce the whole blood impedance response to 5ug/ml collagen by 50% (IC_{50}), and 0.2ng/ml was required for 25uM ADP. The impedance slope and amplitude were inhibited in parallel (Table 3.12). In PRP from six healthy normal volunteers, the IC_{50} for Iloprost in turbidometric aggregation was 0.73ng/ml (SD=0.29) for 5uMol/l ADP. Thus the whole blood impedance method is more sensitive to Iloprost in ADP aggregation than turbidometric PRP methods. Ingerman-Wojenski and colleagues (1982) found that native prostacyclin was detected less readily in whole blood than in PRP. However, they used a slightly different method, and Gresele et al (1984) have found approximately equal sensitivity to PGI_2 between the methods, although a modified whole blood method was used. The half life of Iloprost in citrated whole blood was found to be 130 minutes when incubated at either 4 or 37°C, whereas prostacyclin itself gave a half life of 20 minutes at 37°C, and 120 minutes at 4°C (mean of two experiments).

	SLOPE	**OHMS**
MEAN	53.7	55.5
SD	26.0	32.0
RANGE	14-110	6-138

Table 3.12 - The Effect of Iloprost on Whole Blood Aggregation In Vitro. Aggregation to 5ug/ml collagen was inhibited by 2.5ng/ml Iloprost and results were expressed as a percentage of the control response (n = 20).

3.7.4 ILOPROST INFUSION INTO NORMAL VOLUNTEERS

6 healthy normal volunteers (age 24-52 years), received a two hour infusion of Iloprost. 1ml vials of 0.1 ng/ml Iloprost were diluted in 500ml of sterile sodium chloride and infused into a peripheral arm vein using an Ivac controller (Ivac Ltd., Harrow, Middlesex), at the appropriate weight-related dose. A venous blood sample was taken at time 0 as the cannula was inserted (sample I) and the infusion commenced at a rate of 0.5 ng/Kg/minute. After 30 minutes the infusion rate was increased to 1.0 ng/Kg/min. At 60 minutes, a second venous sample was taken and the infusion rate increased to 2.0 ng/Kg/min for a further 60 minutes. The infusion was then terminated, a third sample taken (sample III) immediately before its completion. Sample IV and V were taken 60 and 120 minutes after the end of the infusion. All samples were taken by separate venepuncture without stasis from the non-infusion arm.

Ex vivo platelet aggregation responses to ADP and collagen showed some discrepancies between the turbidometric and impedance methods. In the former, each subject showed serial inhibition of PRP aggregation as the infusion progressed with increasing dosage (Fig. 3.18). The pre-infusion baseline response was regained within 60 minutes of terminating the infusion, but some degree of rebound hyperaggregability was seen in each subject; ie. after subsidence of the inhibitory effect of Iloprost, the aggregation response to at least one agonist became more active than the pre-infusion response. Whole blood aggregation showed significant inhibition of aggregation during the first

hour of infusion, but after the increased dose in the second hour, a slight increase in aggregation response was seen in three of the four subjects studied (Fig. 3.19). The discrepancy between PRP and whole blood results should not be due to in vitro erythrocyte binding, since Iloprost, unlike PGI_2 (Willems et al, 1983; Jakubowski et al, 1983) is not inhibited by red cells. Rebound hyperaggregability was also seen in whole blood platelet aggregation. Although rebound hyperaggregability was noted in both aggregation methods, spontaneous aggregation was not detected. Serum thromboxane B_2 levels are shown in Fig. 3.20.

In common with prostacyclin (Russell-Smith et al, 1981), Iloprost has been shown to inhibit PMN aggregation in vitro (Belch et al, 1987). PMN's may respond to this by modifying their eicosanoid metabolism, and producing more thromboxane B_2 or other platelet activating substances.

There was a positive correlation between the extent of inhibition of platelet aggregation by in-vitro Iloprost (added to the pre-infusion sample) and the ex-vivo inhibition observed in each subject during the infusion. The correlation was strongest for in-vitro inhibition by 1.0 and 1.5 ng/ml with ex-vivo results obtained at one hour into the infusion (1.0 ng/Kg/min), $r=0.96$ and 0.95, respectively, $p<0.01$).

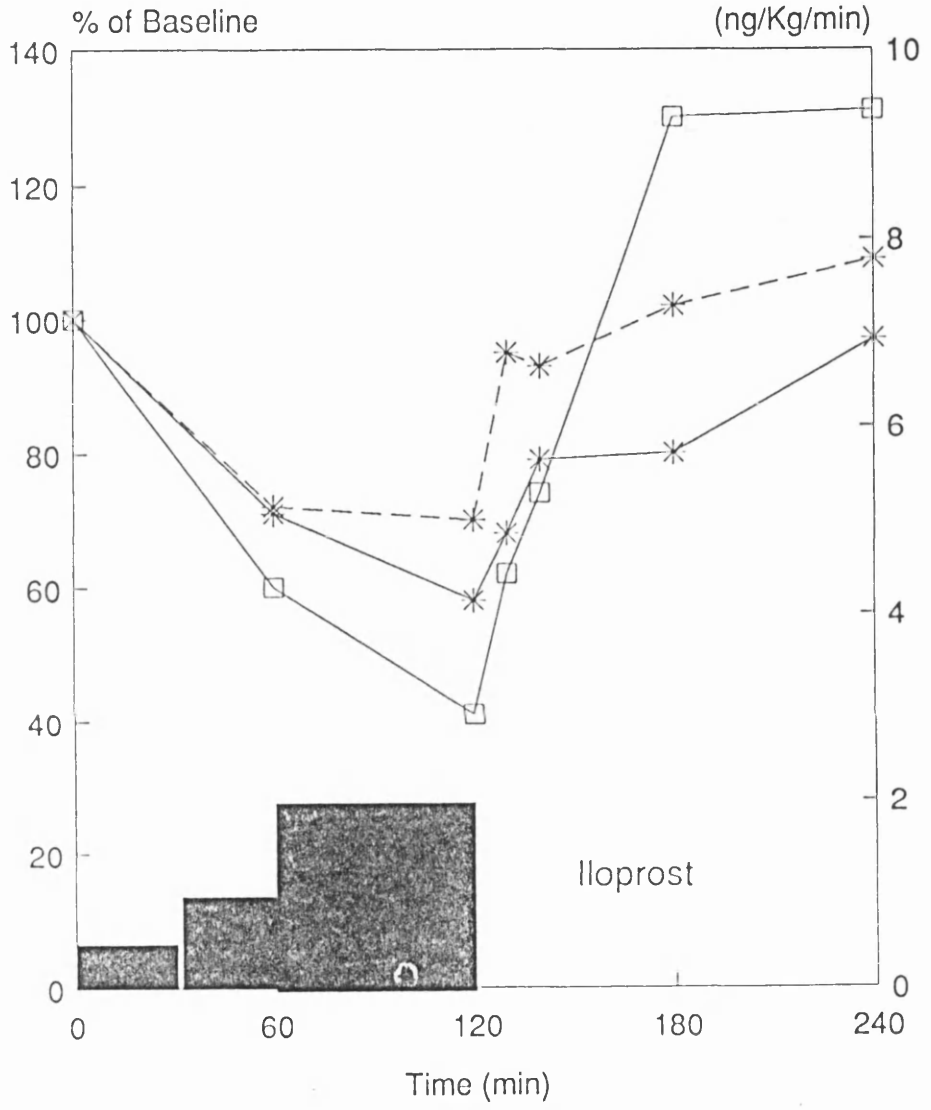

Fig. 3.18. The Effect of Iloprost Infusion Into Normal Volunteers on Turbidometric PRP Aggregation. Results are expressed as a percent of the baseline response. Squares indicate aggregation to 1uM ADP, asterisks and solid line = 5uM ADP; asterisks and broken line = 4ug/ml Collagen.

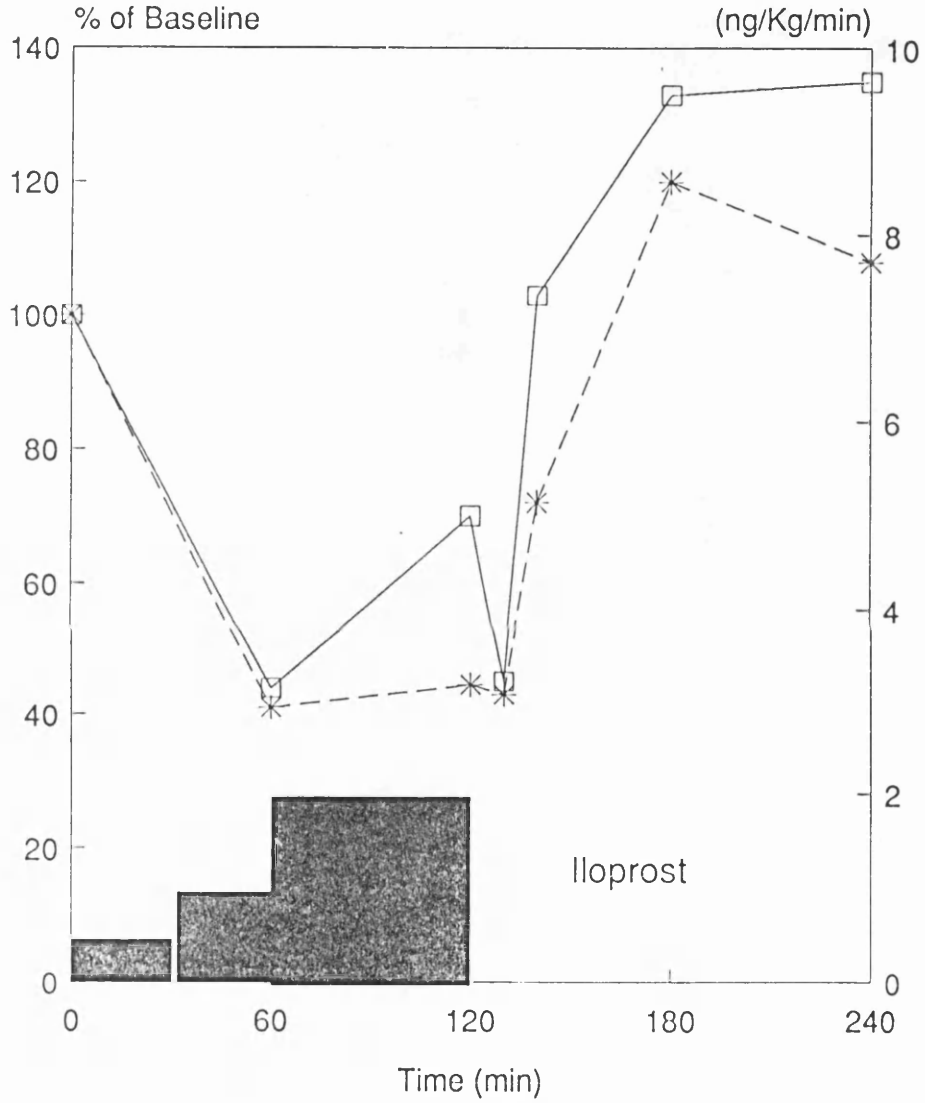

Fig. 3.19. The Effect of Iloprost Infusion into Normal Volunteers on Whole Blood Impedance Aggregation. Results are expressed as a percentage of the baseline response. Squares and solid line indicates aggregation to 12.5uM ADP, asterisks and broken line indicate 1ug/ml Collagen.

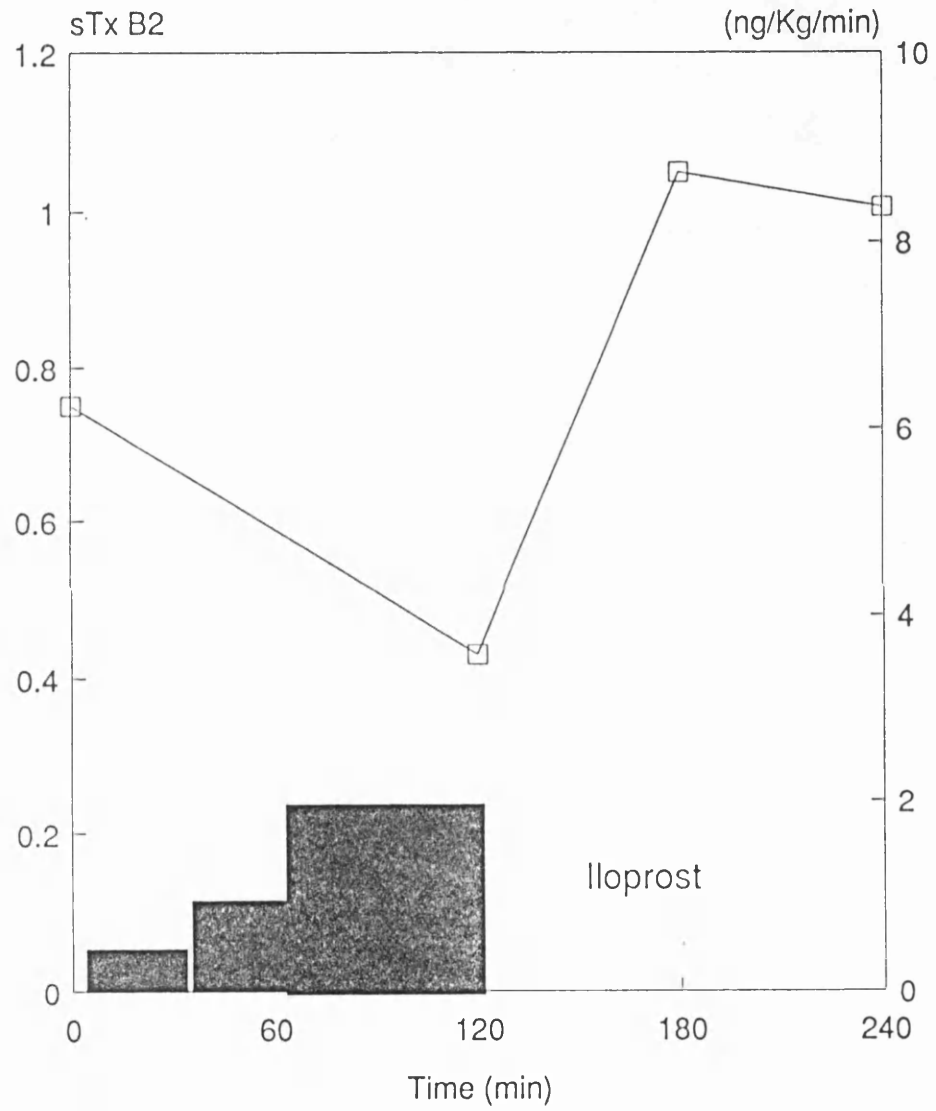

Fig. 3.20. Serum Thromboxane B_2 Levels in Normal Volunteers Receiving Iloprost Infusions. Results are expressed as ng/10^6 Platelets.

3.8 CHEMI-LUMINESCENCE AND PLATELET RELEASE

The simultaneous measurement of platelet nucleotide release and aggregation was carried out with a 2-channel whole blood lumi-aggregometer and Chronolume luciferase reagent (see chapter 2). When collagen was used to stimulate platelets, the amount of ATP released depended on the agonist dose, but did not correlate with the aggregation response. ATP release was detected about 30 seconds after the addition of collagen, and also preceded aggregation by about 30 seconds (Fig. 3.21). In PRP studied by turbidometric techniques, ATP release coincided with the aggregation response to collagen (Fig. 3.22). The pattern of responses seen with bovine thrombin were similar to those with collagen, luminescence changes preceding aggregation, but only occurring after lag phase (not shown).

When ADP was used to induce aggregation in whole blood, graded responses of aggregation and ATP release were obtained over the range of 5-25uM ADP (Fig. 3.23). With 5uM ADP, very weak responses were obtained, and in some subjects there was no detectable response. In samples from normal volunteers, the amount of ATP released correlated with the aggregation response obtained ($r=0.82$, $p<0.01$). In whole blood, nucleotide release in response to ADP occurred immediately, and preceded aggregation, whereas in PRP there was immediate aggregation and ATP release was delayed, occurring at the time of secondary aggregation (Fig. 3.24).

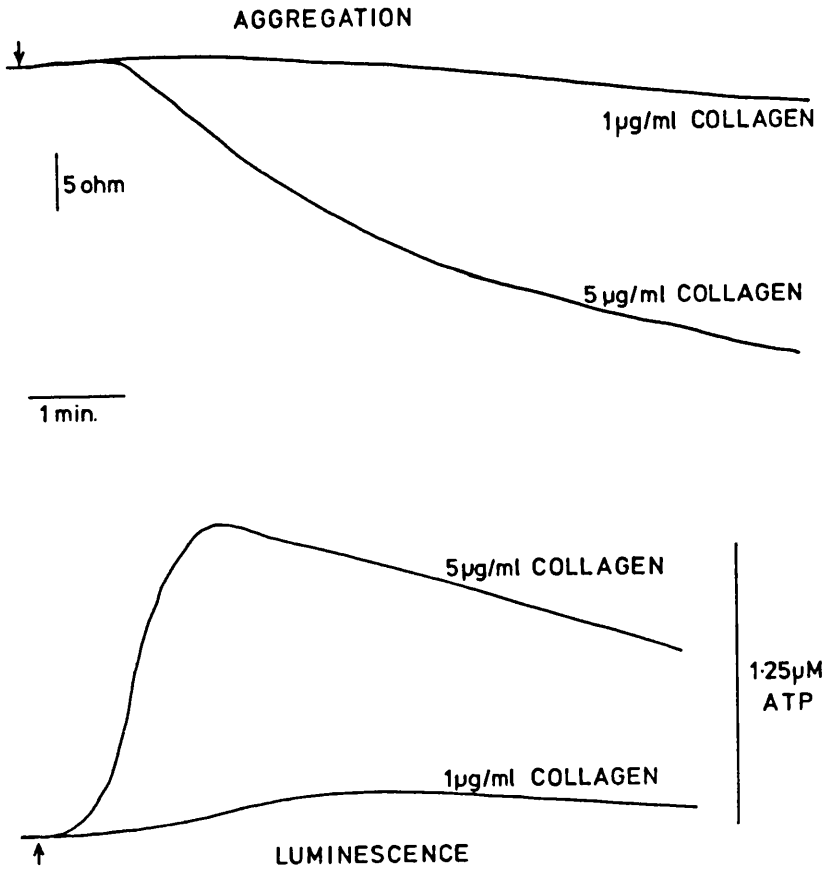

Fig. 3.21. Simultaneous Measurement of Aggregation and Luminescence (ATP release) in Response to Collagen in Whole Blood.

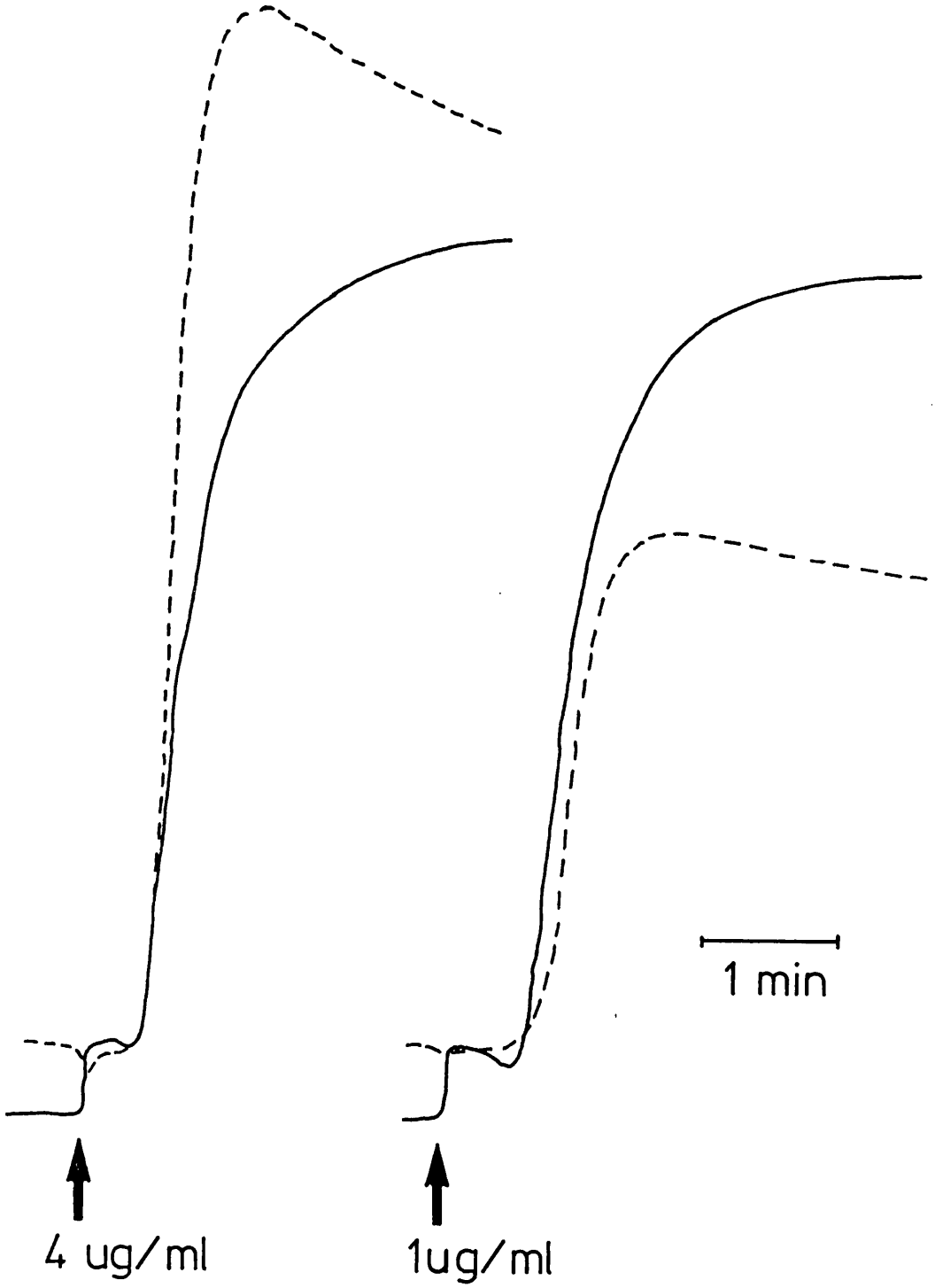

Fig. 3.22. Simultaneous Measurement of Aggregation (solid lines) and Luminescence (ATP release, broken lines) in Response to Collagen in PRP by the Turbidometric Method.

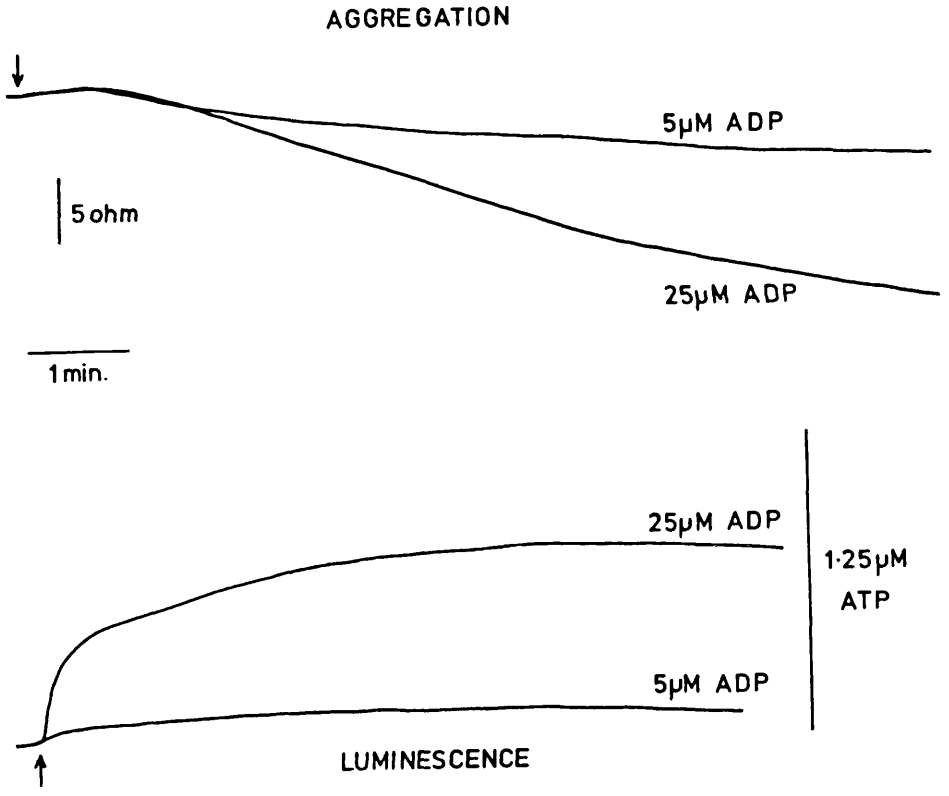

Fig. 3.23. Simultaneous Measurement of Aggregation and Luminescence (ATP release) in Response to ADP in Whole Blood by the Impedance Method.

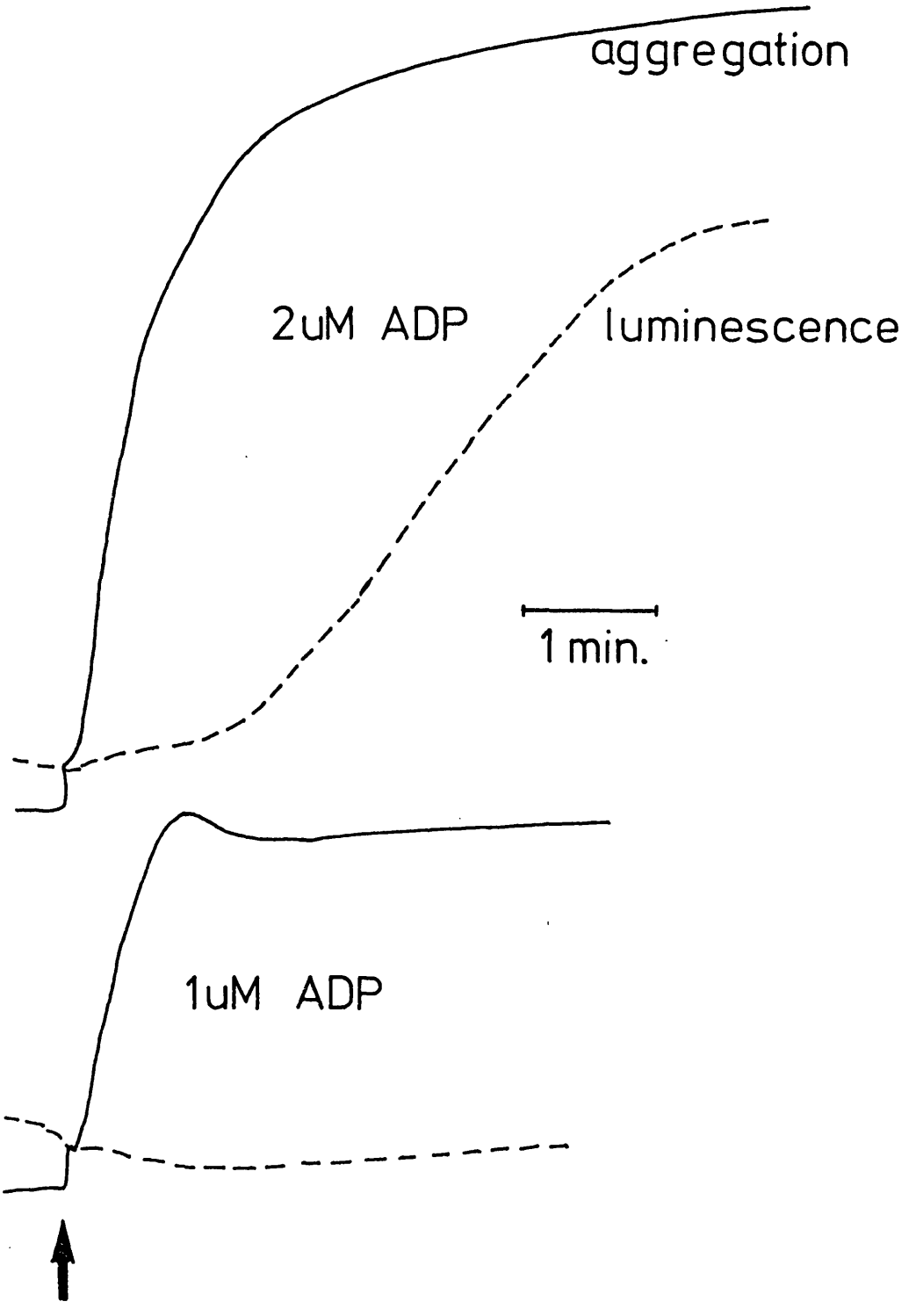

Fig. 3.24. Simultaneous Measurement of Aggregation (solid lines) and Luminescence (ATP release, broken lines) in Response to ADP in PRP by the Turbidometric Method.

The immediate nucleotide response to ADP in whole blood may have been due to the high concentrations that were necessary with this agonist in order to evoke a response. It did not appear to be related to ATP contamination of the ADP, as no increase in luminescence was seen when doses of 25uM ADP were added to Chronolume in buffer, or to thrombocytopaenic blood. In addition, there was a wide range of luminescence responses obtained to ADP in different individuals, and a good correlation between the ATP release to collagen (5ug/ml) and ADP (25uM), although the latter was much lower (Mean ATP 9.29 and 6.19nM/10^9 Platelets, respectively, in 20 normal volunteers and patients with a variety of platelet defects; $r=0.82$, $p<0.005$). It is possible that the high ADP doses used in whole blood caused immediate dense granule release, however aggregation was not detected by the impedance method until about 30 seconds later.

The use of Chronolume reagent did have some platelet activating effects, as aggregation responses to collagen and ADP were higher (Table 3.13) when chronolume was present than when an equivalent volume of saline was used ($p<0.01$ and $p<0.05$, respectively). This increase in aggregation response was accompanied by the formation of a large fibrin clot after about five minutes in all normal volunteers studied, but did not appear to be associated with increased ATP release as judged by comparisons with levels of ATP released from PRP and measured in a luminometer. The heightened aggregation was not inhibited by Iloprost, a stable analogue of prostacyclin, or acetyl salicylic acid. A patient with storage pool defect and very low intra-platelet levels of ADP and ATP, showed a very

weak luminescence response to collagen, but the aggregation response was normal in the presence of chronolume, despite being slightly decreased in the absence of this reagent (Fig. 3.25).

		SALINE	CHRONOLUME
5ug/ml Collagen	**No.**	28	28
	Mean	31.6	46.7
	SD	16.0	17.5
25uM ADP	**No.**	9	9
	Mean	17.2	28.5
	SD	12.6	19.9

Table 3.13 - The Influence of Chronolume Reagent on Platelet Aggregation. Results are expressed as aggregation slope, from a variety of normal controls and patients with platelet defects.

Ingestion of acetyl salicylic acid (600mg) decreased the aggregation response to collagen, but had a lesser effect on ATP release (Fig. 3.26). A similar pattern of impedance and luminescence results were obtained with collagen, following Iloprost infusion.

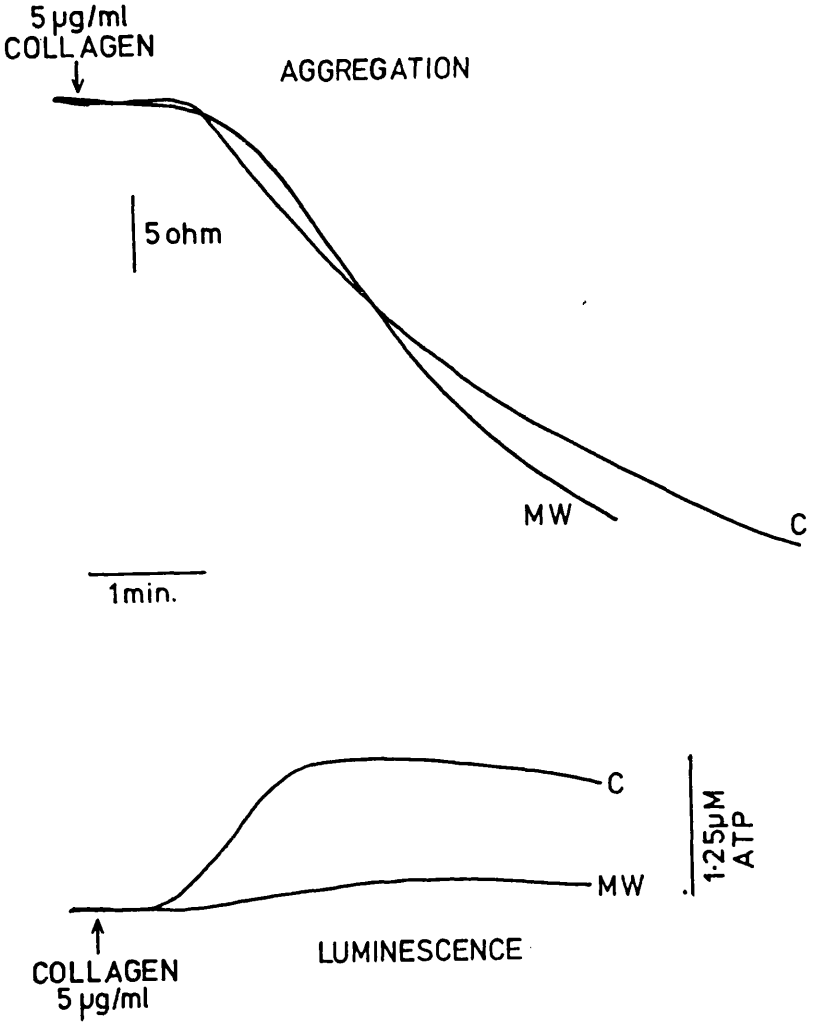

Fig. 3.25. Simultaneous Measurement of Aggregation and Luminescence (ATP release) in Response to Collagen in Whole Blood in a Patient With Storage Pool Defect.

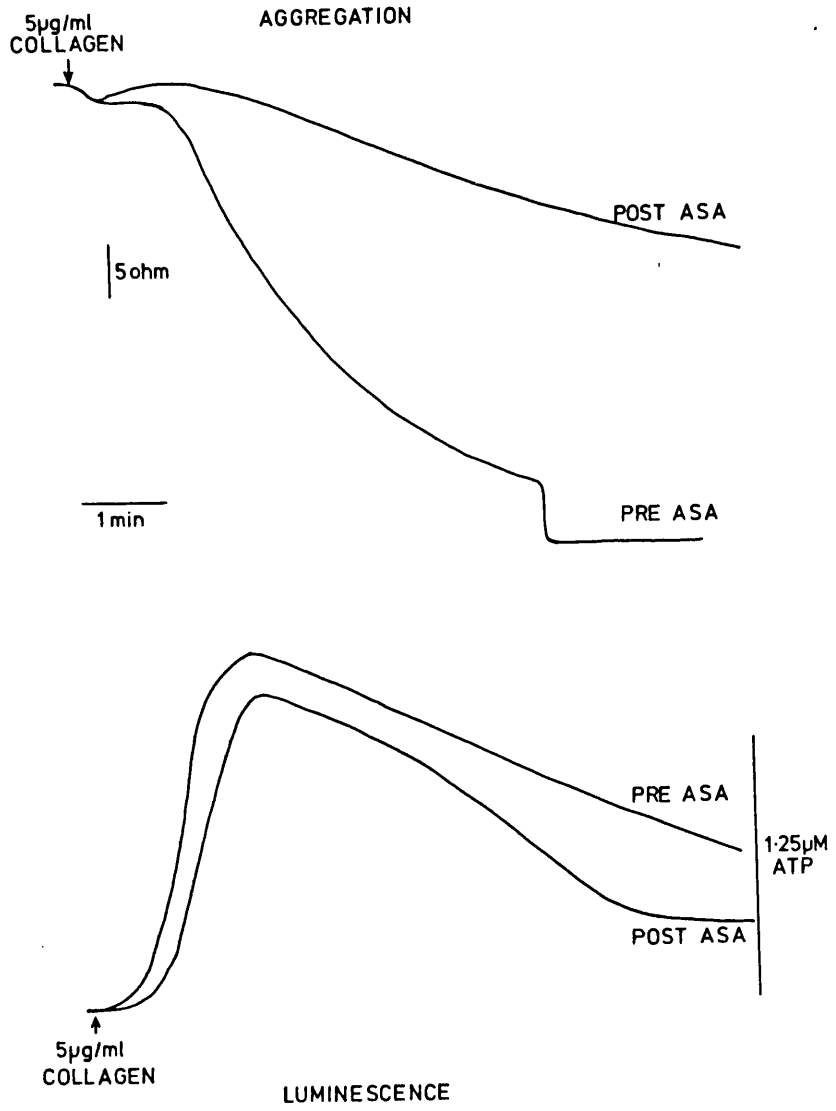

Fig. 3.26. Simultaneous Measurement of Aggregation and Luminescence (ATP release) in Response to Collagen in Whole Blood Before and After an Oral Dose of ASA.

3.9 DISCUSSION

The study of platelet aggregation in whole blood proved to be possible by the impedance method, although the nature of the responses was slightly different to those seen by other methods (Born, 1962; Lumley & Humphrey, 1981; Saniabadi et al, 1983). The aggregation responses obtained generally correlated with the dose of platelet agonist added, and a reduction in impedance response was seen with known inhibitors of platelet function. However a number of variables influenced the technique and required investigation and standardisation.

3.9.1 STIRRER SPEED AND CUVETTE TYPE

The influence of stirring speed on impedance changes was anticipated, since stirring increases the number of collisions between platelets (Born, 1962) which are essential for aggregates to form. The detrimental effect of high stirring speeds may be due to the interference of other blood elements on platelet aggregate formation, or the breaking up of aggregates and removal of adherent platelets from the electrode due to a vortex effect. In samples with very high sedimentation rates, a settling effect can occur despite the stirring, and an increased stirring rate may be necessary despite the possibility of aggregate loss from the electrode. The instrument was initially supplied with glass cuvettes, and most of the early experiments were performed with these. Suitable polystyrene cuvettes were subsequently found, and on comparison gave weaker responses. This suggested that activation of both platelets and plasma was occurring on the cuvette wall, and acting synergistically with the added

agonist, even though it was insufficient alone to cause aggregation. The more recent experiments were performed using polystyrene cuvettes, and appropriate normal ranges have therefore been expressed in the results sections of the chapters of this thesis.

3.9.2 PLATELET COUNT

Mixing studies showed that the impedance response of citrated blood was not significantly affected by low platelet counts, except below approximately $50*10^9$/L. When thrombocytopaenic patients were studied, a correlation between platelet count and impedance response could be demonstrated, at platelet counts below $150*10^9$/l. The correlation remained positive, but the slope of the regression line increased when patients with platelet counts below 100 or $50*10^9$/l were studied. This was because the plateau of response achieved at the higher counts was removed. There was a lack of correlation in normal subjects and patients with platelet counts above $150*10^9$/l. The AML subgroup of thrombocytopaenic patients, had weaker aggregation responses for a given platelet count when compared to ITP patients, and the regression line showed a lower slope. It may be important to identify such thrombocytopaenic individuals, with decreased platelet function, as they may have a higher risk of haemorrhage and require earlier support with platelet concentrates. Some of these patients also had a marked reduction in leucocytes, but there did not appear to be a correlation between absent aggregation and high or low leucocyte counts, since the latter varied between 0.3 and $74*10^9$/l in this group. Platelet size

also appears to play an important role, patients with higher MPV values had better aggregation responses, while AML patients tended to have smaller and less reactive platelets. The turbidometric technique is not very effective for diagnostic purposes below $100*10^9/l$, and is then very sensitive to the presence of lipaemia and contaminating red cells or their fragments. The impedance method therefore has a potential clinical application in patients with mild thrombocytopaenia, as well as patients with haemolytic disorders and a risk of haemorrhage.

3.9.3 THE INFLUENCE OF HAEMATOCRIT

Citrated whole blood had a lower resting impedance than blood diluted with saline or plasma, and should therefore be more sensitive to changes in its environment; however aggregation responses were better in diluted blood than in blood with a haematocrit above 0.3-0.35 l/l. This suggested that there was a mechanical influence of red cells rather than any effect due to changes in the basal impedance. It has been demonstrated that mechanical collision of red cells with platelet aggregates interferes with their growth, particularly under low shear conditions (Machi et al, 1984), but red cells may also impede aggregate accretion to the electrode, or play some biochemical role. The improvement in aggregation remains fairly constant as the haematocrit is lowered, until the platelet count becomes the limiting factor, and there was no correlation between the starting (before dilution) haematocrit or initial platelet count and the response obtained in blood from healthy normal individuals. Since there is a wide

variation of haematocrit in clinical samples it was important to standardise this parameter, and the results show that a haematocrit of 0.3 l/l or less is suitable, since the platelet count is not significantly reduced, and suits most clinical situations. Saline was the diluent of choice giving a steeper and more regular trace than PPP, and because it was immediately available, rather than requiring centrifugation or addition of non-autologous material. However, this may have disadvantages in situations where plasma factors are expected to play an important role.

3.9.4 AGGREGATION WITH DIFFERENT COLLAGEN TYPES

Similar dose response curves were obtained with tendon collagen (the reagent normally used throughout this thesis) in whole blood impedance aggregation as those used in whole blood counting and PRP turbidometric methods. However, when a preparation of skin collagen was used, aggregation could not be demonstrated in whole blood, even at 200 times the tendon collagen dose. With PRP, skin collagen only provoked responses when used at concentrations of 100 times those effective with tendon collagen, and then long lag periods were observed. Unlike tendon collagen, which is a suspension of fibrils, the skin collagen used was in a soluble form. Fibrillar forms of collagen are necessary to cause platelet aggregation (Jaffe & Deykin, 1974), and the time required for fibrils to form in plasma may explain the observed long lag periods. The two collagen preparations may also have a different composition with respect to collagen subtypes. Although collagen types I, III, IV, and V are able to cause platelet aggregation (Barnes

et al, 1980), the nature of the quaternary structure is important, and only types I and III appear to have this effect under physiological conditions (Sixma, 1987). The reason for the discrepancy between PRP and whole blood aggregation is unclear, but it may be that the skin collagen cannot polymerise into fibrils so readily in whole blood as in PRP, or that the binding characteristics to cells other than platelets are different between the collagen subtypes.

3.9.5 ADRENALINE-INDUCED AGGREGATION

Whole blood impedance aggregation responses to adrenaline were absent or very weak, which supports the view that the substance is not an important initiator of platelet aggregation. Other workers have made similar observations, and have found synergy with other aggregating agents in whole blood impedance aggregation studies (Joseph et al, 1987; Swart et al, 1984); surprisingly, Mannucci et al (1988) found comparable responses and dose ranges for adrenaline and ADP between whole blood impedance and PRP optical methods.

3.9.6 IMPEDANCE RESPONSES AND OTHER AGGREGATION METHODS

Reversible aggregation and biphasic responses to ADP, as seen in turbidometric aggregometers could not be demonstrated in WBIA, in agreement with the observations of Ingerman-Wojenski and colleagues (1983), even when wide ranges of ADP concentrations were used. The EC_{50} concentration of ADP for impedance aggregation was approximately 10 fold higher than that used in traditional PRP optical aggregation. These supraphysiological concentrations of ADP may be required

because of competitive uptake of ADP by red and white cell pathways. However, this is unlikely since the EC_{50} value for ADP aggregation measured by platelet counting in whole blood was shown to be similar to that seen in PRP by turbidometry, as has previously been reported (Lumley & Humphrey, 1981). The presence of red cells and/or leucocytes apparently has no effect on the amount of released ATP measured (Ingerman et al, 1979). The type of aggregate formed in whole blood, as well as the way in which that aggregate is detected by the impedance aggregometer probably accounts for the observed differences.

When aliquots of blood were removed from the impedance cuvette and fixed prior to cell counting, a rapid fall in numbers of single platelets was observed immediately after addition of ADP or U46619. 80-90% of free platelets disappeared without any significant change in erythrocyte or leucocyte counts. However, the impedance response lagged behind the platelet count response by 1-2 minutes, and the impedance trace reached a maximum long after the platelet count had reached a plateau. Similar results were obtained with collagen, except that there was a short lag period during which only a slow fall in platelet count occurred, before the platelet count fell rapidly. Only a single phase of impedance increase was observed, again lagging after the changes in free platelet count. Furthermore, the addition of low doses of ADP (1-3uM), which failed to cause any change in impedance, gave a detectable aggregation response by platelet counting. Ingerman-Wojenski and colleagues (1983) made similar observations using a ^{125}I-labelled monoclonal antibody to a platelet surface membrane glycoprotein instead of platelet

counting. After aggregation, the cells were fixed, and the blood partitioned into 3 layers, PRP, buffy coat, and red cells. Impedance was found to lag several minutes behind aggregate formation, and an accretion of ^{125}I-labelled platelets to the electrode coincided with the impedance response.

In plasma, platelets form small clumps within seconds of adding the aggregating agent, but there is little change in the turbidity of the PRP (Born, 1962; O'Brien 1962); the increase in light transmittance is caused mainly by larger aggregates (O'Brien, 1962). In whole blood, these large aggregates are clearly formed, but are not immediately detected, and take some time to adhere to the impedance electrode and cause an impedance change. This conflicts with the early work of Russel-Smith et al (1981), who suggested that the impedance method was very sensitive to small platelet aggregates. However, aggregate size may not be the only determinant of adhesion to the electrode and impedance change, since doses of ADP were found which gave a strong aggregation response as detected by platelet counting, but no impedance response. It is possible that the type of release reaction, or presence of suitable adhesive proteins on the platelet membrane may be a requirement for binding to the impedance electrode. The aggregate size may be important if single platelets and small aggregates have a tendency to circulate around the wall of the cuvette, rather than in a homogeneous fashion, due to rheological effects When larger aggregates are formed, the situation would differ, and the aggregates may tend to flow in the centre of the cuvette, thus having access

to the electrode, and adhering, with the resultant increase in impedance; such rheological effects in aggregometry have not been studied. Galvez et al (1986) have suggested that blood in circular movement is continuously striking the immersed electrodes, and since the accumulation of platelets at a surface involves both biochemical processes and fluid dynamic factors (Baumgartner, 1973; Badimon et al, 1986), the rate of aggregate growth on the electrode may be inhibited. In addition, flow forces delay thrombus growth (Regent & Born, 1970; Richardson, 1973).

3.9.7 PLATELET ATP SECRETION AND AGGREGATION

When doses of approximately 25uM ADP are used in whole blood impedance lumi-aggregometers, an immediate release of ATP is observed, before aggregation is detected by the impedance electrode. Optical and impedance methods using PRP, as well as whole blood counting methods, show aggregation as soon as ADP is added, but any changes in luminescence or ATP concentrations usually lag behind, occurring just before or during the secondary wave. However, at very high doses of ADP, optical methods also show immediate nucleotide release, and the secondary wave is indistinguishable from the primary. In whole blood, the rapid rise in ATP levels appears to be due to the high dose of ADP causing an immediate release reaction, since removal of aliquots of blood for platelet counting showed that the number of free platelets fell at about the same time as ATP levels increased. Ingerman-Wojenski et al (1982 & 1983) found a similar pattern of events using a ^{125}I-labelled monoclonal antibody to glycoprotein IIb/IIIa, and

separating platelet aggregates from PRP by sedimentation, before gamma counting. This implies that "secondary aggregation" was occurring very quickly, and yet an increase in impedance was not observed for over a minute. In addition, a patient with storage pool defect showed decreased ATP release, but aggregation was still present. This suggests that impedance aggregation is not just dependent on the formation of large aggregates, or dense granule release and secondary aggregation, but that some other factors are involved. These other factors may be the expression of particular membrane receptors, presence of cytoadhesins, or co-aggregation of other cell types.

3.9.8 THE LAG PERIOD IN IMPEDANCE AGGREGATION

It is possible that other blood cells mechanically interfere in accumulation of platelet aggregates at the electrodes. The addition of red cells even at 2% haematocrit has been shown to prevent the growth of platelet aggregates (particularly at low shear) in a different system involving a closed loop and aggregate detection by ultrasound (Machi, 1984). However, increasing the platelet count at constant haematocrit does not shorten the lag period unless very low platelet counts are being used; and it has been shown that red cells do not hinder the accretion of labelled platelets to the electrodes during aggregation (Ingerman-Wojenski et al, 1982 and 1983). Red cells are invariably observed in the platelet aggregate adhering to the impedance electrode after aggregation, but these may well be merely trapped in the platelet mass, and not actually co-adhering. Of course

mechanisms other than simple physical obstruction may still be involved, such as the uptake and release of biologically active substances by red and white cells.

During the lag period before impedance aggregation induced by ADP or collagen, there is often a transient decrease in impedance, before aggregation is detected as an increasing impedance. This effect is unlikely to be due to a change in platelet shape, which should not alter the impedance of the solution. It has been suggested that it is due to relatively high concentrations of red cells in the electrode area, and that it is most prominent at haematocrits greater than 0.43 l/l, and disappears after dilution of the blood with an equal volume of saline or plasma, (Ingerman-Wojenski et al, 1983). The results shown above indicate a marked effect of red cells on platelet aggregation responses, particularly when the haematocrit is above 0.3 l/l. However, a slight fall in impedance values during the lag period to collagen was still seen at low haematocrits. Another group (Musumeci et al, 1987) have recently suggested that the impedance decrease is related to the poor conductivity of red cells and that at high haematocrit, stirring causes an increase in red cell concentration around the electrode where red cell aggregates may form. The change in impedance produced by platelet accretion on the electrodes may be blunted or even reversed by a reduction in baseline impedance produced by the displacement of red cells from the electrodes.

An alternative hypothesis is that platelets themselves are responsible for both the decrease in impedance, and the increase that follows. The initial decrease in impedance

occurred with high doses of ADP (although not as marked) as well as with collagen, and in both cases, coincided with an immediate change in the numbers of free platelets was seen. The changes in platelet number seen after addition of collagen may well be associated with adherence of platelets to collagen fibrils before aggregate formation, since they were less rapid than those seen later, or with ADP. The change in number of free platelets in solution would cause fewer platelets to circulate through the electrodes, and may cause a decrease in the basal impedance of the whole blood. As larger aggregates are formed, they start to adhere to the electrode and therefore cause an increase in the impedance value.

3.9.9 PLATELET FUNCTION ANTAGONISTS

A further discrepancy between turbidometric and impedance aggregation is the influence of acetyl salicylic acid (ASA). Parallel inhibition of impedance aggregation to collagen was obtained both in vitro and ex vivo, with stronger inhibition at lower doses of collagen, similar to turbidometric results (Packham & Mustard, 1977). Riess et al (1985) detected the effects of a single oral dose of ASA for longer in whole blood than in PRP; and De La Cruz et al (1987) also found greater ex vivo inhibition in whole blood than PRP, after ASA was taken daily for 10 days, as well as more effective in vitro inhibition. This was in contrast to some previous results (Ingerman-Wojenski et al, 1982; Ingerman-Wojenski & Silver, 1984), where no in vitro inhibition of impedance aggregation by ASA was found at collagen doses above 2ug/ml, although these authors did not dilute the blood for aggregation, which

may have decreased the sensitivity, and masked the inhibitory effects. These workers also found a decrease in ATP release after similar oral doses of ASA, but no inhibition of impedance responses to 2.5ug/ml collagen. However in PRP, ASA has little effect on the amplitude of aggregation, and no effect on the rate, since only secondary aggregation is affected. This was not true for whole blood impedance aggregation where high (25uM) and low doses of ADP could be completely inhibited by ASA in vitro. This suggests that impedance aggregation may be dependent on thromboxane generation, or its consequences, since it was shown above that impedance aggregation did not correlate with dense granule release or primary aggregation to ADP. The endoperoxide analogue U46619 induced impedance aggregation in a dose dependent manner, but responses lagged behind aggregation as measured by disappearance of free platelets in counting methods using aliquots of blood removed from the impedance cuvette. U46619 impedance aggregation responses could only partially be inhibited by ASA, with a residual resistant component, which was presumably due to the direct effects of the thromboxane A_2 formed. The partial inhibitory effect was likely to be due to blockade of feedback arachidonate liberation and conversion to the endoperoxides PGG_2 and PGH_2. As expected, the thromboxane receptor antagonist AH23848 had a potent inhibitory effect on aggregation to U46619 in impedance, counting, and turbidometric methods, although in ex vivo samples, the effects of the receptor antagonist were far more potent in impedance and turbidometric methods.

In two individuals studied before and after both in

vitro, and ex vivo (24 hours after ingestion) ASA, ADP aggregation showed no ex vivo inhibitory effects of ASA, despite potent inhibition in vitro; paradoxically there was potentiation of ADP aggregation by ASA in ex vivo samples. ASA acetylates and thus inactivates platelet cyclo-oxygenase (Carvalho & Rao, 1987), and since platelets lack a nucleus and lose the ability to synthesise protein soon after detachment from the parent megakaryocyte, they are unable to replace the cyclo-oxygenase enzyme. Some other mechanism within the platelet must therefore be influenced to compensate for this inactivation, or alternatively another cell type which has the capacity to overcome the action of ASA by new protein synthesis may be involved, and influence platelet aggregation. A candidate for the latter function may be polymorphonuclear leucocytes.

Prostacyclin and its stable analogue Iloprost, caused a dose dependent inhibition of whole blood impedance aggregation to collagen and ADP, with parallel depression of rate and amplitude. The IC_{50} value for prostacyclin on ADP was lower for impedance than turbidometric aggregation. These results were similar to those of others (Riess et al, 1985; Splawinski et al, 1984; Gresele et al, 1984), who found that prostacyclin was more active against platelet aggregation in whole blood than PRP; but in contrast to those of Ingerman-Wojenski et al (1982 & 1983) who found poor sensitivity of the impedance method to prostacyclin, and a decreased rate but unaffected amplitude.

When Iloprost was infused into healthy normal volunteers, significant inhibition of impedance aggregation was seen in

the first hour of the infusion, but after increasing the Iloprost dose during the second hour, a slight increase in impedance aggregation was detected. In PRP, turbidometric aggregation showed a progressive inhibition throughout the period of the infusion. Both methods showed a rebound hyperaggregability, with responses being greater than the pre-infusion baseline, after stopping the infusion, but no spontaneous aggregation was detected. The hyperaggregability coincided with an increase in serum thromboxane B_2 levels, and this may explain the earlier tailing off of inhibition by impedance rather than turbidometry methods, if thromboxane has an important role in the impedance method. The cause of the observed hyperaggregability and increase in serum thromboxane levels remains unknown. The sensitivity of each donor to Iloprost seemed to be similar in vitro to ex vivo.

3.10 CONCLUSIONS

The time course, shape of the aggregation curve, and dose response relationship to certain agonists in whole blood impedance aggregation differed from other methods of studying platelet aggregation. The dose response relationship with collagen was similar in the aggregation methods studied, and this was therefore considered a useful agonist for studying the various factors influencing impedance aggregation.

There was no significant effect of platelet numbers on the impedance response with counts of $50\text{-}600*10^9/l$; below this range a linear relationship was observed between platelet count and impedance response for normal and ITP platelets. Certain patients with haematological malignancy and

thrombocytopaenia had weaker than expected platelet function for their platelet count. The impedance method could be useful for identifying patients at risk of bleeding, who would not necessarily receive platelet concentrates because of their platelet count alone.

Haematocrit values greater than 0.35 l/l depressed the aggregation response and caused poor reproducibility, and it was necessary to dilute whole blood to a constant 0.3 l/l or less. It is likely that this effect of red cells was due to mechanical interference.

Strong responses could be obtained to collagen, arachidonic acid, and a synthetic endoperoxide analogue. Little or no aggregation was obtained with adrenaline, and high doses of ADP were required. Only a single wave of aggregation was seen. When platelet aggregation was assessed by removing aliquots of blood from the impedance cuvette and counting free platelets, the fall in platelet count followed a similar time course to turbidometric aggregation measurement of PRP, but preceded detection of aggregation by impedance. Similar results were obtained to a variety of agonists, and low doses of ADP (1-3uM) caused a fall in free platelets, but no change in impedance. The release reaction also preceded impedance aggregation in whole blood, and a patient with storage pool defect with absent ADP and ATP release had detectable impedance aggregation. The formation of irreversible aggregates appears to be essential for impedance aggregation, but aggregate size, and presence of surface associated proteins may be important. The production of thromboxane A_2 may also be required, since acetyl salicylic

acid blocked impedance responses to high and low doses of ADP, whereas the endoperoxide analogue U46619 was only partially blocked; and a thromboxane receptor blocker had potent inhibitory effects against impedance aggregation.

The initial decrease in the impedance aggregation tracing correlates with the fall in free platelet numbers, and may be due to an alteration in the basal impedance of the solution due to platelets aggregating. As the larger aggregates stick to the impedance electrode, the pattern is reversed, with impedance between the two platinum electrodes increasing.

The impedance method was more sensitive to prostacyclin than turbidometric methods. Normal volunteers infused with the stable prostacyclin analogue Iloprost initially showed inhibition of aggregation in the whole blood impedance method and PRP turbidometric method. Later in the infusion, inhibition was no longer detected in whole blood, although still present in PRP. Both methods showed rebound hyperaggregability, coinciding with increased levels of serum thromboxane B_2, after stopping the Iloprost infusion.

Doses of ADP and collagen in the upper part of the dose response curves were selected for clinical investigations, since there was room for increased aggregation detection as well as decreased responses, and it was impractical to perform aggregation at a number of doses for each reagent. Collagen was used as the agent of choice where only limited numbers of responses could be obtained, since it is a natural platelet activator, and ADP requires supra-physiological doses for impedance aggregation, while arachidonate and U46619 stimulate platelets by acting as substrates for specific platelet

enzymes. Whole blood impedance aggregation has a role in the study of platelet function in thrombocytopaenia. Patients could be subdivided into those with responsive or dysfunctional platelets, and it is clear that it would be an advantage to monitor their platelet function as a guide to modifying therapy and implementing support with platelet concentrate infusions. Platelet responses to anti-platelet drugs differed in whole blood as compared to other systems, and so there is a clear role in prothrombotic patients and drug compliance studies.

Obviously it would be desirable to study platelet function in a completely natural milieu, and this can be taken a step closer by measuring platelet function in the presence of extracellular calcium ions, and thus allowing thrombin generation. The method was therefore further developed so that there was no calcium chelation, ie. a method using non-anticoagulated blood; this is presented in the following chapter.

CHAPTER 4

IMPEDANCE AGGREGATION IN NON-ANTICOAGULATED WHOLE BLOOD

4.1 INTRODUCTION

In order to understand the pathophysiological processes involving platelets in vivo, we require laboratory tests and models which approximate the natural situation as closely as possible. Several approaches have been tried for the study of platelets in whole blood with conditions close to the natural state. Citrated blood is far from ideal, since citrate chelates extracellular calcium ions and reduces the pH (Tsao et al, 1976; Rogers, 1972). Calcium ions are required for the activation of most of the coagulation zymogens as well as many of the systems involved in platelet activation, release, and aggregation. In addition, calcium ions are an essential requirement for certain leucocyte processes such as aggregation (Russell-Smith et al, 1981). Some workers have used blood anticoagulated with heparin, but this substance binds to, activates and promotes aggregation of platelets, as well as activating neutrophils (Zucker, 1975; Thomson et al, 1973; Lazarowski et al, 1986). The elimination of anticoagulant allows thrombin generation to occur in the presence of physiological levels of calcium ions, and the measurement of platelet aggregation in whole blood becomes very close to the physiological situation; only local rheological factors are missing. There are a special set of problems associated with the elimination of anticoagulant and the use of non-anticoagulated blood. Rapid transport and handling of the sample are necessary before coagulation occurs, and fibrin formation may interfere with the method of

aggregate detection; aggregation must therefore be measured before the blood clots.

One of the earliest events in primary haemostatic plug formation is adhesion and activation of platelets with the consequent release of substances involved in thrombin generation, and the provision of an organising surface on which coagulation reactions can take place. In vitro, platelets aggregate in non-anticoagulated blood before significant fibrin is generated, and it is therefore possible to study platelet aggregation in non-anticoagulated blood. Although under the conditions used, platelets did not aggregate 'spontaneously' before blood clotted in the cuvette, aggregation could be induced by suitable agonists within the clotting time. The effects of a variety of agonists and inhibitors on non-anticoagulated whole blood platelet aggregation has been investigated.

4.2 NON-ANTICOAGULATED WHOLE BLOOD TECHNIQUE

A dual channel impedance aggregometer, chart recorder and ancillary equipment (see Chapter 2) were mounted on a trolley so that they could be moved close to the subject being bled. Blood was collected by venepuncture with minimal stasis, and the first 5ml discarded, 2ml of blood was then drawn into a small syringe and immediately ejected into a polystyrene tube prewarmed to 37°C. Duplicate 650ul volumes of this blood were pipetted into plastic 9*44mm cuvettes containing 325ul isotonic saline and a teflon coated stir bar, stirring (at 600 rpm) at 37°C, in the aggregometer. The electrodes were inserted, and aggregation was monitored on a chart recorder at

a chart speed of 2cm/minute, until clot formation occurred. The chart was calibrated by making a 5 Ohm signal equal to 10 small chart divisions (2.5cm). The lag time from blood collection to a measurable increase in impedance was determined, as well as the clotting time, initial slope of response, and extent of aggregation (Fig. 4.1). In the initial experiments, the whole blood clotting time was also determined, by tilting identical plastic tubes containing the same volumes of blood and saline, in a 37°C waterbath.

In the initial studies, blood was sampled through a 19 or 21 gauge 'Butterfly' cannula (Abbott Laboratories Ltd), kept patent with sterile isotonic saline. The first 5ml of blood was always discarded, and the next 2ml used for studying non-anticoagulated whole blood aggregation. The cannula was then flushed with saline, and the latter was continuously pushed through the Butterfly until the next sample time. The effect of using glass or polystyrene cuvettes on the aggregation response was also studied. Aggregation responses of non-anticoagulated whole blood were also compared to those obtained with citrated whole blood.

Impedance aggregation responses to ADP and collagen (Hormon-Chemie, Munchen) were studied by adding 25ul of agonist to the prewarmed saline in the cuvette 2 minutes before adding the blood. Similarly, the effects of inhibitors were studied by adding them to the cuvette and reducing the saline by an equivalent volume; a final concentration of 2u/ml hirudin (Sigma Chemical Co.) was added to prevent fibrin formation by thrombin; 1ug/ml colchicine was used to prevent neutrophil degranulation; and various doses of acetyl

salicylic acid (ASA), Iloprost (Schering Chemicals Ltd), or AH23848 (Glaxo Ltd) were added to inhibit platelets.

4.3 PLATELET AGGREGATION AND CLOT FORMATION

The process of collecting blood, adding it to cuvettes, and starting to monitor the aggregation trace took approximately 15 seconds. With the exception of patients on intravenous heparin therapy, clot formation could be detected in all samples studied by NWBIA as a sharp increase in impedance (Fig. 4.1 & 4.2), often preceded by a slight dip in the aggregation trace. Clots could be visually detected after these changes in aggregation trace (but not before), by removing and inspecting the electrodes, or inserting orange wood applicator sticks. The clotting times measured in this way were similar to those obtained by traditional tube tilting techniques, with diluted blood in plastic tubes. The clotting time was prolonged in patients with congenital coagulation defects as well as those receiving intravenous heparin.

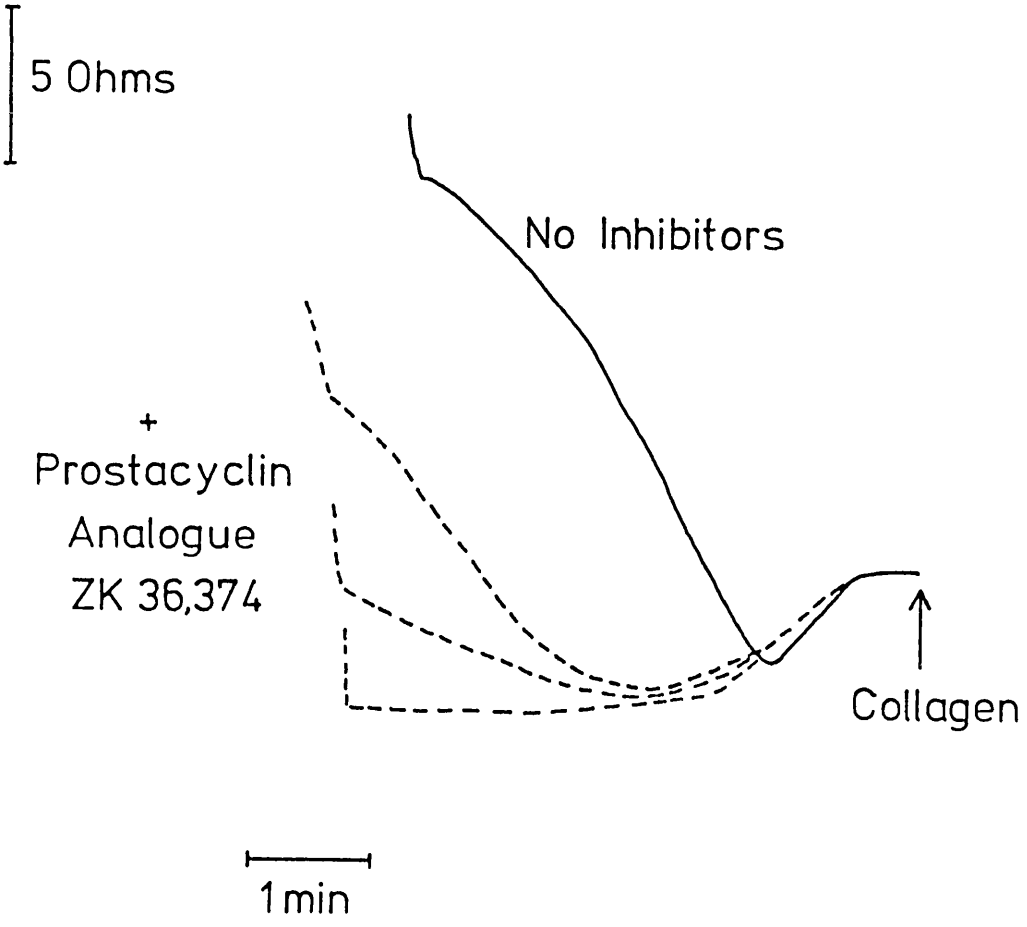

Fig. 4.1. Parameters Measured in Non-Anticoagulated Whole Blood Aggregation. A typical response to 5 ug/ml collagen: a = lag phase (min), b = clotting time (min), c = aggregation rate or slope (acute angle measured in degrees), d = amplitude or extent (Ohms); arrow indicates blood collection.

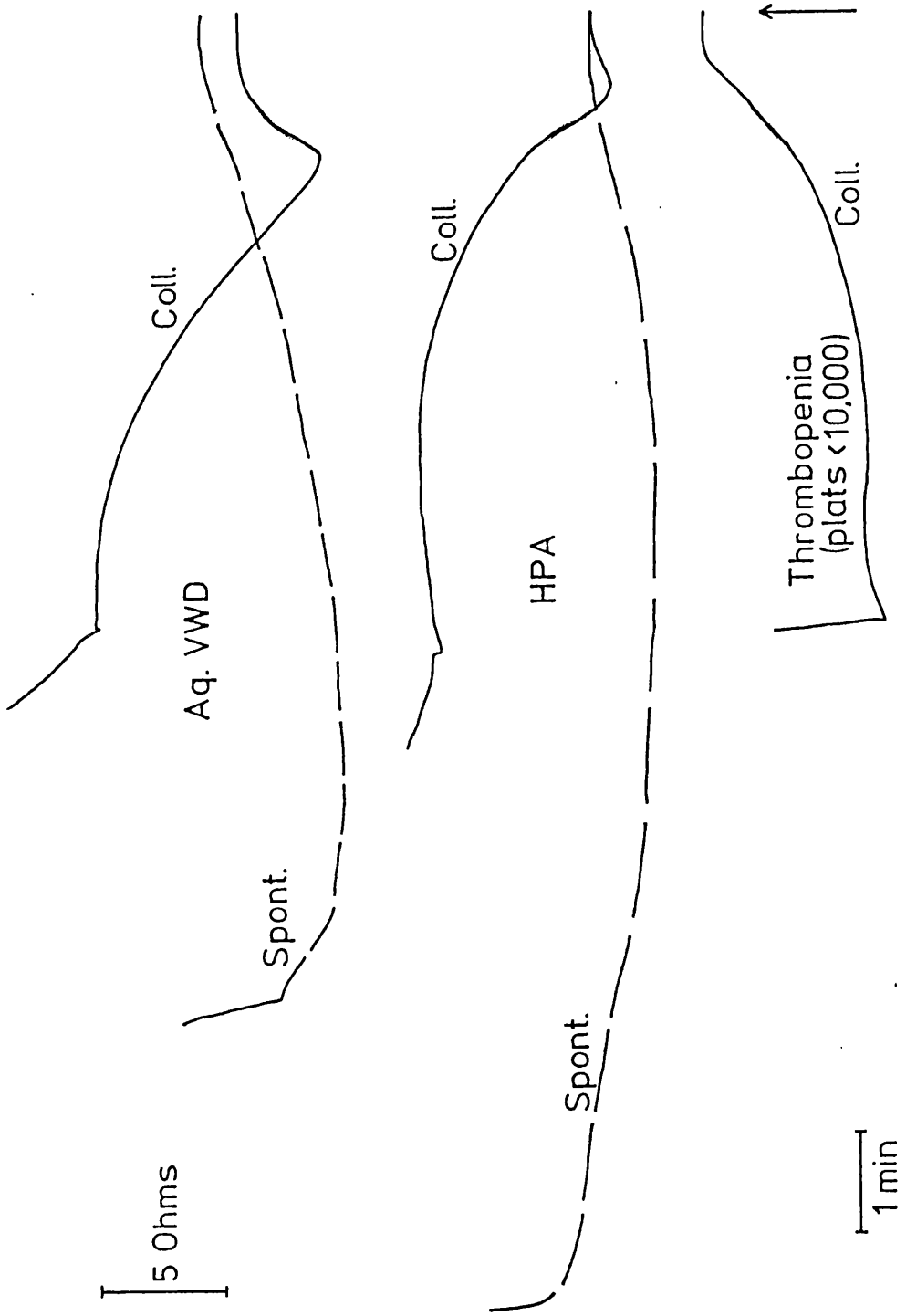

Fig. 4.2. Spontaneous Aggregation in Non-Anticoagulated Blood Samples From Patients With Haemophilia, Acquired von Willebrands Disease, and Thrombocytopaenia.

Platelet aggregation was measured as a progressive increase in impedance usually occurring within a few minutes of blood collection. In normal subjects spontaneous aggregation in the absence of exogenous agonist was not detected before clot formation even if plastic cuvettes were used, so that the clotting time was prolonged (see below). This lack of aggregation was presumably due to the absence of subendothelial tissues as well as the ADP and other substances released from damaged cells, which would normally contribute in the activation of platelets after in vivo trauma. In addition, the impedance method appears to detect a particular type of aggregate (Chapter 3). In haemophiliacs, where the whole blood clotting time is prolonged, some spontaneous aggregation was detected after several minutes (Fig. 4.2), when blood would have clotted in normal samples. These results also suggested that responses to collagen can be measured independently from any background aggregation component induced by thrombin. The clotting time in normals and haemophiliacs was shortened by the addition of collagen, probably due to activation of the contact system and the platelet release reaction. As expected, aggregation was not observed in patients with severe thrombocytopaenia, although clotting still occurred (Fig. 4.2).

4.4 BLOOD SAMPLE COLLECTION AND AGGREGATION CUVETTES

The use of glass cuvettes caused an enhancement of responses (also observed with citrated blood, see Chapter 2) and a shorter clotting time, indicating contact activation of the sample. This was undesirable since aggregation could only

be observed in a very short time before clot formation, and the presence of a glass surface introduced a further variable; all subsequent work was therefore carried out using plastic cuvettes.

Repeated venepunctures are unpleasant and may be stressful, so attempts were made to collect serial blood samples through 'Butterfly' cannulae, kept patent by flushing with isotonic saline between blood samples, and discarding the first 5ml of blood drawn. No differences were seen between initial samples collected by 'Butterfly' cannulae or those taken by ordinary venepuncture with a hypodermic needle of the same gauge. However, if the 'Butterfly' was then flushed with saline and multiple samples collected over a period of up to 60 minutes, steeper aggregation slopes and sometimes reversed dose response curves were obtained. In addition the 'Butterfly' devices often became occluded, and their use in this fashion was undesirable. All samples were therefore collected by separate venepunctures in the rest of the study.

Precision was tested by reproducibility studies in one volunteer who was experienced with and tolerant to venepunctures. A coefficient of variation of 7% was obtained for the aggregation slope. The reproducibility of the extent of aggregation was not as good, since the aggregation response did not always reach its plateau before clot formation took place. The precision figures obtained with citrated whole blood are also relevant here, since the principle of measuring the accretion of platelet aggregates to the electrode and increasing the impedance is basically the same in both types of blood. Twenty eight impedance responses using citrated

blood samples from three volunteers, gave a coefficient of variation of 9% for the aggregation slope (Chapter 3).

4.5 AGGREGATION RESPONSES

Platelet aggregation to ADP and Collagen could be detected in a dose dependent manner before clot formation took place. These responses could be inhibited by Iloprost, a stable analogue of prostacyclin, and acetyl salicylic acid (see below). More consistent results were obtained with collagen rather than ADP-induced aggregation, and the results in 20 healthy normal laboratory staff (10 male, 10 female) are shown in Table 4.1. There were no significant differences between responses in blood samples from males and females.

	HCt	Plats	LAG	CT	SLOPE
Mean	275	192	1.25	4.07	55.0
SD	20.6	55.8	0.27	0.57	9.6
Range	240	121	0.75	3.0	33
	-305	-356	-1.7	-5.3	-71

Table 4.1 - Non-Anticoagulated Whole Blood Impedance Aggregation To 5ug/ml Collagen (n=20). Final haematocrit (HCt) and platelet count (Plats) in the cuvette are shown. Lag period (LAG) is expressed in minutes, clotting time (CT) also in minutes, and aggregation rate as the slope in degrees.

There was a good correlation between the slope and extent of aggregation ($r=0.7$, $p<.005$), but neither of these parameters correlated with the Haematocrit, platelet count, Lag period, or clotting time, suggesting that they have little influence on aggregation responses within the limits studied. However, extreme platelet counts and haematocrits may well influence the results as seen in citrated whole blood aggregation.

	LAG		SLOPE		EXTENT	
	NWB	CWB	NWB	CWB	NWB	CWB
MEAN	1.26	1.34	57.5	49.8	10.8	14.2
SD	0.27	0.43	8.6	13.0	4.1	4.1

Table 4.2. Comparison of Non-anticoagulated (NWB) and Citrated (CWB) Whole Blood Impedance Aggregation to 5ug/ml collagen in 10 Normal Volunteers. Lag measured in minutes, slope in degrees, and extent in Ohms.

4.6 COMPARISON OF IMPEDANCE RESPONSES IN NON-ANTICOAGULATED AND CITRATED WHOLE BLOOD

Platelet aggregation in non-anticoagulated and citrated whole blood were compared in 10 of the volunteers. Both ADP and collagen gave shorter lag periods and steeper slopes in non-anticoagulated blood (Table 4.2, Fig. 4.3). The extent of aggregation appeared lower in non-anticoagulated blood since clot formation often preceded maximal aggregation. The increased aggregation in non-anticoagulated blood was not due to differences in haematocrit or platelet count, since citrated whole blood was diluted by the same amount, but could have been due to a substance with very short half life, although citrated whole blood aggregation was performed immediately after the non-anticoagulated whole blood response (ie. within 10 min).

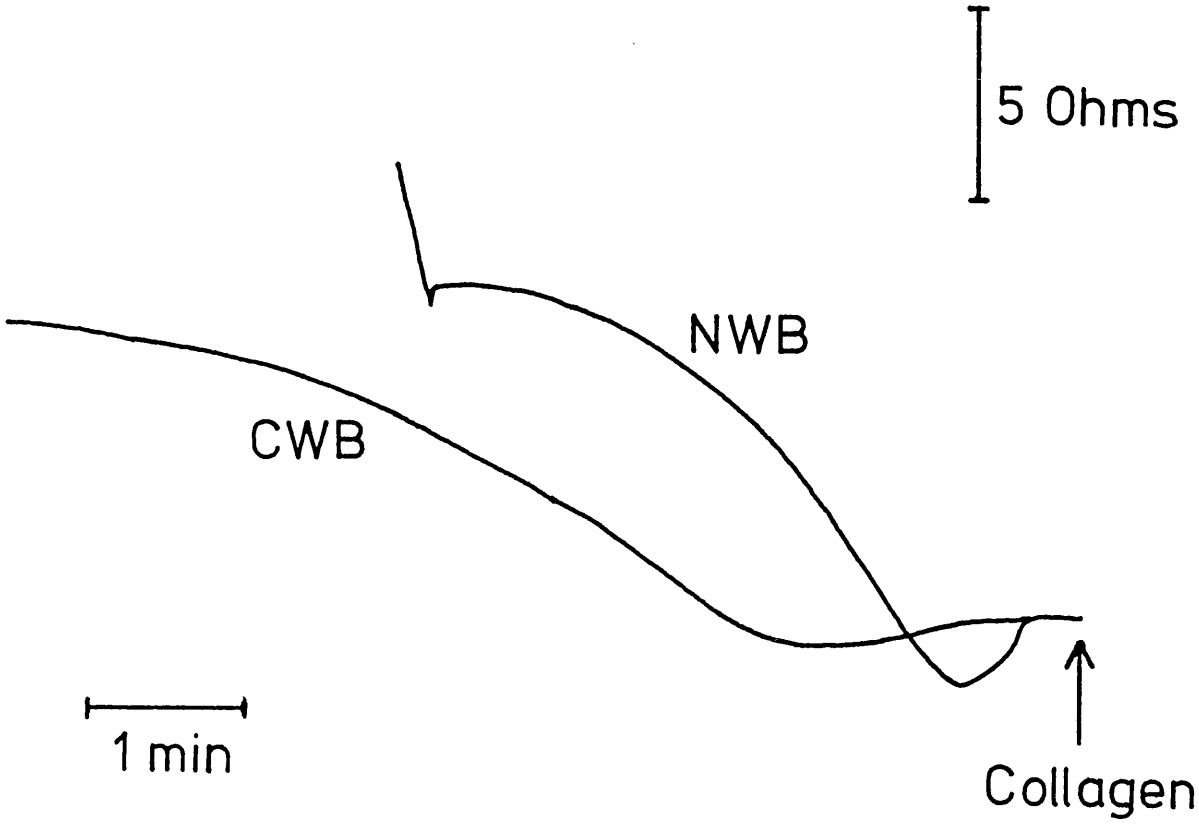

Fig. 4.3. Comparison between Impedance Aggregation in Citrated and Non-Anticoagulated Blood.

In a similar method, Zwierzina and Kunz (1985) have shown that there is a lower threshold for collagen induced impedance aggregation in non-anticoagulated than in citrated whole blood. However this study used multiple venepunctures, which may be stressful and thus activate platelets, and undiluted whole blood was used. The importance of diluting human blood for impedance aggregation (Mackie et al, 1984) is discussed in Chapter 3, and has recently been confirmed by Johnson & Davis (1986).

4.7 CALCIUM IONS AND THE INFLUENCE OF THROMBIN

When citrated whole blood was recalcified to give a final concentration of 5mM Ca^{++}, an increase in aggregation was seen with no change in the lag period (Table 4.3). These results show that calcium dependent processes may influence impedance aggregation. The most likely candidate to explain the phenomenon is thrombin; although thrombin alone could not account for the observed increase in aggregation, since no aggregation appears in the absence of collagen during this time, and the lag period was not shortened. There may be a synergistic effect between thrombin and collagen, or there may be other compounds generated in the presence of calcium ions and thrombin which enhance platelet aggregation.

	Lag		**Slope**	
Calcium	**-**	**+**	**-**	**+**
MEAN	1.25	1.10	39.3	48.8
SD	0.30	0.09	8.02	3.31
p	NS		0.03	

Table 4.3. The Influence of Recalcification on Impedance Aggregation Using Citrated Blood. Aggregation responses to 5ug/ml collagen were measured in the presence (+) and absence (-) of 5mM Ca^{++}, and analysed by non-parametric statistics.

Hirudin is a small protein (<20kd) purified from the saliva of the European medicinal leech (Markwardt, 1970), which acts as a potent inhibitor of thrombin. When mixed with saline diluent prior to adding blood, to give a final concentration of at least 2U/ml, hirudin had a weak, but statistically significant ($p<0.01$) effect on non-anticoagulated whole blood aggregation responses, causing a decreased slope and prolonged lag phase and as expected, a marked prolongation of the time for clot formation (Table 4.4). When final concentrations of less than 1U/ml hirudin were used, no inhibition of the aggregation response was observed. These results explain the apparent discrepancy with the data of Zwierzina & Kunz (1985), who found no statistically significant differences when hirudin or heparin at a final concentration of 0.025u/ml were mixed with non-anticoagulated whole blood prior to collagen aggregation. Apart from the methodological differences, these authors used much lower hirudin concentrations, which may have been swamped by the thrombin generated. No effects were observed in similar experiments on aggregation in citrated whole blood. It appears that in normal individuals, only a small part of the increased aggregation seen in non-anticoagulated whole blood and in recalcified citrated whole blood is due to thrombin generation, and the direct effects of thrombin on platelets.

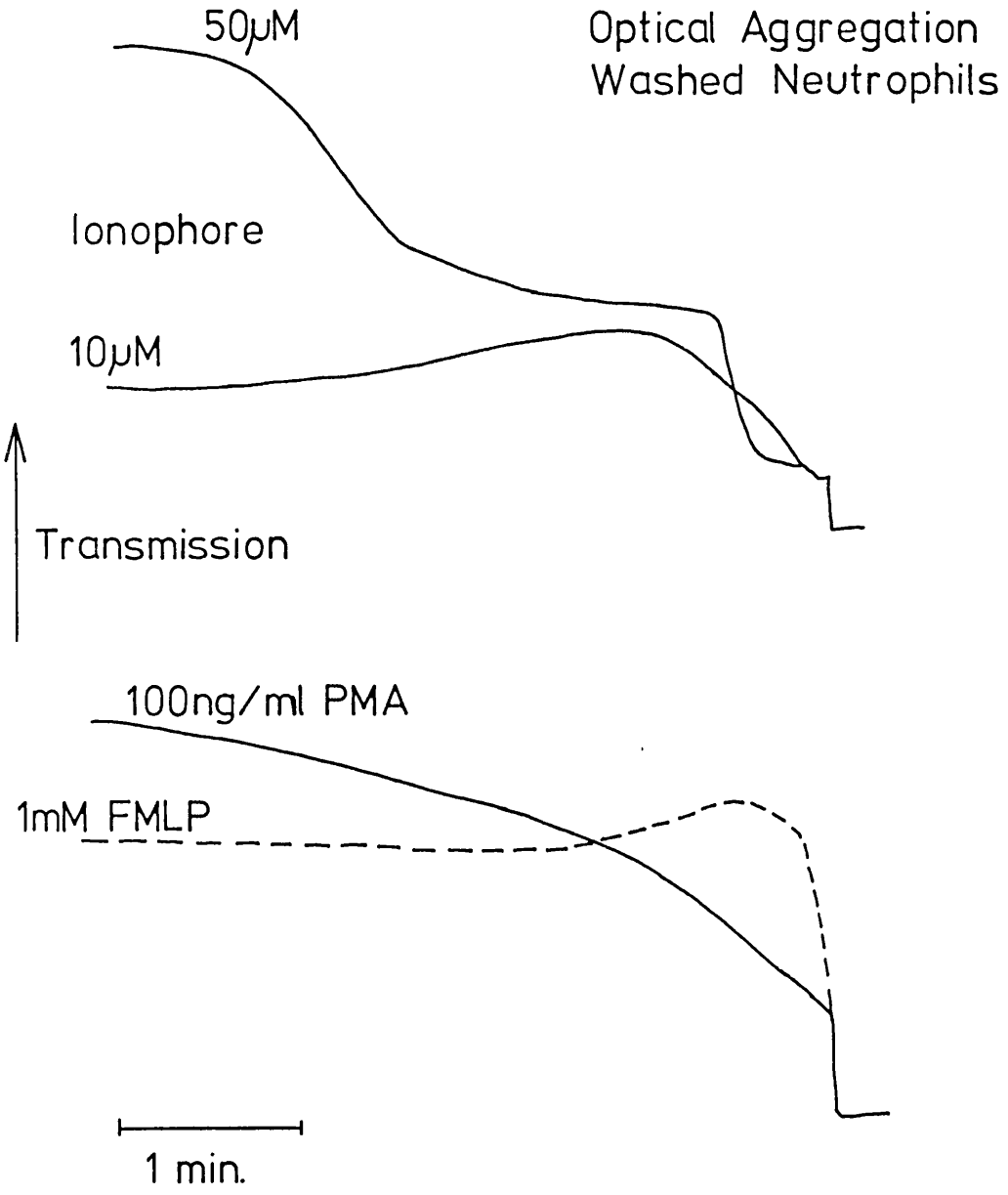

Fig. 4.4. Neutrophil aggregation in a washed cell suspension measured by a turbidometric technique. Final neutrophil count $6*10^9/l$, the final concentrations of agonists used were: 10 & 50uM Calcium Ionophore A23187; 1mM FMLP; 100ng/ml PMA.

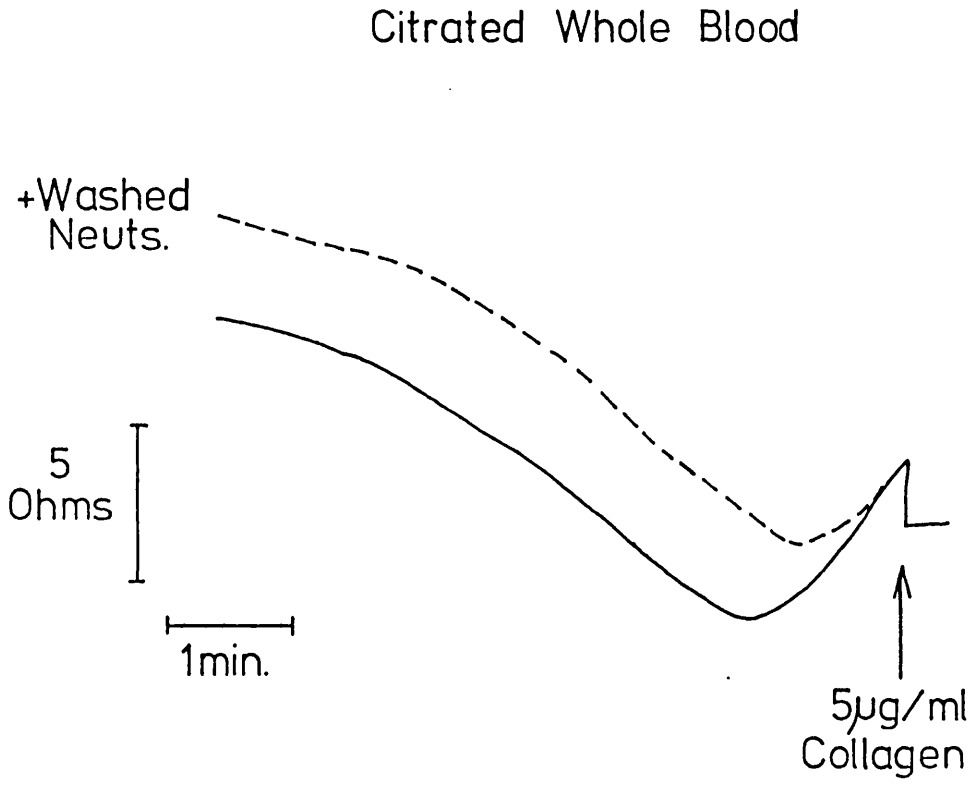

Fig. 4.5. Aggregation responses in mixtures of citrated whole blood and washed neutrophils.

SUBJECT	LAG	SLOPE	C.T.
HL	100	81	166
MW	62	108	200
IM	131	87	154
KW	140	79	186
JR	100	86	180
DC	115	98	176
TM	143	94	130
RW	129	79	143
Mean	115.0	89.0	166.9
SD	27.1	10.3	23.4
P	NS	0.04	0.01

Table 4.4. - The Influence of Hirudin on Aggregation in Non-Anticoagulated Blood. Aggregation to 5ug/ml collagen was measured in the presence and absence of hirudin (2u/ml f.c.); results are expressed as a percentage of the saline control, and were analysed by non-parametric statistics.

4.8 LEUCOCYTES AND NON-ANTICOAGULATED WHOLE BLOOD AGGREGATION

The increased aggregation in non-anticoagulated whole blood may be mediated through leucocyte activation and release. PMN's aggregate in response to certain chemotactic factors and other reagents such as N-Formyl-l-Met-l-Leu-l-Phe (FMLP, a bacterial cell wall peptide), complement C_5 fragments, calcium ionophore A23187, PAF-acether, Leukotriene B_4, phorbol myristate acetate (PMA), and thrombin (Issekutz & Ripley, 1986; Craddock et al, 1978; O'Flaherty & Ward, 1978; Camussi et al, 1981; Ford-Hutchinson et al, 1981; Ricevuti et al, 1987). The mechanism of PMN aggregation is poorly understood, but probably reflects cell membrane rather than cytoskeletal changes. PMN adhesion and aggregation frequently precedes degranulation, but the latter can occur independently. PMN aggregation differs from platelet aggregation in several ways; the response is dependent on extracellular calcium flowing into the cytoplasm and no aggregation occurs in response to ADP, adrenaline, or collagen

(Ricevuti et al, 1987). Most publications have used cells from laboratory animals rather than humans.

PMN aggregation could not be induced in citrated whole blood, but impedance aggregation of washed neutrophils could be demonstrated with FMLP, PMA, and calcium ionophore (Fig. 4.4). The addition of washed normal polymorphonuclear neutrophils (PMN's) to citrated whole blood caused potentiation of the aggregation response to collagen (Fig. 4.5), shortening the lag period and increasing maximal impedance.

	A)			**B)**		
SUBJECT	**LAG**	**SLOPE**	**C.T.**	**LAG**	**SLOPE**	**EXTENT**
IM	150	78	114	-	-	-
TM	125	87	100	-	51	44
AC	136	87	93	110	77	70
NL	83	94	93	222	57	43
MW	129	88	118	110	72	59
RF	89	109	100	100	113	139
Mean	**118.7**	**90.5**	**103.0**	**135.5**	**74.0**	**71.0**
SD	**26.8**	**10.4**	**10.6**	**57.9**	**24.2**	**39.6**
p	NS	<0.05	NS	NS	<0.05	NS

Table 4.6. - The Influence of Colchicine on Whole Blood Aggregation. 1ug/ml colchicine was added to: A) non-anticoagulated blood, and B) citrated blood, before aggregation with 5ug/ml collagen. Results are expressed as a percentage of a saline control.

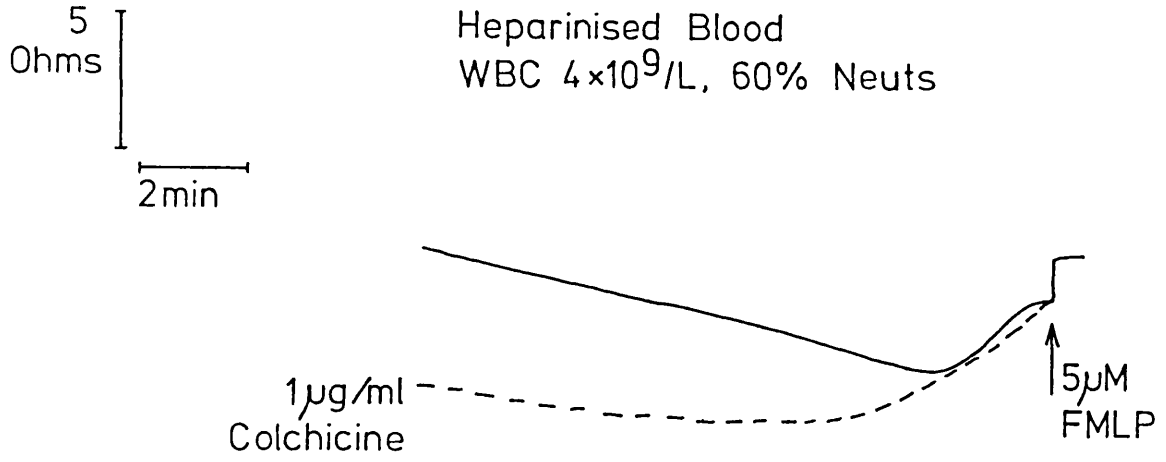

Fig. 4.6. The Influence of Colchicine on Neutrophil Impedance Aggregation to FMLP in Heparinised Blood.

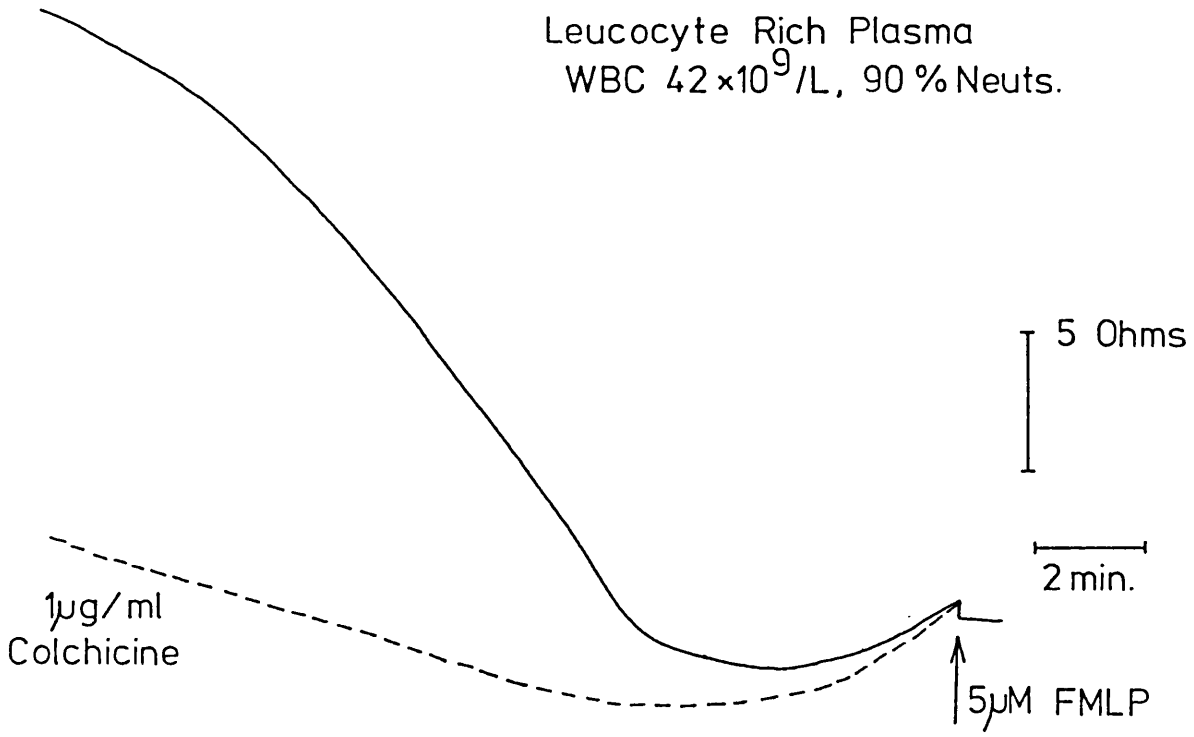

Fig. 4.7. The Influence of Colchicine on Neutrophil Impedance Aggregation to FMLP in Leucocyte Rich Plasma.

Colchicine is an anti-tubular agent, which blocks PMN degranulation (O'Flaherty & Ward, 1978), and may inhibit adhesion and aggregation (Russell-Smith et al, 1981), although the latter is controversial (O'Flaherty & Ward, 1978; Penny et al, 1966). The addition of 1ug/ml colchicine to heparinised blood or leucocyte rich plasma immediately prior to the addition of FMLP, caused inhibition of the neutrophil impedance aggregation response (Fig. 4.6 & 4.7) When neutrophils were counted before and immediately after maximal aggregation of heparinised (10 u/ml) blood with 5uM FMLP, the presence of colchicine (1ug/ml) inhibited (by 10-45%) the fall in free neutrophils, but had no effect on monocyte or lymphocyte counts. Colchicine paralyses platelet microtubules, and inhibits shape changes during activation, but using doses said to block PMN degranulation, there is no effect on platelet aggregation (O'Flaherty & Ward, 1978; Russell-Smith et al, 1981). When a similar dose (1ug/ml) was incubated with platelets in PRP prior to turbidometric aggregation, there was no change in the response to a range of doses of ADP, collagen, or the endoperoxide analogue U46619 (not shown). In contrast, colchicine had a marked effect on collagen induced impedance aggregation in both non-anticoagulated and citrated whole blood (Table 4.5). The lag phase was prolonged, and the aggregation slope and amplitude consistently decreased, whereas the clotting time of NWB remained unchanged.

These results implied that PMN were at least in part responsible for the increased impedance responses of non-anticoagulated blood. Washed neutrophils increased citrated whole blood responses to collagen, yet extracellular calcium

(required for neutrophil aggregation) was chelated by citrate, and collagen does not induce neutrophil aggregation; however, there may have been minimal activation of the neutrophils during washing. In addition, neutrophil aggregation could not be stimulated by agents such as FMLP and PMA in citrated whole blood. Impedance aggregation was decreased by colchicine in citrated and non-anticoagulated whole blood, yet the aggregation of platelets in plasma is not inhibited. This suggests that some other blood cell is involved, and if PMN's are responsible, the effect may occur independently of aggregation and degranulation. There may be cohesion of neutrophils and other leucocytes with platelets forming a mixed mass coating the electrode. Scanning electron microscopy of platelet aggregates formed in response to collagen in citrated whole blood showed that they contained red cells and leucocytes amongst the aggregated platelets (Joseph et al, 1989a). Suitable stimuli can induce the production of thromboxane B_2 by PMN's (Ricevuti et al, 1987), and this may increase platelet aggregation. A variety of eicosanoids are produced by PMN's and many of these have effects on other cell types, and could be responsible for the increased platelet aggregation. PMN's also produce PAF-acether, a potent platelet agonist, and release can be triggered by thrombin. In addition, aggregating platelets are thought to release lyso-PAF, which might be taken up by PMN's and converted into PAF-acether. It has been demonstrated that very high concentrations of PAF-acether can transform erythrocytes from their normal biconcave disk shape to a spherocyte, with the concomitant release of ATP and ADP (Joseph et al, 1989b). This

released ADP could act synergistically with collagen on platelets.

4.9 PLATELET AGGREGATION INHIBITORS

Although Iloprost always prolonged the lag phase to collagen, there was great variability in the aggregation response, although there was a reduction in slope in the majority of cases (Table 4.6). In ten of these subjects, the effects of Iloprost on non-anticoagulated and citrated whole blood were compared. There was significantly less inhibition of aggregation rate in non-anticoagulated whole blood than in citrated whole blood (P=0.05) (Table 4.7), but the two methods showed similar increases in the lag period. There was no correlation between lag periods or rate of aggregation in non-anticoagulated and citrated whole blood. The weaker effect of Iloprost in non-anticoagulated than in citrated whole blood is consistent with synergism between thrombin and collagen in causing aggregation, since it would offset the effect of Iloprost.

	LAG	SLOPE
Mean	229.4	61.8
SD	72.5	42.9
Range	100-400	1-119

Table 4.6. The Influence of Iloprost on Aggregation in Non-Anticoagulated Blood. 2.5ng/ml Iloprost or isotonic saline were added to blood before aggregation with 5ug/ml collagen; results are expressed as a percentage of the saline control (n=16).

	LAG		SLOPE	
	NWB	CWB	NWB	CWB
Mean	220.6	228.6	71.1	31.0
SD	68.2	78.2	41.6	30.6
Range	100-330	125-400	1-119	1-82

Table 4.7. Comparison of Inhibition of Collagen Aggregation in Non-Anticoagulated and Citrated Blood by Iloprost. Results are expressed as a percentage of the saline control (n = 10).

In 4 normal subjects, incubation with 2mM acetyl salicylic acid in vitro produced a prolonged lag phase to 5ug/ml collagen in both citrated and non-anticoagulated whole blood, but had variable effects on aggregation slope (Table 4.8).

1uM AH 23848 caused no change in aggregation to 5ug/ml collagen in non-anticoagulated whole blood. Iloprost, ASA, and AH23848 had no effect on the clotting time of non-anticoagulated whole blood.

	NWB		CWB	
SUBJECT	**LAG**	**SLOPE**	**LAG**	**SLOPE**
CM	136	95.4	127	89.1
DW	100	100	138	127.3
CS	125	87.3	85	93.5
MW	113	109.3	167	79.0

Table 4.8. The Effect of Acetyl Salicylic Acid (ASA) on Aggregation to Collagen in Non-Anticoagulated and Citrated Blood. ASA was used at 2mM and collagen at 5ug/ml; results are expressed as a percentage of the saline control.

4.10 CONCLUSIONS

Platelets behaved differently in non-anticoagulated compared to citrated whole blood, with increased aggregation rates. Although this was partly due to the effects of generated thrombin, which is itself an agonist for platelet activation, some other component(s) was involved, since the inhibitor hirudin decreased the aggregation responses, but did not depreciate them to the level of the citrated whole blood response. Recalcification of citrated whole blood had similar effects, and the addition of washed neutrophils to citrated blood increased the aggregation response to collagen. Neutrophils and/or other leucocytes may well play a role, since aggregation was decreased in the presence of colchicine, which blocks neutrophil degranulation and inhibits aggregation, without affecting platelet aggregation. Neutrophils may act by releasing potent platelet activators such as PAF-acether or thromboxane B_2, or by cohering with platelets on the impedance electrode. The activation of washed neutrophils by FMLP or activated complement, in the presence of platelets, causes the formation of giant mixed PMN/platelet aggregates (Redl et al, 1983). Platelets and PMN's may both be important in prothrombotic states; mixed masses of the two cell types have been detected in the lungs of laboratory animals subjected to shock (Connell et al, 1975). Mixed aggregates may also be formed in vitro in blood from patients with thrombotic states (Silbergleit, 1970).

The marked and consistent effects of ASA and Iloprost on the lag period, but variable effects on the slope in both non-anticoagulated and citrated whole blood suggest that impedance

aggregation does not monitor the early phases of platelet aggregation, since these agents have been well characterised as inhibitors of platelet aggregation; these findings support the data presented in Chapter 3.

The impedance tool measures more than just platelet aggregation; it may provide an in vitro model of thrombogenicity. One limitation is the requirement for samples from fresh venepunctures for each pair of aggregation curves, but this may be overcome by the use of more suitable catheters and infusion solutions, such as that used in a recent study (Strauss et al, 1988). In the latter, samples were collected by venepuncture and catheter, for BTG and PF4 levels (sensitive indicators of platelet release), and no differences were found in the assays between the two sample types. The study of platelet function in non-anticoagulated whole blood allows a global evaluation of haemostasis at the bedside, which may be particularly important for monitoring some of the new generation of anticoagulant drugs such as recombinant hirudin and synthetic peptide anti-thrombins.

CHAPTER 5

PLATELETS IN PROTHROMBOTIC STATES

5.1 GENERAL INTRODUCTION

Platelet function was studied in whole blood using samples collected from a variety of patients with diseases associated with an increased risk of thromboembolism. The prothrombotic states investigated included: critically ill patients with adult respiratory distress syndrome (ARDS), myeloproliferative diseases, recurrent thrombosis and/or familial thrombophilia, and peripheral arterial disease where there was intermittent claudication and exercise associated pain. The latter patients were studied before and after infusion of Iloprost, a stable analogue of prostacyclin with potent anti-platelet effects, with the aim of improving blood flow and patient exercise tolerance.

5.2 MYELOPROLIFERATIVE DISEASE

5.2.1 INTRODUCTION

The myeloproliferative disorders occur when there is an abnormal bone marrow proliferation of a blood cell line. There may be gross changes in platelet biochemistry and function, but platelet defects may also be secondary to increased blood viscosity, elevated red cell mass, and increased numbers of circulating platelets.

The myeloproliferative disorders comprise four groups of diseases which frequently overlap and become indistinct: polycythaemia rubra vera (PRV), myelofibrosis (MF), essential thrombocythaemia (ET), and chronic granulocytic leukaemia

(CGL). Paradoxically, both bleeding and thrombotic events, sometimes occur in the same patient, and are prominent complications in all of the myeloproliferative diseases (Wasserman & Gilbert, 1963, 1966; Ward & Block, 1971; Weinfeld et al, 1975). In CGL, bleeding complications are most common in patients with thrombocytopaenia, but also occur in some cases with normal platelet counts, whereas thrombosis is unusual (Mason et al, 1974). In patients with PRV, thromboembolic complications occur in 14-63% of cases, and cause death in 10-40%, whereas bleeding occurs in 15-35%, and is associated with a lower mortality (Rigby & Leavell, 1960; Wasserman & Gilbert, 1963, 1966; Berger et al, 1973). The thromboembolic tendency has been attributed to many different factors, including: hypervolaemia with an increased red cell mass, stasis of blood, capillary distension, abnormal clot formation, thrombocytosis, and qualitative platelet abnormalities (Gilbert, 1975). The haemorrheological abnormalities found in PRV are absent from ET, and yet both haemorrhagic and thrombotic complications, often associated with spontaneous platelet aggregation, occur frequently in ET. Patients with MF and elevated platelet counts commonly have bleeding problems, but thrombosis is relatively uncommon (Laszlo, 1975; Takacsi-Nagy & Graf, 1975). Thrombocytosis by itself does not seem to be an important determinant of these haemostatic complications, since they are rare in patients with reactive thrombocytosis (McClure et al, 1966; Zucker & Mielke, 1972; Ginsberg, 1974, 1975). An exception to this latter generalisation are patients with thalassaemia, sideroblastic anaemia, hereditary non-spherocytic haemolytic

anaemia, and haemoglobinopathies, following splenectomy, who have a 13% incidence of thrombosis (Hirsh & Dacie, 1966). These clinical observations as well as the bleeding encountered in myeloproliferative patients with a normal platelet count, blood volume, and viscosity, suggests that qualitative disorders rather than quantitative abnormalities determine the risk of thrombotic and haemorrhagic complications.

5.2.2 PATIENTS STUDIED

Thirty three patients with myeloproliferative disorders were studied at presentation, or before treatment:- 6 had PRV, 3 had CML, and 24 had ET. Three of the latter group had a PRV component to their disease, with a high haematocrit and increased red cell mass. Some patients were followed up after treatment, at later clinic visits.

Control blood samples were obtained from 20 healthy normal laboratory staff; and 6 patients with secondary polycythaemia.

	ET				Normals			
	MPV	**Plats**	**HCt**	**WBC**	**MPV**	**Plats**	**HCt**	**WBC**
Mean	8.2	1062	0.39	10.9	8.0	279	0.42	6.6
SD	1.2	399	0.08	3.6	0.7	51	0.04	1.9
Min.	6.3	445	0.24	6.3	7.0	203	0.35	4.0
Max.	10.6	2060	0.51	19.9	9.9	401	0.49	11.0

Table 5.1 - Blood Count Parameters in 24 Essential Thrombocythaemia Patients and 20 Healthy Normal Controls. The units were as follows:- mean platelet volume (MPV) - fl, platelets (Plats) - $*10^9/l$, haematocrit (HCt) - l/l, white cell count (WBC) - $*10^9/l$.

5.2.3 RESULTS IN ESSENTIAL THROMBOCYTHAEMIA

As expected, patients with ET had significantly higher platelet counts than healthy normals ($p<0.001$), and certain individual patients had an increased mean platelet volume (MPV) (Table 5.1); there was also a small, but significant ($p<0.01$) increase in leucocyte numbers.

15/24 ET patients showed spontaneous aggregation in undiluted citrated blood, but this was not associated with significantly higher haematocrit, white cell count, increased platelet number or MPV (Table 5.2). Spontaneous aggregation disappeared in 4 of these patients when the blood was diluted to a haematocrit of 0.3 l/l (but not in 6 others) and in five, spontaneous aggregation was blocked when Iloprost (a stable analogue of prostacyclin) was added to the blood at 0.5-2.0 ng/ml before stirring. The final platelet count was significantly lower in samples where spontaneous aggregation disappeared on dilution than in those where it remained (mean count 498 and $903*10^9$/l, respectively, $p<0.05$).

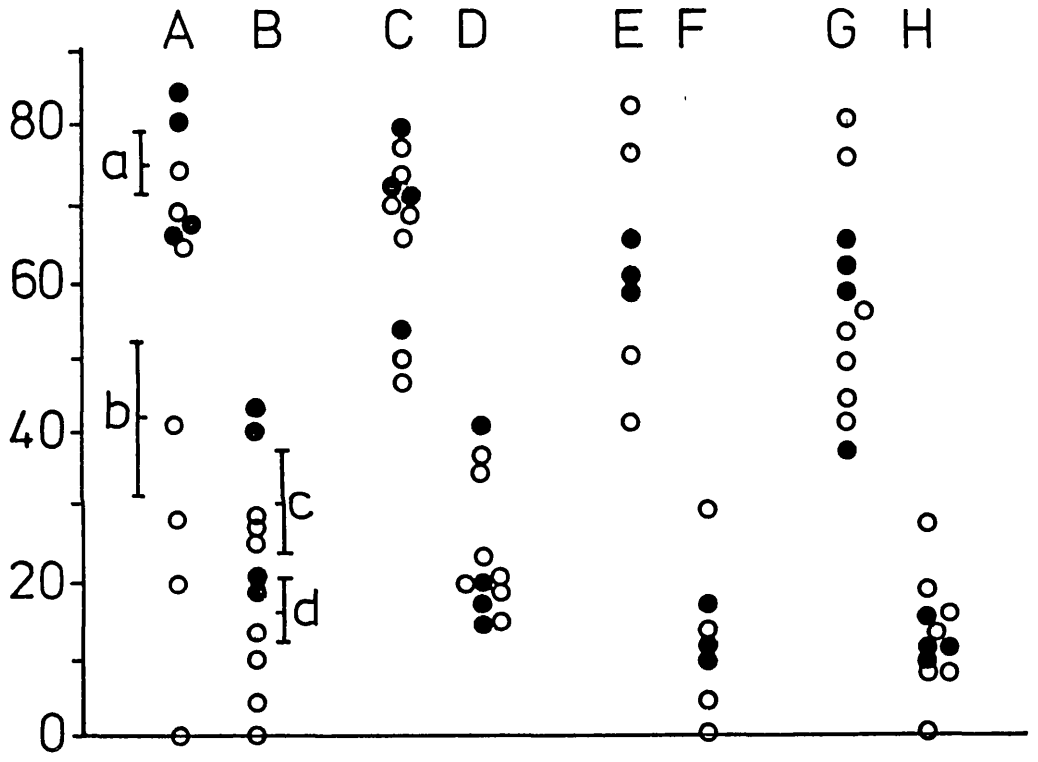

Fig. 5.1 - Whole Blood Impedance Aggregation in Essential Thrombocythaemia. The slope and amplitude of responses to: 1ug/ml Collagen (A & B), 5ug/ml Collagen (C & D), 12.5uM ADP (E & F), and 25uM ADP (G & H) were measured. Results were divided according to the presence (closed circles) or absence (open circles) of spontaneous aggregation before dilution of blood. The mean +/- SD are indicated by a) and c) for the group with spontaneous aggregation; and b) and d) for the group without spontaneous aggregation.

	A)				B)			
	MPV	**Plat**	**HCT**	**WBC**	**MPV**	**Plat**	**HCT**	**WBC**
Mean	8.2	1081	0.39	11.5	8.1	1032	0.38	9.8
SD	1.4	411	0.08	4.1	1.0	427	0.08	2.4

Table 5.2 - Results in Essential Thrombocythaemia.
The patients were divided into: A) those with (n=15), and B) those without (n=9) spontaneous aggregation in citrated blood. units of measurement were as described in Table 5.1.

Whole blood impedance aggregation to low dose collagen in patients who had exhibited spontaneous aggregation in undiluted blood tended to be higher than in normals and in patients without spontaneous aggregation (Fig. 5.1), although this did not achieve statistical significance. Aggregation was not abnormally increased with ADP, or when a higher dose of collagen was used, probably because maximal aggregation had been achieved, suggesting that the threshold for activation had been lowered.

A few patients exhibited abnormally low responses to collagen. With low concentrations of ADP, the ET group showed significantly higher rate (but not amplitude) of aggregation when compared to healthy normals (mean 62.9 and 44.4 respectively, $p<0.05$). There were no significant differences when higher doses of ADP were used, but 2 patients gave rate values greater than normal. Impedance aggregation to collagen was studied in non-anticoagulated blood in 4 patients, and all had normal responses.

Eleven ET patients showed spontaneous aggregation in both whole blood and PRP by impedance and turbidometric methods respectively; five were negative by both methods, two were positive in whole blood but negative in PRP, while four only showed spontaneous aggregation in PRP (two were not tested).

The latter could be abolished in PRP in all except one patient by diluting the platelets with autologous platelet poor plasma. The platelet count at which this was achieved varied between 200 and $1180*10^9/l$.

Three patients were studied after treatment with busulphan, two of them still had spontaneous aggregation in whole blood although not in PRP, despite the platelet count having reduced to 196 and $357*10^9/l$. The third patient had a platelet count of $936*10^9/l$, yet still had no detectable spontaneous aggregation. In two further patients treated with acetyl salicylic acid (ASA) and busulphan, one spontaneous aggregation was blocked in one patient, but remained in the other (platelets $508*10^9/l$). There was a tendency for aggregation responses to ADP and collagen in both PRP and whole blood to increase within the normal range following busulphan or ASA and busulphan treatment. There were no significant correlations between whole blood and PRP aggregation to ADP or collagen. PRP turbidometric aggregation (Table 5.3) to ADP was significantly decreased; 4 patients gave abnormal responses with absence of a secondary wave even at 5uM ADP. PRP aggregation to low dose (1ug/ml) collagen was also significantly decreased (Table 5.4), with abnormal responses in 12/18 patients; 10 of these had normal responses at the higher dose of 4ug/ml. There were no significant differences from normals in arachidonate aggregation responses of ET patients, and normal responses were obtained in all except one of the patients with reduced collagen aggregation.

	I) 1uM		5uM		II) 1uM		5uM	
	S	A	S	A	S	A	S	A
Mean	48.2	18.5	74.7	45.6	65.8	21.7	80.2	58.9
SEM	7.7	5.3	2.2	5.3	3.6	4.1	0.8	2.8
Min	0.0	0.0	57.0	9.0	26.0	4.0	72.0	26.0
Max	81.0	71.0	85.0	75.0	80.0	63.0	86.0	75.0
p	*	NS	*	*				

Table 5.3. Turbidometric Aggregation to ADP in Essential Thrombocythaemia. PRP aggregation was measured with two doses of ADP in:- I) ET patients (n=17), and II) normal controls (n=20). S=slope and A=amplitude of response, *=p<0.05.

	I) 1ug/ml		4ug/ml		II) 1ug/ml		4ug/ml	
	S	A	S	A	S	A	S	A
Mean	32.3	18.9	69.6	54.4	74.4	57.1	77.3	59.7
SEM	6.3	5.0	5.4	5.8	2.3	2.7	2.6	2.8
Min	0.0	0.0	0.0	0.0	47.0	22.0	35.0	35.0
Max	75.0	60.0	83.0	85.0	83.0	72.0	83.0	76.0
p	**	**	NS	NS				

Table 5.4 Turbidometric Aggregation to Collagen in Essential Thrombocythaemia. PRP aggregation was measured with two doses of Collagen in:- I) ET patients (n=18), and II) normal controls (n=20). S=slope and A=amplitude of response, **=p<0.01.

	I) MPV	Plats	HCt	WBC	II) MPV	Plats	HCt	WBC
Mean	8.5	336	0.49	7.7	8.3	260	0.54	7.2
SD	0.7	121	0.05	1.7	0.5	47	0.03	2.1
Min.	7.7	255	0.42	6.5	7.3	213	0.50	5.8
Max.	9.1	514	0.54	10.2	8.7	336	0.57	11.1

Table 5.5 Blood Count Results Polycythaemia Patients. I) PRV, and II) secondary polycythaemia patients; units were as described in Table 5.1.

5.2.4 RESULTS IN POLYCYTHAEMIA PATIENTS

PRV and secondary polycythaemia patients had higher haematocrits ($p<0.05$ and $p<0.001$ respectively), but similar leucocyte and platelet counts to the normal controls (Tables 5.1 & 5.5). Whole blood platelet aggregation responses to collagen were significantly lower in both groups of patients compared to normal controls (Table 5.6). There were no significant differences in collagen induced impedance

aggregation between PRV and secondary polycythaemia patients. Responses to ADP (Table 5.7) were also lower in PRV patients compared to normals (secondary polycythaemia not tested), but there was a wide scatter of responses within the patient group.

	A)		B)		C)	
	SLOPE	**OHMS**	**SLOPE**	**OHMS**	**SLOPE**	**OHMS**
Mean	45.5	16.2	39.5	13.7	63.0	24.0
SD	10.8	5.6	10.1	5.1	8.4	6.1
Min	27.0	8.0	27.0	7.0	45.0	14.0
Max	59.0	25.0	59.0	22.0	79.0	41.0
p	<0.01	<0.05	<0.01	<0.01		

Table 5.6. Whole Blood Impedance Aggregation to Collagen in Polycythaemia. Aggregation to 5ug/ml collagen was measured in:- A) PRV patients (n=6); B) secondary polycythaemia patients (n=6); C) normals (n=20).

	A)		B)	
	SLOPE	**OHMS**	**SLOPE**	**OHMS**
Mean	24.2	5.0	49.5	16.1
SD	20.0	4.4	11.4	7.3
Min	0.0	0.0	31.0	9.0
Max	58.0	12.0	75.0	32.0
p	<0.01	<0.01		

Table 5.7. Whole Blood Impedance Aggregation to ADP in Polycythaemia. Aggregation to 25uM ADP was measured in: A) PRV patients (n=5), and B) normal controls (n=20).

Only one patient with PRV had an abnormal PRP turbidometric response to ADP, with a decreased secondary wave; the group showed no significant difference from normal controls. Collagen aggregation of PRP was reduced in 3 patients, correcting in one of these at 4ug/ml (Table 5.8). PRP aggregation was studied in only two patients with secondary polycythaemia, but responses in these were normal to ADP and collagen.

	1ug/ml		4ug/ml	
	S	**A**	**S**	**A**
Mean	55.0	42.0	52.5	41.5
SEM	14.8	15.4	16.0	16.2
Min	14	7	15	5
Max	80	78	83	76
p	*	NS	*	NS

Table 5.8. Turbidometric Aggregation of PRP in Response to Collagen in 4 PRV Patients. S=slope, A=amplitude, *=p<0.05.

5.2.5 RESULTS IN CML PATIENTS

Two of the 3 patients with CML showed abnormalities of impedance aggregation to ADP and collagen, while the third was normal; abnormalities in one of these patients may have been related to a low platelet count of $36*10^9/l$. The other patients had normal platelet counts, and all patients had decreased haematocrits and increased leucocyte counts. PRP aggregation was studied in only one of these patients, and decreased responses to collagen, but normal ADP and arachidonate responses were seen (not shown).

5.2.6 DISCUSSION

The majority of ET patients showed spontaneous aggregation in undiluted whole blood, and this has been described previously in PRP (Waddel et al, 1981; Hoagland & Silverstein, 1978; Wu, 1978). The aggregation disappeared in 4 patients when the blood was diluted to a haematocrit of 0.3 l/l with isotonic saline. In these patients, spontaneous aggregation appeared to be related to the platelet count, which was obviously reduced by dilution. However, neither the platelet count, MPV, haematocrit, or leucocyte count appeared to determine whether a patient exhibited a spontaneous response or not. Wu (1978) found no correlation between

spontaneous aggregation in PRP and platelet count. The phenomenon could be blocked by adding the stable prostacyclin analogue Iloprost, which binds to platelets and causes an increase in intracellular cyclic AMP levels, thus inhibiting platelet activation. The observed spontaneous aggregation may be due to the high platelet count bringing platelets into close proximity to one another and causing activation. Patients with myeloproliferative disorders have been noted to have abnormal platelet lipoxygenase activity, and increased thromboxane production (Okuma & Uchino, 1979). Eleven of the patients displaying spontaneous aggregation in whole blood had the same phenomenon in PRP, this could be abolished in all except one patient by dilution with plasma. In some patients the spontaneous response disappeared at a platelet count of approximately $1000*10^9/l$, while in other patients the platelets had to be diluted to less than $300*10^9/l$. Two patients had spontaneous responses in whole blood, but not in PRP, and 4 patients showed the reverse.

Close proximity of platelets and thromboxane generation may not be the only explanation, since certain patients with high platelet counts lack spontaneous aggregation. ET patients may have more reactive platelets than normal, and when they are brought into close proximity, a small amount of thromboxane generation could provoke massive platelet activation. Others have said that platelets in myeloproliferative disorders have abnormalities of membrane proteins such as GpI and GpIV (Bolin et al, 1977), and of receptors for external regulators such as alpha adrenergic agonists (Kaywin et al, 1978), and prostaglandin D_2 (Cooper &

Ahern, 1979).

ET patients gave variable responses to ADP and collagen in whole blood, some having increased and some decreased aggregation. In PRP, there was a significant reduction in ADP and collagen aggregation, as previously observed by others (Berger et al, 1973; Waddell et al, 1981; Inceman & Tangun, 1972; Phadke et al, 1981), but only one patient showed abnormal arachidonic acid responses. This shows that defects of the thromboxane pathway of aggregation, which have previously been observed in certain myeloproliferative patients (Gerrard et al, 1978; Jubelirer et al, 1980; Pareti et al, 1982) were not the sole cause of the decreased ADP and collagen responses. It has been suggested that the decreased PRP aggregation results from an acquired storage pool defect due to a generalised intravascular platelet aggregation and release (Zahavi & Marder, 1974; Boughton et al, 1977).

In both PRV and secondary polycythaemia patients, whole blood platelet aggregation was reduced in response to ADP, low and high dose collagen. This suggested that the defect was related to increased red cell numbers rather than to an inherent defect in the myeloproliferative platelets, such as abnormal thromboxane generation. However blood was diluted to a constant haematocrit before aggregation, and decreased responses were also detected in PRP. This may be explained by high plasma adenine nucleotide levels due to the increased red cell count. Released ADP may make platelets temporarily refractory to subsequent stimulation with ADP as an agonist. When released into plasma, adenine nucleotides are rapidly

converted to adenosine (Mills, 1966), and this substance has been shown to inhibit platelet aggregation in PRP (Steer & Salzman, 1980), and acts by activating adenylate cyclase and thus increasing cyclic AMP levels (Haslam & Lynham, 1972). Adenine nucleotides and adenosine could be released from the closely packed red cells during sample handling, as there were no abnormalities of platelet aggregation studied in non-anticoagulated blood (and tested immediately) in 4 patients with abnormalities in citrated whole blood and PRP; however, erythrocytes and other cells normally scavenge adenosine avidly from plasma (Schrader et al, 1972).

5.2.7 CONCLUSIONS

Spontaneous platelet aggregation, as well as abnormal responses to ADP and collagen have been well documented in myeloproliferative disordered using PRP and turbidometric methods. Similar abnormalities occurred in whole blood, but there were some important differences. In ET, the dilution of PRP with autologous plasma, to reduce the platelet count usually abolishes spontaneous aggregation. However, in whole blood spontaneous aggregation was sometimes still present after dilution of the blood sample. Furthermore, the presence of spontaneous aggregation did not always correlate with raised platelet counts. ADP and collagen aggregation responses were variable, and it is likely that the pattern of in vitro results depends on the degree of activation of platelets in vivo. Since the methods of studying platelet aggregation detect different types of aggregate, the response obtained may depend on the physical state of the platelet before agonist

addition. In ET platelets may have been chronically exposed to low grade activation, and have become refractory to in vitro stimulation; in certain cases, a higher level of activation may have caused granule secretion, creating an acquired storage pool defect.

In polycythaemia, abnormalities of platelet function were indirectly related to the increased red cell numbers. Decreased platelet aggregation to ADP and collagen was seen in whole blood after dilution to a constant haematocrit, and in PRP, in both PRV and secondary polycythaemia patients. Some biochemical effector released by red cells would account for these effects, and likely candidates are ADP, causing subsequent refractoriness, or adenosine causing increased platelet levels of inhibitory cAMP. However the aggregation responses were normal in non-anticoagulated whole blood and the abnormal responses in polycythaemia may therefore be purely an in vitro artifact.

5.3 RECURRENT VENOUS THROMBOSIS

5.3.1 INTRODUCTION

Young patients (less than 40 years old) with unexplained, recurrent venous thrombosis are often investigated in the interval between thrombotic episodes, when they may have a prothrombotic state. Some of these patients have a defect of one of the control mechanisms of haemostasis, such as deficiency of a serine protease inhibitor, or defective fibrinolysis. There is an association between venous thrombosis and deficiency of AT-III, protein C, or protein S deficiency (Egeberg, 1965; Griffin et al, 1981; Comp et al, 1984). One recent study (Engesser, 1988) looked at 203 unrelated European patients with venous thrombophilia, and concluded that only 31% of cases with familial thrombophilia, and 8% of isolated cases could be explained by diseases known to cause thrombophilia. This still leaves a large percentage of defects undiagnosed; presumably these can be accounted for by fibrinolytic and platelet defects. There is also a strong association between the presence of anti-phospholipid antibodies and thrombosis in patients with SLE or anti-phospholipid syndrome (Mueh et al, 1980; Boey et al, 1983). The incidence of anti- phospholipid antibodies in normal and thrombophilia populations remains unknown. This is in part due to the lack of a universally recognised, sensitive, and well standardised test.

Platelet hyperactivity can be assumed to be the cause of thrombosis, or at least a contributory factor, in some patients. The difficulty is that it may be a primary abnormality, or a secondary result of some underlying

prothrombotic condition. The relatively unsophisticated techniques available for the study of platelets in these conditions also makes the identification of such groups difficult.

5.3.2 PATIENTS STUDIED

26 patients with recurrent venous thrombosis (14 female, 12 male) were studied on attendance at a thrombophilia clinic. Each patient had recurrent venous thrombosis and/or a strong family history of thrombosis. Most were under 45 years, and except for two patients with SLE, and one with PNH, they had no obvious predisposing factors. After investigation 2 were found to have a congenital dysfibrinogenaemia, 1 had antithrombin III deficiency, 1 had defective fibrinolysis with high levels of plasminogen activator inhibitor, and the 2 SLE patients had lupus anticoagulant and cardiolipin antibodies. Protein C and protein S were not measured at the time of study for most of the patients.

Various other patients were also investigated:- 4 with severe, relapsing TTP, 2 with severe vasculitis, 1 patient with primary parathyroidism, 4 with haemoglobinopathies (1 HbSS, 1 HbSC, and 2 B-Thallassaemia Intermedia).

5.3.3 RESULTS AND DISCUSSION

Details of full blood and platelet counts are given in Table 5.9. Three patients showed weak spontaneous aggregation in undiluted whole blood, which disappeared on dilution of blood to a haematocrit of 0.3 l/l. These patients had normal platelet counts and haematocrits; one had high normal collagen

aggregation, but none of the three had spontaneous aggregation in PRP. A further patient had spontaneous aggregation in PRP, but not in citrated whole blood.

No individual patient had increased PRP or whole blood aggregation responses, but three patients had abnormal arachidonate and collagen responses compatible with undisclosed medication. As a group, mean aggregation responses to 5ug/ml collagen and 25uM ADP as measured by slope and amplitude (Table 5.10 & 5.11), gave lower responses than normals, achieving statistical significance ($p<0.01$) for collagen slope. These results were due to the subgroup of patients with abnormal responses, reducing the lower end of the observed range of values.

Four patients with TTP who were recovering or in remission, had no abnormalities of whole blood aggregation detected. One patient was also studied when acutely ill, with a platelet count of $28*10^9/l$, and whole blood aggregation to 5ug/ml collagen was markedly reduced. No abnormalities of platelet aggregation were detected in the patients with PNH or haemoglobinopathies.

There was no overall increase in platelet aggregation in this group of patients. Platelet hyperactivity as judged by aggregation studies in the absence of any other detectable reason for a thrombotic history (such as ATIII deficiency or dysfibrinogenaemia), appears to be rare. It probably occurs more frequently as a secondary feature of other hypercoagulable defects. However, new cohorts of platelets released from the bone marrow may be hyperactive, but after circulating in blood, they may undergo minimal activation, and

thus secrete some of their contents, and then continue in the circulation. Thus when a blood sample is taken, and in vitro tests performed, the platelets actually appear to be hypoactive, with decreased responses to agents such as ADP and collagen, and decreased levels of adenine nucleotides, and alpha granule proteins.

	MPV	HCt	Plats	WBC
Mean	8.3	0.38	316	18
SD	1.0	0.07	133	3.7
Min	5.7	0.19	87	3.5
Max	9.9	0.47	622	16.7

Table 5.9. Blood Count Results in Patients With Recurrent Venous Thrombosis. MPV (fl), HCt (l/l), Plats ($*10^9$/l), WBC ($*10^9$/l).

	SLOPE		AMPLITUDE	
	CON	PAT	CON	PAT
Mean	49.5	35.7	16.1	9.7
SD	11.4	18.3	7.3	6.4
Min	31.0	0.0	9.0	0.0
Max	75.0	57.0	32.0	24.0
Wilcoxon	NS		P=0.05	

Table 5.10. Whole Blood Impedance Aggregation to 25uM ADP, in Patients With Recurrent Venous Thrombosis. CON = normal controls (n = 20); PAT = patients (n = 14); statistical analysis by Wilcoxon Rank Sum Test.

	SLOPE		AMPLITUDE	
	CON	PAT	CON	PAT
Mean	63.0	53.1	24.0	19.4
SD	8.4	12.9	6.1	9.3
Min	45.0	21.0	14.0	7.0
Max	79.0	71.0	41.0	40.0
Wilcoxon	p<0.01		NS	

Table 5.11. Whole blood Impedance Aggregation to 5ug/ml Collagen, in Patients With Recurrent Venous Thrombosis. CON = normal controls (n = 20); PAT = patients (n = 26); statistical analysis by Wilcoxon Rank Sum Test.

5.4 CRITICAL ILLNESS

5.4.1 Introduction

Patients with massive trauma, shock, and/or sepsis, frequently develop multi-organ failure (MOF). Many of these patients fulfil the criteria for adult respiratory distress syndrome (ARDS). This is a form of non-cardiogenic oedematous lung injury that can arise in patients with no obvious lung involvement, and was first described 20 years ago (Ashbaugh et al, 1967). It is a profound clinical syndrome occurring in approximately 15,000 cases per year in the UK, and is frequently fatal, having a mortality of 60-70% or higher where sepsis is the predominant underlying condition. ARDS is characterised initially by reduced lung compliance, a high ventilation/perfusion imbalance leading to hypoxia. Later, pulmonary hypertension and increased capillary permeability occur. This causes tissue damage and leads to alveolar flooding and haemorrhage.

Microthrombi may sometimes be detected in the pulmonary circulation in MOF. The initiating mechanisms are unknown, but a variety of cellular elements and vasoactive substances have been implicated, including platelets, neutrophils, and there release products, as well as fibrin, complement, and kinins (Heffner et al, 1987). Platelets, neutrophils, and fibrin strands have been seen enmeshed in the pulmonary vascular bed at postmortem. Animal models suggest that these cells are involved, and their release products (eg. prostaglandins, leukotrienes, thromboxane, and growth factors) are able to simulate much of the pathophysiology of ARDS and MOF. There may also be direct effects of toxins such as oleic acid,

fibrin, histamine, and bradykinin on the lungs (Hofman & Ehrhart, 1984; Gerdin & Saldeen, 1978; Brigham & Owen, 1975; Lewis, 1963).

During respiratory failure, neutrophils sequester in the pulmonary circulation at sites of endothelial injury (Redl & Schlag, 1980; Schlag et al, 1980), and there are increased numbers of degranulated neutrophils in bronchoalveolar lavage fluid (McGuire et al, 1982). In animals, endotoxin or phorbol myristate acetate infusion activates neutrophils, and causes lung oedema and damage (Heflin & Brigham, 1981), but not if the animals are first rendered neutropenic (Shasby et al, 1982; Lloyd et al, 1983). Neutrophils release free radicals (shasby et al, 1982), eicosanoids (Bizios et al, 1983; Goldstein et al, 1977; Spagnuolo et al, 1980), and proteolytic enzymes (Janoff, 1970) which may damage the endothelium and alter permeability, increase vasoconstriction, recruit further neutrophils, and cause a zone of inflammation. Neutrophil activation may be brought about by activated complement in disorders like pancreatitis, which predispose to MOF and ARDS (Craddock et al, 1977; Hyers, 1981). The depletion of complement does not prevent endotoxin induced lung injury (Flick et al, 1986). Other factors have also been implicated such as substances released from alveolar macrophages (Harada et al, 1984) and mast cells (Kennerly et al, 1979).

Platelets are heavily implicated in the pathogenesis, and may be an initiating factor of lung damage in early ARDS. A number of substances associated with conditions predisposing to ARDS are also potent activators of platelets. Endotoxin binds to specific membrane receptors on the platelet and could

cause aggregation during sepsis related respiratory failure (Carvalho, 1985; Hawiger et al, 1975; Morrison & Ulevirch, 1978). Thrombin may be generated in septicaemia, which frequently is associated with a consumptive coagulopathy. Infection may also involve the presence of viral bodies and immune complexes in the circulation, as well as PAF released from neutrophils and macrophages. In trauma, blood may be exposed to, tissue factor and procoagulants due to massive tissue damage. The reticuloendothelial system is frequently blocked by the massive demands on it and this may cause poor clearance of these foreign bodies, activated substances and effectors. Activated platelets release various substances which could be implicated in the disease process. They contain the highest concentration of serotonin except for the intestine, and serotonin is the most potent pulmonary vasoconstrictor known in humans, having the strongest effect on the precapillary pulmonary vessels (Malik, 1983). Platelet derived growth factor (PDGF) is a mitogen for mesenchymal cells such as fibroblasts and smooth muscle cells, is chemotactic for neutrophils and monocytes, stimulates neutrophil aggregation and release of free radicals and granules (Dueul et al, 1982; Dueul & Huang, 1984). Thromboxane A_2 is a potent pulmonary vasoconstrictor and 12-HETE or 12-HPETE increase airway mucous production, as well as promoting neutrophil activation and chemotaxis (Voelkel, 1985; Goetzl, 1980).

In ARDS, sequestered platelets may be detected by histological or radiochemical methods, in the pulmonary circulation (Carvalho, 1985; Pietra et al, 1981). Progressive

thrombopenia occurs in 50% of non trauma ARDS cases, and the degree of thrombopenia parallels the worsening hypoxia. Biopsies and autopsies on half of the patients with early ARDS show pulmonary artery occlusions due to platelets, neutrophils, and fibrin (Hill et al, 1976; Pratt et al, 1979). The infusion of collagen, thrombin, or platelet antiserum to laboratory animals causes marked microembolisation to the pulmonary vasculature (Bo & Hognestad, 1972; Vaage et al, 1976; Malik & van der Zee, 1977; Binder et al, 1980), and these microthrombi are composed of leucocytes, platelets and fibrin (Garcia-Szabo et al, 1984; Huval et al, 1984). Prior removal of platelets does not prevent increased permeability (Binder et al, 1980). Fibrin may be involved, since it can cause direct damage or trap leucocytes (Malik et al, 1982); inhibition of fibrinolysis makes the oedema worse. Neutropenic animals show no increase in permeability after thrombin infusion, so perhaps fibrin traps neutrophils, which then modulate platelet release causing increased capillary permeability (Johnson & Malik, 1985; Tahamont & Malik, 1983).

Infusion of human platelets and PAF-acether into isolated perfused rabbit lungs causes a marked rise in pulmonary artery pressure (Heffner et al, 1983). Platelets are likely to contribute to the increased pulmonary pressure in these models by releasing vasoactive substances, rather than by aggregates occluding vessels. There is no correlation between the degree of pulmonary hypertension and either the number of circulating platelets or the number trapped in the pulmonary vasculature (Vaage, 1982). TXA_2 is a potent stimulator of platelet aggregation, but is also vasoactive,

inducing pulmonary artery vasoconstriction (Bowers et al, 1979). Elevated plasma concentrations have been noted in patients dying of septic shock with ARDS (Reines et al, 1982), and in animal models following endotoxin, or PAF-acether infusion (Heffner et al, 1983; Watkins et al, 1982). These latter responses could be reduced by the prior administration of cyclo-oxygenase or thromboxane synthetase inhibitors (Snapper et al, 1983; Kubo et al, 1985). The source of the TXA_2 may not be entirely from platelets, since thrombocytopaenia only reduces TXA_2 production in sheep following endotoxin infusion by 50%, and has no significant effect on pulmonary hypertension (Wanders et al, 1981). Alveolar macrophages and neutrophils can synthesise and release small quantities of TXA_2 (Cook et al, 1982; Goldstein et al, 1977). Endothelial cells from large branches of the pulmonary artery synthesise small amounts of TXA_2 compared to prostacyclin (McDonald et al, 1983), but ex vivo evidence suggests that injured microvascular endothelial cells may synthesise more TXA_2 than prostacyclin (Ingerman et al, 1980; Serneri et al, 1983; Cooper et al, 1980). The exact contribution of platelets, leukocytes, alveolar macrophages, and endothelial cells to the final plasma concentration of TXA_2 in ARDS remains to be elucidated, as does its actual pathophysiological role. In addition, relatively little work has been performed in humans, and the results may be quite different to those obtained in animal models.

5.4.2 Patients Studied

8 critically ill patients with MOF were studied within 24 hours of admission to the intensive therapy unit (ITU), and then followed for varying intervals of time, depending on clinical progress and access to patients. 6 of the patients were clinically diagnosed as having ARDS (radiological evidence of interstitial or alveolar infiltrate; hypoxaemia on mechanical ventilation with PaO_2 <9.0 kPa on FiO_2 > 0.6 or PaO_2/FiO <15; pulmonary artery wedge pressure < 18mmHg). 4 patients developed ARDS after surgery, one after head injury, and the other had septic shock (Table 5.12). The other two patients had respiratory failure, but lacked the full criteria for ARDS; one had septic shock post-prostatectomy, and the other had SLE and developed acute renal failure after surgery for an aortic aneurysm.

PATIENT	SEX	AGE	UNDERLYING CONDITION
TI	M	68	Encephalopathic, post mediastinotomy (benign growth on lung) ARF
BH	M	53	Head injury and pneumonia
DH	M	82	Post abdominal surgery, ARF, pneumonia.
AD	M	69	Post coronary artery bypass grafting, ARF, liver failure.
AA	M	37	Septic shock
RB	M	73	Haemorrhage post-prostatectomy
BS	M	73	Post colon repair, septic shock, ARF
DE	F	35	SLE, ARF, after aortic aneurysm

Table 5.12. Clinical Information on Critically Ill Patients ARF=acute renal failure; SLE=systemic lupus erythematosus.

5.4.3 RESULTS

Data on individual samples from the different patients is shown as well as the mean values (where applicable), since each patient differs considerably with respect to degree of organ failure, drug treatment, and underlying disease state.

None of the patients studied had acute DIC (Table 5.13), although the prothrombin time was often prolonged, thrombin times were normal or only slightly raised (unless patients were on heparin infusion because of haemofiltration procedures, where thrombin times were long, but corrected by protamine sulphate), and fibrinogen levels were normal or high, whereas the chronic mild to moderate thrombocytopaenia was compatible with respiratory distress. Leucocyte counts were invariably raised, with neutrophilia and the appearance of immature forms in the peripheral blood (left shift).

Very strong aggregation responses in non-anticoagulated whole blood were frequently obtained (Fig. 5.2 and Table 5.14). These excessive responses appeared to be generally associated with a poor or deteriorating clinical condition.

DAY		CLINICAL CHANGE	WBC	NTS	PLTS	PT/C	TT/C
TI	1		24.1	18.8$	206	17/12	61/13*
	2	Worse	34.0	29.9$	145	24/12	14/12
	5	Improved	29.4	20.3$	80	13/13	14/11
	9	Awake	17.4	15.5	81	13/12	14/12
	14	Well, off D & V	11.3	7.9	159	16/13	15/13
	16	Relapse, RF, on V	9.4	7.4	123	15/12	15/14
BH	1		13.6	11.6$	49	20/13	14/15
	2		17.9	15.6$	52	16/14	15/14
	3	RF	15.5	13.2$	60	23/14	48/14*
	4		35.4	32.9$	84	18/14	13/13
	7	Worse, died 4/7 later	27.4	23.8$	249	17/13	13/12
DH	1		2.1	ND	148	19/13	11/13
	4		8.3	7.1	102	21/15	11/13
A	1		9.8	8.9$	169	16/14	15/14
AA	1		16.4	14.2	96	22/13	17/13
	4	Deteriorating	13.3	10.7$	82	25/11	19/13*
	7	Worse, RF	28.9	22.5$	61	30/11	23/13*
RB	1		4.2	3.4	46	24/13	15/13
BS	1		21.8	17.4$	191	16/12	20/13*
	2		26.2	24.9$	184	15/12	12/12
	5	Awake	20.9	11.7$	416	15/13	11/11
	14	Well	13.3	11.2	ND	15/13	24/13*
DE	1		18.4	ND	32	18/13	18/15
	2	Improving	24.1	22.2$	22	16/13	23/15*
	3	Improving	22.4	20.2$	49	15/14	14/14
	4	For Discharge	27.9	ND	39	19/13	13/13

Table 5.13. Blood Count and Coagulation creening Results in Critically Ill Patients. key:- D = Dialysis; V = Ventilator; RF = renal failure; NTS = Neutrophil count; Plts = Platelet count; $ = with left shift; * = Thrombin Time (TT) corrected with 1mg% protamine sulphate; PT = Prothrombin Time; C = Control time; cell counts are shown as $*10^9/l$, and clotting times in seconds.

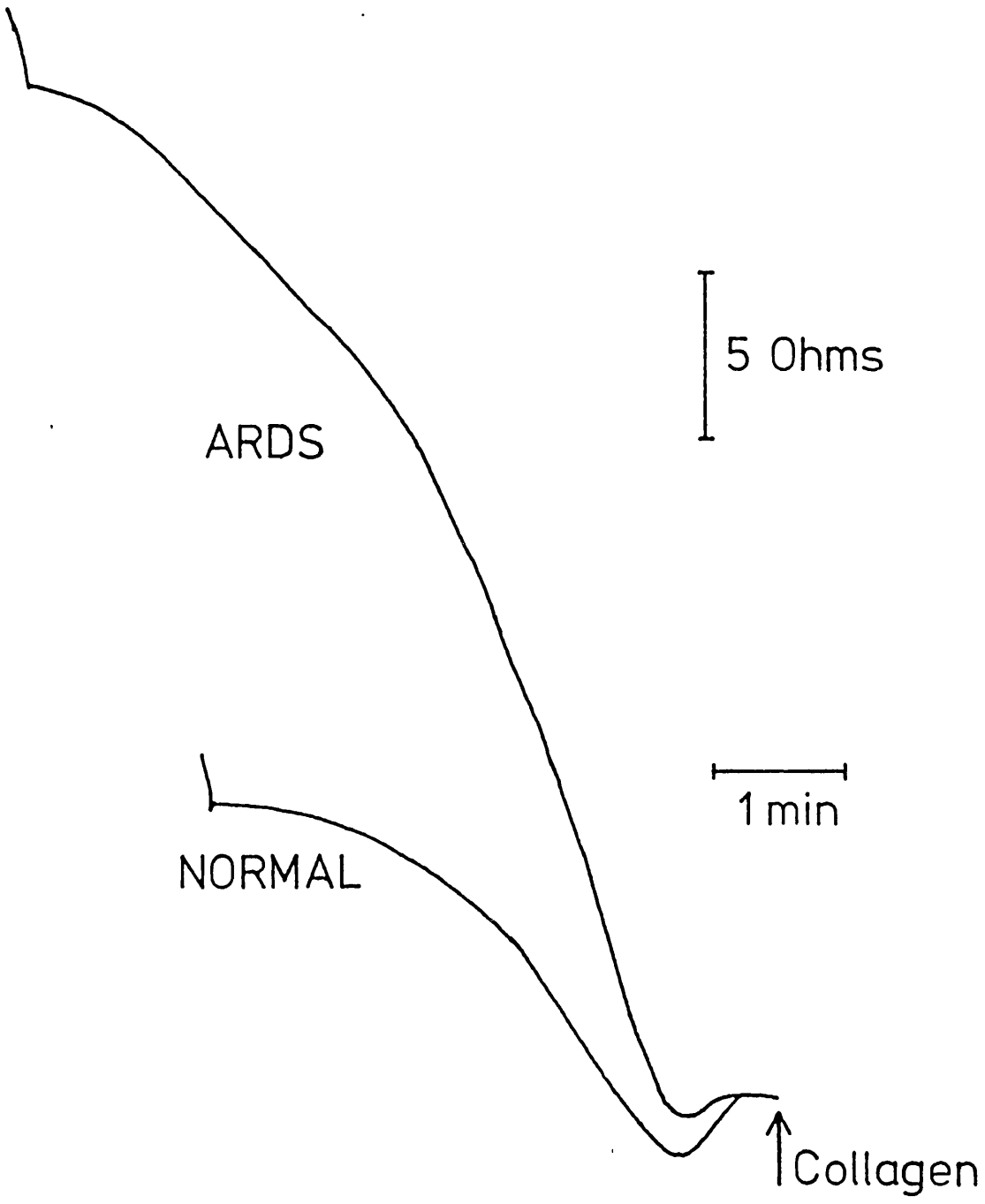

Fig. 5.2 - A Typical Aggregation Response in Non-Anticoagulated Whole Blood From a Patient With ARDS. Aggregation to 5ug/ml collagen in patient and healthy normal control.

The overall mean values of non-anticoagulated whole blood aggregation in critically ill patients were significantly higher than a healthy normal control group for slope and total impedance measurements, although the lag period was similar (Table 5.15). The increased aggregation occurred in spite of a much lower platelet count after dilution of the blood for aggregation than in the normal controls (Mean 78.1 and 191.8 respectively, $p<0.01$). Aggregation slope in non-anticoagulated whole blood correlated with WBC and neutrophil counts in the patients ($r = 0.57$, $p<0.01$; and $r = 0.55$, $p<0.01$, respectively), but not with platelet count. The clotting times obtained in the non-anticoagulated whole blood technique were significantly longer in the critically ill patients than in normals ($p<0.001$), which probably reflects the use of heparin in some of the patients. There was no obvious difference in any of the haemostatic parameters studied, between the patients with and without ARDS.

CASE	DAY	DRUG	fPlat	Lag	CT	Slope	MI
TI	1	H PRE I_2	137	0.7	>8	72	>80
	2	H I_2	97	1.5	>8	78	>90
	5	H I_2 L	53	?	>8	77	>90
	9	H	54	2.0	5.0	76	66
	14	NIL	106	3.0	6.0	19	7
	16	POSI$_2$/H	82	0.8	6.5	65	60
BH	1	E_1/P L	33	nd	nd	nd	nd
	2	E_1/P L	35	2.0	6.7	66	35.6
	3	E_1/P H	40	1.5	>8	70	>50
	4	E_1/P	56	1.0	4.2	74	38
	7	E_1/P	166	0.8	6.2	74	36.7
DH	1	NIL	99	1.5	5.7	60	19
	4	NIL	68	1.2	3.7	60	14.5
AD	1	NIL	113	1.3	5.5	64	26
AA	1	NIL	64	2.0	5.5	54	14
	4	NIL	55	1.7	7.5	76	>50
	7	H I_2 L	41	1.7	>7.5	81	>60
RB	1	BLOOD	31	0.7	4.7	54	>14
BS	1	H I_2	127	1.0	5.0	70	>55
	2	H I_2	123	1.5	3.0	70	>70
	5	H	277	0.5	3.5	71	40
	14	H	nd	0.5	5.0	67	37
DE	1	H I_2 L	21	1.5	6.5	78	>55
	2	H I_2 L	15	1.4	7.0	79	>50
	3	Nil	33	1.5	5.5	67	28
	4	Nil	26	1.5	6.5	76	44

Table 5.14. Non-anticoagulated Whole Blood Aggregation in Critically Ill Patients. Aggregation to 5ug/ml collagen was performed after dilution to give a haematocrit of less than 0.3 l/l. Key:- fPlat = final platelet count after dilution; MI = maximum impedance; H = Heparin infusion for haemofiltration; L = parenteral feeding with Intralipid; I_2 = Prostacyclin infusion; E_1/P = Prostaglandin E_1 or placebo (patients on drug trial).

When aggregation was studied in citrated whole blood, the responses showed no differences from the normal control group, for lag period, slope, or maximum impedance (Tables 5.15 and 5.16), and spontaneous aggregation was not detected. The non-anticoagulated whole blood aggregation responses in critically ill patients were significantly higher than their citrated blood responses for slope and maximum impedance ($p<0.01$,

p<0.01), but not for lag period.

		NWB			CWB		
		LAG	**SLOPE**	**MI**	**LAG**	**SLOPE**	**MI**
a)	**Mean**	1.37	67.9	45.2	1.57	52.1	19.3
	SD	0.58	12.6	23.3	0.50	13.6	6.8
	Min	0.5	19.0	7.0	0.7	22.0	9.0
	Max	3.0	81.0	90.0	3.0	73.0	33.6
b)	**Mean**	1.25	55.0	11.4	1.29	52.4	16.1
	SD	0.27	9.6	4.1	0.40	11.1	4.1
	Min	0.8	33.0	7.5	0.7	28.0	7.0
	Max	1.7	71.0	19.0	2.0	70.0	26.0
a) v b)		NS	*	*	NS	NS	NS

Table 5.15. Non-anticoagulated (NWB) and Citrated (CWB) Whole Blood Impedance Aggregation to 5 ug/ml Collagen in Critical Illness. a) 25 critically ill patients (only 15 for CWB), and b) 20 healthy normals. NS = non significant difference between patients and normals, * = p<0.001.

CASE	**DAY**	**Lag**	**Slope**	**MI**
BH	1	3.0	47	16.0
	2	2.0	37	14.0
	3	1.5	22	9.0
	4	1.3	68	31.5
	7	0.7	73	33.6
DH	1	1.20	61	19.5
	4	1.60	59	22.0
AD	1	1.50	49	17.5
AA	1	1.50	63	24.0
	4	1.70	64	19.5
	7	1.70	55	22.0
RB	1	1.70	36	11.5
DE	1	nd	nd	nd
	2	1.5	46	16.5
	3	1.2	54	19.5
	4	1.4	48	13.0

Table 5.16. Aggregation in Citrated Whole Blood in Critically Ill Patients. Aggregation to 5ug/ml collagen was performed at the same HCt as for non-anticoagulated whole blood responses (samples from cases TI and BS were not tested).

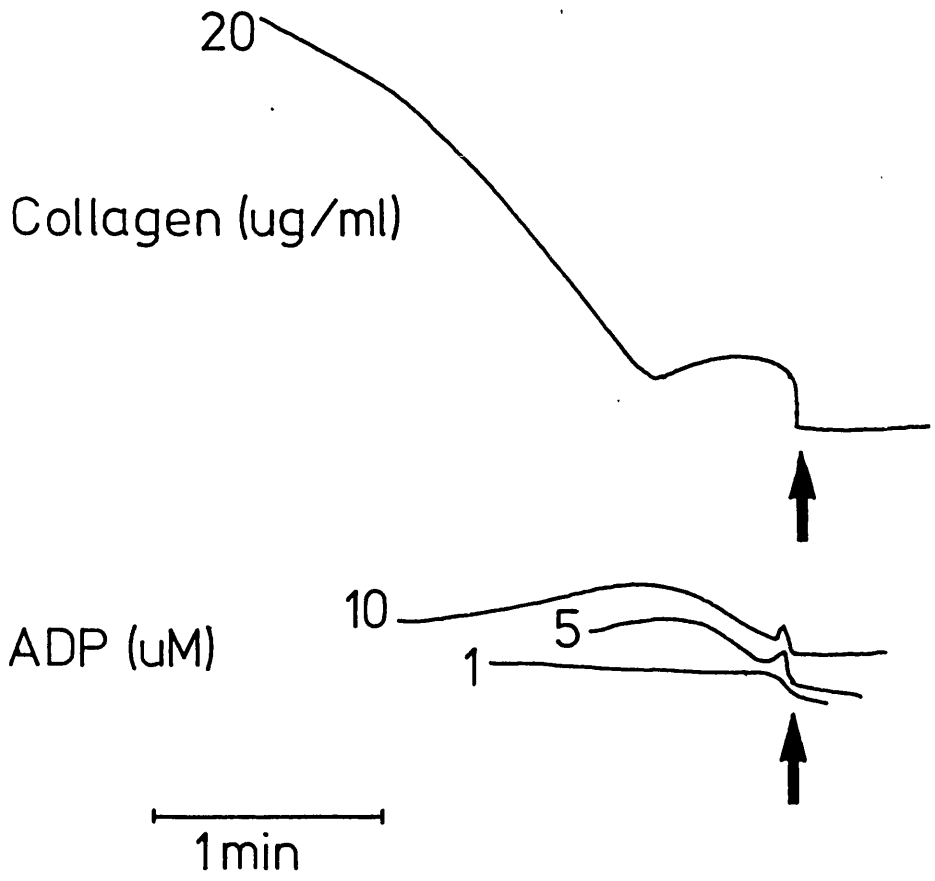

Fig. 5.3 - Turbidometric Aggregation Using PRP in ARDS. Results are shown using ADP and Collagen with PRP from an ARDS patient who had shown increased aggregation in non-anticoagulated whole blood.

Citrated whole blood from the patients was recalcified by adding 5mMol/l calcium chloride, to simulate the non-anticoagulated whole blood responses in 2 patients on 4 separate occasions. The results obtained were similar to those obtained in citrated whole blood which had not been recalcified, and did not reproduce the elevated responses of non-anticoagulated blood. The concentration of calcium chloride added was sufficient to cause blood coagulation in 3.0-6.5 minutes, which was in fact slightly shorter than that obtained in blood without anticoagulant.

Turbidometric aggregation on PRP was performed on one patient with excessively high non-anticoagulated whole blood aggregation, on two occasions, and found to be abnormally low (Fig. 5.3), although the platelet count on these occasions was only 86 and $101*10^9$/l (slightly higher than the count in EDTA blood).

Various inhibitors of platelet function were added to blood immediately after collection to study the influence on aggregation. Iloprost was used at a final concentration of 2.5ng/ml in 4 patients in 16 different blood samples. The lag period was prolonged in the majority of samples ($p<0.05$), but the aggregation slope was only inhibited in a few (Table 5.17). Acetyl salicylic acid was used at a concentration of 2mM in 2 patients with increased non-anticoagulated whole blood aggregation, and had no effect on the responses. 2 mM EDTA abolished all responses in normal and patient blood. Colchicine which is a leucocyte inhibitor, caused a decrease in the aggregation of patient blood in both non-anticoagulated (Table 5.17) and citrated whole blood (not shown).

		Saline			Iloprost		
		Lag	**Slope**	**MI**	**Lag**	**Slope**	**MI**
a)	Mean	1.35	68.9	57.4	1.78	65.8	51.6
	SD	0.69	14.0	24.2	0.97	13.2	27.0
		Saline			**Colchicine**		
		Lag	**Slope**	**MI**	**Lag**	**Slope**	**MI**
b)	Mean	1.53	66.3	33.3	2.4	49.3	32.0
	SD	0.57	14.3	23.1	0.5	22.5	25.5

Table 5.17. Effects of Platelet and Leucocyte Function Inhibitors on Aggregation in Non-Anticoagulated Blood. Addition of a) 2.5ng/ml Iloprost (n=16); or b) 1ug/ml Colchicine (n=4), compared with isotonic saline to non-anticoagulated whole blood immediately after collection.

Various associated haemostatic parameters were studied and are shown in Table 5.18. Serum Thromboxane B_2 levels were not increased in any of the patients studied, and were actually decreased in some samples. Levels of the complex of neutrophil elastase with its plasma inhibitor were markedly elevated, and the levels tended to correlate with the clinical course, being highest at the start of the critical illness, and decreasing as the patient improved clinically, or increasing again as the patient deteriorated. There was no correlation of elastase complex levels with aggregation, WBC or neutrophil counts. Antithrombin III levels were decreased in all patients studied, and showed an inverse correlation with non-anticoagulated whole blood aggregation slope ($r = -0.64$, $p<0.05$). Fibrinogen and von Willebrand factor levels were consistently increased above the normal range.

	DAY	NE:A1AT	sTXB2	AT-III	Fg	VWF
TI	1	228	ND	0.58	4.76	3.86
	2	252	0.05	0.35	5.75	4.52
	5	83	0.15	0.44	4.32	4.63
	9	96	0.06	0.30	3.04	4.68
	14	175	0.53	0.74	4.76	4.95
	16	154	ND	0.45	3.40	3.84
BH	1	870	0.43	0.20	4.02	3.37
	2	307	0.79	0.18	5.95	4.56
	3	307	0.01	0.27	6.25	2.44
	4	913	0.67	0.46	7.59	3.93
	7	>2000	0.83	0.39	6.15	4.25
DH	1	798	1.01	0.51	6.05	4.20
	4	463	0.41	0.53	4.66	4.74
AD	1	482	0.87	0.31	6.55	11.83
AA	1	>3000	ND	0.35	ND	3.84
	4	608	ND	0.19	ND	3.66
	7	350	ND	0.10	ND	5.66
RB	1	506	ND	0.52	ND	0.67
BS	1	nd	ND	ND	ND	ND
	2	nd	0.26	ND	ND	ND
	5	nd	0.50	ND	ND	ND
	14	nd	0.31	ND	ND	ND
DE	1	ND	0.54	ND	ND	ND
	2	ND	0.19	ND	ND	ND
	3	>2500	0.27	ND	8.05	2.78
	4	745	0.41	ND	7.34	4.61
Normal Range		9-197	0.89-1.87	0.8-1.2	1.50-4.0	0.50-2.0

Table 5.18. Other Parameters Measured in Critically Ill Patients. NE:A1AT = Neutrophil elastase:alpha-1-antitrypsin complexes; sTXB2 = Serum Thromboxane B_2 (ng/10^6plats; AT-III = Antithrombin-III (iu/ml); Fg = Fibrinogen (g/L); VWF = von Willebrand factor (u/ml); nd = not done

5.4.4 DISCUSSION

The impedance aggregation responses observed in non-anticoagulated whole blood from critically ill patients were significantly more rapid and had a greater amplitude than healthy normals. In fact the responses were much greater than in any other group studied, with the possible exception

patients with essential thrombocythaemia. However, impedance aggregation in citrated whole blood was normal even after recalcification.

In essential thrombocythaemia, excessive impedance amplitudes are associated with the typical high platelet counts and hyperaggregability, and decrease as the platelets are diluted out, so that the increase in impedance is related to the mass of platelets adhering to the electrode. In the case of critically ill patients, the platelet count was invariably low, and could not account for the increased impedance response. This view is supported by the lack of evidence for heightened platelet aggregation from citrated whole blood, or from turbidometric measurement of PRP aggregation. The peripheral platelet count is thought to be low in respiratory distress because of consumption rather than failure of megakaryocyte maturation or sequestration in the spleen. This may either be due to DIC, for which there was no evidence of an acute episode in the patients studied here, or to local consumption in the pulmonary microcirculation, which is a common phenomenon in ARDS. For this to occur, the platelets must either be activated in the circulation by soluble factors, or activate on passage through the pulmonary circulation. If the peripheral platelets were already activated one would expect to see increased impedance aggregation in citrated whole blood, which is particularly sensitive at detecting aggregation in thrombopaenic samples, yet none was seen. Thus it is more likely that the platelets become activated and trapped locally in the lungs, and that other cell types or factors are involved in the increased non-

anticoagulated whole blood impedance responses.

Serum thromboxane B_2 ($sTXB_2$) levels were normal or decreased, suggesting that platelets were not activated in the circulation, as appears to be the case in certain arterial disease patients (see below). The lack of elevation of $sTXB_2$ in the patients studied is at variance with previous reports, although these have centred on animal models, or plasma rather than serum TXB_2 has been measured. It may be that the stimulation of platelet release by allowing blood to clot causes such large TXB_2 production, that any high plasma levels are masked. However, it must be remembered that in the patients studied here, the platelet count was frequently low, and the TXB_2 level/10^6 platelets was often decreased; if there was a high background level, one would expect it to appear as an apparent increase in $sTXB_2$. The contribution of the thromboxane pathway to the increased non-anticoagulated whole blood aggregation is also doubtful, since acetyl salicylic acid apparently had no effect on the aggregation response.

There was little effect of Iloprost on non-anticoagulated whole blood aggregation in most blood samples, except for a significant increase in the lag time. This would suggest that although the mechanism of this increased impedance measurement is not affected by Iloprost, at least the initiation of it is to some degree platelet dependent.

The observed heightened responses depended to some extent, or at some stage on a calcium dependent process. Aggregation was normal in citrated whole blood, where plasma calcium ions were sequestered, and was abolished in the presence of EDTA, which is a stronger chelator, and can remove

calcium ions from cell membranes and certain intracellular pools. However, restoring the calcium ion concentration did not reproduce the heightened aggregation. Since the non-anticoagulated whole blood responses were always obtained first (before the blood clotted!), citrated blood responses were not measured until 5 to 10 minutes later, and it may be that a substance with a very short half life is involved.

5.4.5 Leucocytes and Hyperaggregability

Neutrophils are able to aggregate under suitable conditions, and this response can be measured by impedance, counting, and turbidometric methods (Russell-Smith et al, 1981; Belch et al, 1987; Craddock et al, 1978). This process is said to depend on the presence of extracellular calcium ions and a suitable stimulus (Russell-Smith et al, 1981; O'Flaherty & Ward, 1978). Examples of such stimuli are the bacterial cell wall peptide, N-Formyl l-Methionyl -l-Leucyl l-Phenylalanine (FMLP), thrombin, calcium ionophore A23187, and phorbol myristate acetate (PMA). FMLP is a popular agent for this purpose, and is thought to cause neutrophil aggregation by stimulating a receptor which triggers phosphatidyl inositol metabolism. Neutrophil aggregation to this agent can be detected in heparinised or non-anticoagulated blood from normal donors by impedance methods, but occurs very slowly, and gives a weak response. If the neutrophil concentration is increased, which can be achieved in vitro by density gradient centrifugation, stronger responses are obtained. These responses can be completely blocked by colchicine, an inhibitor of microtubule formation.

This agent has little effect on platelet aggregation at the doses used to block neutrophil aggregation, as shown here, and by others (Russell-Smith et al, 1981). In non-anticoagulated whole blood experiments, detection of a neutrophil impedance aggregation response to FMLP depends largely on the clotting time, and since the response is slow in onset, often only the beginning of the neutrophil aggregation response can be detected before the sample clots.

If platelets are stimulated at the same time as neutrophils, or a combination of activators are present, the reaction may of course be quite different. Colchicine had no effect on aggregation responses to ADP, collagen, or endoperoxide analogues, when added to PRP in vitro, even at 4 fold higher doses, but reduced the impedance aggregation response in both normal volunteers (Chapter 4) and critically ill patients. This suggests that even in normal circumstances, there may be some contribution of neutrophils to platelet aggregate formation. The additional observations of a colchicine effect in citrated whole blood suggests that neutrophils may cohere with platelets in a process independent of extracellular calcium ions. Alternatively, neutrophils may be aggregating in the presence of calcium ions released from platelet dense granules. The increase in non-anticoagulated whole blood aggregation correlated with both the white blood cell and neutrophil counts in critically ill patients. Intravascular activation of neutrophils and an increase in turnover was also indicated by the increased levels of neutrophil elastase:alpha-1-antitrypsin complexes, and presence of immature granulocytes on the peripheral blood

film. However, there was no correlation between neutrophil count and elastase complexes, so that neutrophils were probably releasing their contents in the pulmonary circulation rather than having increased release during sample collection. Platelets and neutrophils are found as mixed masses in the lungs of laboratory animals given anaphylotoxin or with experimental shock (Connell et al, 1975; Stimler et al, 1980). Some studies have shown that platelets are trapped in alveolar capillaries before neutrophils are accreted (Barry et al, 1985). The addition of platelets to neutrophil preparations in vitro considerably increases their adhesion and aggregation responses to stimuli (Rasp et al, 1981; Redl et al, 1983). In addition, platelets potentiate the phagocytic activity (and presumably superoxide radical release) of activated neutrophils (Sakamoto et al, 1984). Platelets may therefore promote neutrophil induced pulmonary damage, by increasing neutrophil chemotaxis, adherence, phagocytosis, and aggregation. Platelet arachidonic acid and TXA_2 promote leucocyte adherence, aggregation, and chemotaxis in vitro, while cyclo-oxygenase inhibitors decrease neutrophil entrapment in the lungs and limit the resulting oedema.

In neutrophils the main pathway for arachidonate metabolism is through 5-lipoxygenase to form 5-hydroxy acids and leukotrienes. Several leukotrienes have potential for producing pulmonary hypertension, lung oedema, bronchoconstriction, and potent neutrophil chemotactic, and aggregating properties. Platelets labelled with radioactive arachidonate and stimulated and added to unlabelled neutrophils produce several labelled eicosanoids that are not

produced by platelets alone. This suggests that stimulated platelets release arachidonate and 12-hydroxy acids, which are taken up by neutrophils, and further metabolised by their 5-lipoxygenase systems. The leukotrienes generated from these mixtures are at far higher concentrations than obtained from stimulated neutrophils alone. There is also the potential for neutrophils to synthesise and release products which promote platelet aggregation. Leukocytes have been shown to produce TXA_2, leukotrienes C_4 and D_4, as well as PAF-acether, which induce or enhance platelet aggregation.

The interaction of platelets and neutrophils may therefore partly explain the observed increase in aggregation. Sepsis is relatively common amongst critically ill patients, and endotoxin at concentrations similar to those found in septic shock causes platelet aggregation in heparinised or citrated whole blood, as detected by impedance or counting free platelets (Csako et al, 1988). Aggregation was not seen in PRP, and it is likely that the effect was mediated by endotoxin stimulation of monocytes and/or neutrophils.

5.4.6 Thrombin Involvement in Hyperaggregability

AT-III levels in critically ill patients were very low, and showed an inverse correlation with impedance aggregation in non-anticoagulated whole blood, ie. aggregation was stronger in patients with lower AT-III concentrations. Decreased AT-III levels are associated with recurrent venous thrombosis in patients with congenital deficiency (Egeberg, 1965); these patients usually have levels in the range of about 0.4-0.7 IU/ml, and are presumed to be heterozygotes.

Patients with completely absent ATIII due to congenital deficiency have not been seen, and it is assumed that the homozygous state is incompatible with life. These facts illustrate the likely importance of decreased ATIII levels in the pathophysiology of critically ill patients. In addition, deficiency of another plasma thrombin inhibitor, Heparin cofactor II has been described in a similar group of patients (Mackie et al, 1989). Previous studies (Velasco et al, 1986; Kinsey et al, 1991) and parallel experiments on some of the patients studied here (Walshe et al, 1988) have shown that in critically ill patients, particularly those with ARDS and sepsis, there is activation of the contact system (factor XII and Prekallikrein), with consequent reduction in the levels of AT-III and other protease inhibitors such as alpha-2-macroglobulin. Excessive enzyme generation can therefore occur unchecked, potentially causing the activation and/or damage of platelets, endothelial cells, and leucocytes. Once the haemostatic system is triggered, in non anticoagulated whole blood, large amounts of thrombin and other proteases may be generated and able to activate platelets and other blood cells without being rapidly inactivated, which would be the normal occurrence.

The reactions leading to thrombin generation are calcium dependent and do not therefore occur in citrated whole blood, which may explain the normal responses in this medium. Many of the patients, however, were receiving heparin, which is an antithrombin, and had prolonged clotting times. Heparin requires AT-III to exert its full anticoagulant activity, and the latter was decreased in these patients; heparin inhibits

thrombin relatively slowly on its own. It may also be that thrombin generation occurred at cell membrane sites, local to thrombin receptors on the cell surface and thus acquired some protection from heparin. It is also known that the tenase and prothrombinase complexes are protected from inactivation by AT-III:heparin complexes when they are bound at platelet membranes (see chapter 1). The affinity of thrombin for cell surface receptors may also be greater than that for heparin. The increased aggregation of non-anticoagulated whole blood cannot be explained solely by increased thrombin generation and its action on platelets. The recalcification of citrated whole blood led to thrombin generation as shown by the blood clotting at times similar to or shorter than non-anticoagulated blood, but did not reproduce the exaggerated aggregation responses seen with non-anticoagulated blood. Patients not receiving heparin did not have a noticeably shorter lag period or clotting time.

Plasma levels of the adhesive proteins fibrinogen and von Willebrand factor were increased in critically ill patients, probably due to them both being acute phase reactants. In systems involving washed red cells and platelets (chapter 3), collagen induced platelet aggregation occurred independently of fibrinogen, but was increased as the fibrinogen level was increased to about 0.4 g/l. Above this level, there was little change in the response, and the von Willebrand factor level appeared to have no influence on the result. No correlation was found between either fibrinogen or von Willebrand factor and aggregation response. Fibrinogen may however be important in the setting of increased thrombin generation and

availability. If thrombin is generated slowly and protected at the cell surface, fibrin formation may occur locally, allowing the gradual formation of fibrin strands bound to platelets and other cells. Red cells might also become trapped in such a mass, adding to the accretion on the impedance electrode. This scenario could well occur in blood from critically ill patients because of the reduced levels of AT-III and other protease inhibitors.

5.4.7 CONCLUSIONS

If platelet aggregation had only been studied by turbidometric methods or in citrated whole blood by impedance, it would have appeared that there were no heightened interactions of platelets to explain the clinical and post mortem observations of ARDS. In non-anticoagulated blood, excessive aggregation responses were obtained and may be related to the occlusion of pulmonary vessels with platelets, neutrophils and fibrin strands (Hill et al, 1976; Pratt et al, 1979) seen in ARDS. Although it was initially thought that the non-anticoagulated whole blood response was due to platelets alone, this does not fit with the platelet count and results from PRP and citrated whole blood studies. There is undoubtedly some interaction between platelets neutrophils, fibrin strands and perhaps red cells, probably at the level of both a cellular aggregate and interchange of biologically active intermediates. Such heightened responses could not be found in other groups of patients studied.

5.5 PLATELET HYPERAGGREGABILITY IN ARTERIOPATHS RECEIVING A PROLONGED CONTINUOUS INFUSION OF ILOPROST

5.5.1 INTRODUCTION

Peripheral arterial occlusive disease and thrombosis are closely interlinked. Thrombi contribute to plaque formation and the progression of atherosclerosis in a process which is usually chronic, occurring over many years. Overt haemodynamic derangements only occur when the arterial lumen is markedly reduced, and result in regions of static blood which predispose to thrombus formation, in a vicious circle. The resulting narrow arterial lumen decreases the perfusion of peripheral vessels, causing ischaemic pain and difficulty in using the limbs. The World Health Organisation definition of atherosclerotic changes (1958) are a local accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue, and calcium deposits, in association with medial changes. These atherosclerotic changes cause an increase in intimal thickness and loss of elasticity of the arterial wall. There are two major hypotheses for the pathogenesis of atherosclerosis: the incrustation theory, and the imbibition hypothesis. The incrustation theory of Rokitansky (1852) was modified by Duguid (1948) and suggests that intimal thickening results from fibrin deposition, with subsequent organisation of fibroblasts and secondary accumulation of lipids. Recent studies have also suggested that endothelial cell injury and deposition of platelets can initiate the intimal proliferation (Stemerman, 1979). The second hypothesis made by Virchow in 1856 (Virchow, 1971) has been modified to become the lipid hypothesis. This infers that

lipids in the arterial wall are derived from blood lipids, which subsequently form complexes with acid mucopolysaccharides. The lipids accumulate in the artery because of the lack of a mechanism for their removal. These two hypotheses have been integrated into a more complex hypothesis that the pathogenesis of atherosclerosis depends on a precise sequence of events occurring in the interaction of blood elements and lipids with the arterial wall. Each of these events may be modified by various risk factors, including: cigarette smoking, genetic factors, hyperlipoproteinaemia, hypertension, and diabetes mellitus. The major events in the development of atherosclerosis appear to be the following:- haemodynamic stress and endothelial cell injury; arterial wall-platelet interaction and smooth muscle cell proliferation; lipid entry and accumulation; altered mechanisms of lipid removal; fibrosis and development of thrombi; and finally, ulceration, calcification, and aneurysm.

The vessel wall can be injured by several substances released from activated platelets, including serotonin, ADP, and a cationic protein that can increase vessel permeability (Nachman et al, 1970). In addition, they release thromboxane A_2, which can cause vasospasm (Ellis et al, 1976). The adherence of platelets at the site of vessel damage, and their degranulation precedes the migration of smooth muscle cells across the internal elastic lamina, and their proliferation in the intima (Ross & Glomset, 1976). A platelet derived growth factor (PDGF) (Antoniades et al, 1979) exerts these properties on smooth muscle cells and fibroblasts in culture (Ross et al, 1974).

Several syndromes arise from arteriosclerotic occlusive disease of the peripheral arteries (Bergan JJ, 1982). Aorto-iliac occlusion begins with an atherosclerotic plaque near the origin of the common iliac artery. As the plaque enlarges, it encircles the vessel and narrows the lumen progressively until thrombosis occurs and the iliac artery becomes occluded. A simultaneous process in the contralateral iliac artery may lead to aortic thrombosis. Aorto-iliac occlusive disease affects mainly males over 40 years old in a slowly progressive disease and usually produces claudication rather than severe ischaemia. There is a subset of patients in whom the severe ischaemia afflicting both lower extremities occurs suddenly (Danto et al, 1972). The most common cause of chronic ischaemia of the lower limbs is occlusion of the superficial femoral artery. The majority of the remainder have occlusion of the mid-popliteal artery behind the knee, and the rest occur at the origins of the tibial arteries or more distally. Spontaneous resolution of these occlusions does not normally occur if they result from arteriosclerotic thrombosis, and surgical intervention is required.

Platelet abnormalities have been implicated in chronic arterial insufficiency (O'Donnell et al, 1978; Hamer et al, 1973; Ward et al, 1974); these studies showed increased platelet sensitivity to ADP and adrenaline, as well as spontaneous platelet aggregation. Several reports have indicated the relief of ischaemic rest pain, improvement of intermittent claudication, and improvement of leg ulcers, by infusions of the potent platelet aggregation inhibitor, prostacyclin (Carlson & Erikson, 1973; Szczeklik et al, 1979;

Gryglewski, 1980). However, not all studies have demonstrated improvements of this condition (Vermylen et al, 1980; Pardy et al, 1980), and the anatomic lesions remained unchanged, suggesting that the capillaries were cleared of obstructing platelet deposits, and transient vasodilatation was induced. Increased muscle blood flow could in some cases be observed six weeks after termination of prostacyclin infusion, suggesting new collateral arterial flow stimulated by the therapy. Prostacyclin has a short in vivo half life and continuous intra-venous infusion is necessary for sustained platelet inhibition.

Iloprost (ZK 36,374, Schering Chemicals) is a carboprostacyclin which is classed as a prostacyclin analogue because of its biological properties. Unlike native prostacyclin, it is stable at room temperature and has a longer in vivo half life. Iloprost has a lower IC50 for ADP aggregation in humans than prostaglandin E_1 (PGE_1) or prostacyclin (PGI_2) (0.06, 2.05, and 0.29 ng/ml respectively). Iloprost and PGI_2 cause a dose dependent increase in cyclic AMP levels of human platelets. The platelet antagonist effects occur at lower concentrations than the cardiovascular effects, which include a lowering of peripheral resistance, increased cardiac output, and a lowering of pulmonary artery pressure. Facial flushing occurs at doses above 1 ng/Kg/min, whereas circulatory effects occur at about 4-8 ng/Kg/min. PGI_2 is about 5 times more potent than Iloprost in the latter respect. The potency of Iloprost for increasing cAMP levels is approximately 2.5 times that of PGI_2. 0.5 ng/Kg/min i.v. Iloprost causes 50% inhibition of platelet aggregation and 2

ng/Kg/min causes 70% inhibition (Chapter 3; and Belch et al, 1983); this platelet inhibition is accompanied by a decrease in plasma Beta-Thromboglobulin and serum thromboxane B_2 levels.

These properties of Iloprost make it theoretically suitable for the treatment of patients with vascular disease where platelet hyperaggregability or activation is suspected as a major contributory factor to the disease progress. Little is known about the effects of prolonged continuous infusions of Iloprost, and it was therefore interesting to study the effects in two patients with vascular disease in whom such treatment was thought to have a beneficial potential. The effects of various infusion dose regimes of Iloprost on platelet aggregation were also studied in whole blood in a group of 11 arteriopaths.

5.5.2 PATIENTS AND METHODS

Infusions - 1ml vials of Iloprost 0.1mg/ml (Schering Chemicals, West Sussex) were diluted in 500ml of 0.9% sterile sodium chloride and infused into a peripheral arm vein via an Ivac Controller (Ivac Ltd, Harrow, Middlesex) at the appropriate weight related dose.

Patients - The clinical details of the two patients receiving prolonged infusions of Iloprost are given in the case reports below.

For the study of Iloprost infusion in arteriopaths, 11 male patients were selected randomly from a group of 24 patients in an ongoing study of Iloprost and iscaemic leg

pain. These patients were aged between 50-77 years (mean 65 years) and had intermittent claudication which had been stable in severity for the preceding 2 months. Ischaemia was due to blocked grafts or disease of the common femoral, superficial femoral, or aorto-iliac arteries, or distal artery disease. Patients with ischaemic rest pain and diabetes mellitus were excluded. Where possible, medication known to interfere with platelet function was stopped 2 weeks prior to the study; informed consent was obtained. They received either placebo, 0.5, 1.0, or 2.0 ng/Kg/min Iloprost on each of four visits, in a blind study, in which the order of giving the placebo and each dose level was randomised. The infusion was maintained for 5 hours and blood samples were collected pre-treatment, at the end of the infusion (sample II), and 2 hours after terminating the infusion (sample III)

A control group of 20 healthy laboratory staff (age range 18-55 years), with no known clinical disease, and who had received no medication for at least the previous 10 days, was compared to the pre-infusion samples from the arteriopaths.

Aggregation - Platelet aggregation was measured in platelet rich plasma and whole blood. Whole blood aggregation was measured by the impedance method using citrated blood, and siliconised glass cuvettes (Chapter 2). Blood was diluted to a constant haematocrit of 0.3 l/l using isotonic saline, except for spontaneous aggregation studies, where undiluted citrated blood as well as diluted blood was tested. The effects of exogenous addition of Iloprost in vitro on ADP and collagen aggregation was also studied.

5.5.3 PROLONGED ILOPROST INFUSION

CASE REPORT 1

Mr. WC was a 62 year old man with long standing systemic sclerosis, was admitted because of a fulminant, painful digital ischaemia. He initially received a 9 day infusion of Iloprost, at a starting rate of 0.5ng/Kg/min, increasing by 0.5ng/Kg/min up to a final rate of administration of 3.5ng/Kg/min. There were no adverse clinical effects during the infusion. The infusion was then discontinued for 3 days, thereafter for 5 days he received a daily 5 hour infusion of the drug, at increasing doses as before. At 4.5ng/Kg/min the patient suffered headache and skin flushing, without cardiovascular side-effects, and the remaining two infusions were given at a reduced rate of 4.0 ng/Kg/min. Throughout the treatment period there were no significant changes in the blood count or peripheral platelet count.

Platelet aggregation responses were studied on days 1, 2, 3, 6, 8 and 9 of the prolonged infusion (Fig.5.4). Initially, on days 2 and 3, there was inhibition of platelet aggregation responses to ADP and collagen by the increasing dose of Iloprost infused, but by day 6 the inhibitory effect was no longer detectable, and responses on days 6, 8 and 9 were hyperaggregable as compared to the pre-infusion response, and spontaneous platelet aggregation was observed in the sample taken on day 10, immediately before the termination of the infusion. Serum thromboxane B_2 levels measured during this period mirrored the aggregation

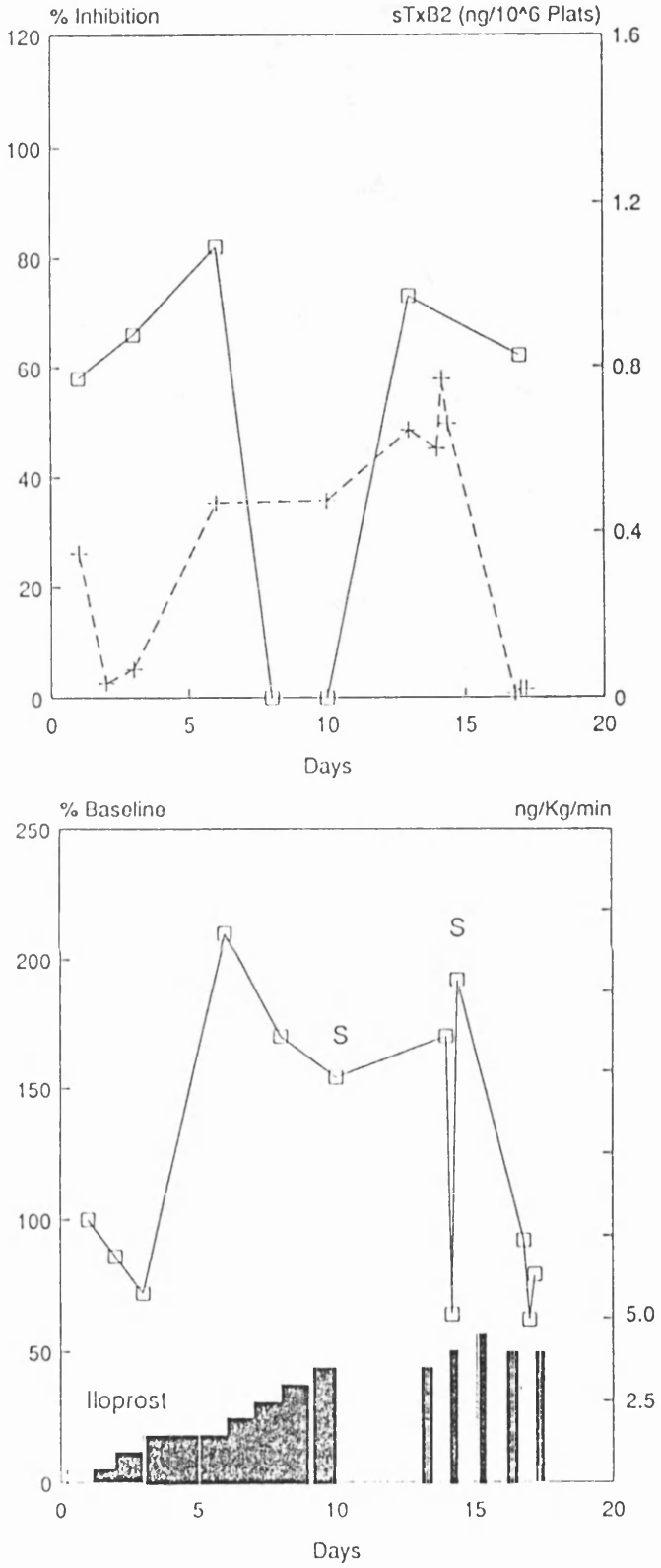

Fig. 5.4 - Platelet Function During Prolonged Continuous Infusion of Iloprost, Case 1 Mr. WC.
Upper panel: in vitro inhibition of ADP aggregation by 1ng/ml Iloprost (squares and solid line); serum thromboxane B_2 concentration (crosses and broken line).
Lower panel: ADP Aggregation expressed as a percentage of the baseline response (squares); S = spontaneous aggregation.

responses, falling markedly during the first 3 days of infusion, but then rising to greater than pre-infusion levels. The level was still raised 3 days after the end of the continuous infusion.

Dipyridamole 100mg tds, was commenced on day 10 because of the potential clinical risks associated with such platelet hyperaggregability, and this was continued for the remainder of the treatment period.

During the following 5 day period, daily 5 hour infusions were administered, commencing on day 13. On day 14 a rebound increase in aggregability was observed to ADP and collagen in a 1 hour post infusion sample as compared to the pre-infusion responses, with spontaneous aggregation again being seen in this sample. In view of this, the patient received ASA 300mg on the following day. Aggregation studies on day 17 revealed no rebound hyperaggregability. Serum thromboxane B_2 levels remained raised until day 15, falling to very low levels following ASA ingestion. There was increasing resistance to the anti-platelet activity of exogenous Iloprost added to PRP as the initial prolonged infusion progressed. Three days after this infusion was terminated the in vitro platelet inhibition by the drug was restored to the pre-infusion level. Such progressive loss of in vitro sensitivity to the actions of Iloprost was not seen during the period of daily interrupted infusions. Whole blood impedance aggregation (not shown) gave similar results for ex vivo Iloprost inhibition, with inhibition of both ADP and collagen aggregation during the first 3 days, but becoming reduced thereafter. However the in vitro addition of Iloprost to whole blood was only minimally

effective at causing inhibition of platelet aggregation.

CASE REPORT 2

JT, a 54 year old man with active rheumatoid disease and a deep indolent malleolar vasculitic ulcer, received an infusion of Iloprost in an attempt to hasten ulcer healing. He first received a continuous 10 day infusion of the drug at a starting dose of 0.5ng/Kg/min, increasing by increments of 0.5ng/Kg/min until a dose of 3.0ng/Kg/min had been reached; this was then maintained for the final 48 hours of the infusion. Throughout this period he continued to take his regular medication of indomethacin 75mg bd and D-penicillamine 375mg daily. He then received no Iloprost for a 72 hour period, after 48 hours of this time the indomethacin was discontinued so that when the infusion was recommenced he had taken none for 24 hours. Iloprost was then restarted on day 15, at a dose of 3.0ng/Kg/min, but after 6 hours the patient was suffering from troublesome headache so the infusion rate was decreased to 2.0ng/Kg/min and continued at this dose for the following 4 days; the patient suffered no other adverse clinical effects.

Throughout both infusion periods there was no significant change in blood count or platelet count. The therapeutic regime and platelet function results are shown in Figs. 5.5 & 5.6. On the first 3 days of the initial infusion, progressive inhibition of ex-vivo platelet aggregation to ADP and collagen was observed in whole blood and PRP. However, from day 4 onwards, the inhibitory effect was diminished in PRP, and by day 10 had entirely disappeared. In whole blood, collagen

aggregation remained decreased in this period, but ADP showed variable responses. No significant hyperaggregability compared with the pre-infusion responses occurred in PRP or whole blood, and serum thromboxane B_2 levels remained low throughout this period. Twenty four hours after stopping the indomethacin therapy, the second infusion was commenced. Platelet aggregation studies after 24 hours (day 16) and 48 hours (day 17) of this infusion showed spontaneous aggregation and hyperaggregable responses to ADP and collagen in both whole blood and PRP compared to the initial pre-infusion response. This was accompanied by a significantly raised thromboxane B_2 level. These hyperaggregable features were completely abolished and the inhibitory effect of infused Iloprost was restored after the patient received acetyl salicylic acid (ASA) 300mg daily on day 18.

The in vitro responses of platelets to exogenous Iloprost varied between the PRP and whole blood methods. In PRP, there was a progressive reduction in the inhibitory action of the drug as the first infusion progressed (Fig. 5.5). After termination of this infusion the inhibitory effect of Iloprost added in vitro started to return over the subsequent 3 days, but platelet sensitivity was again reduced when the second infusion was started. In whole blood, ADP was very sensitive to Iloprost in vitro,

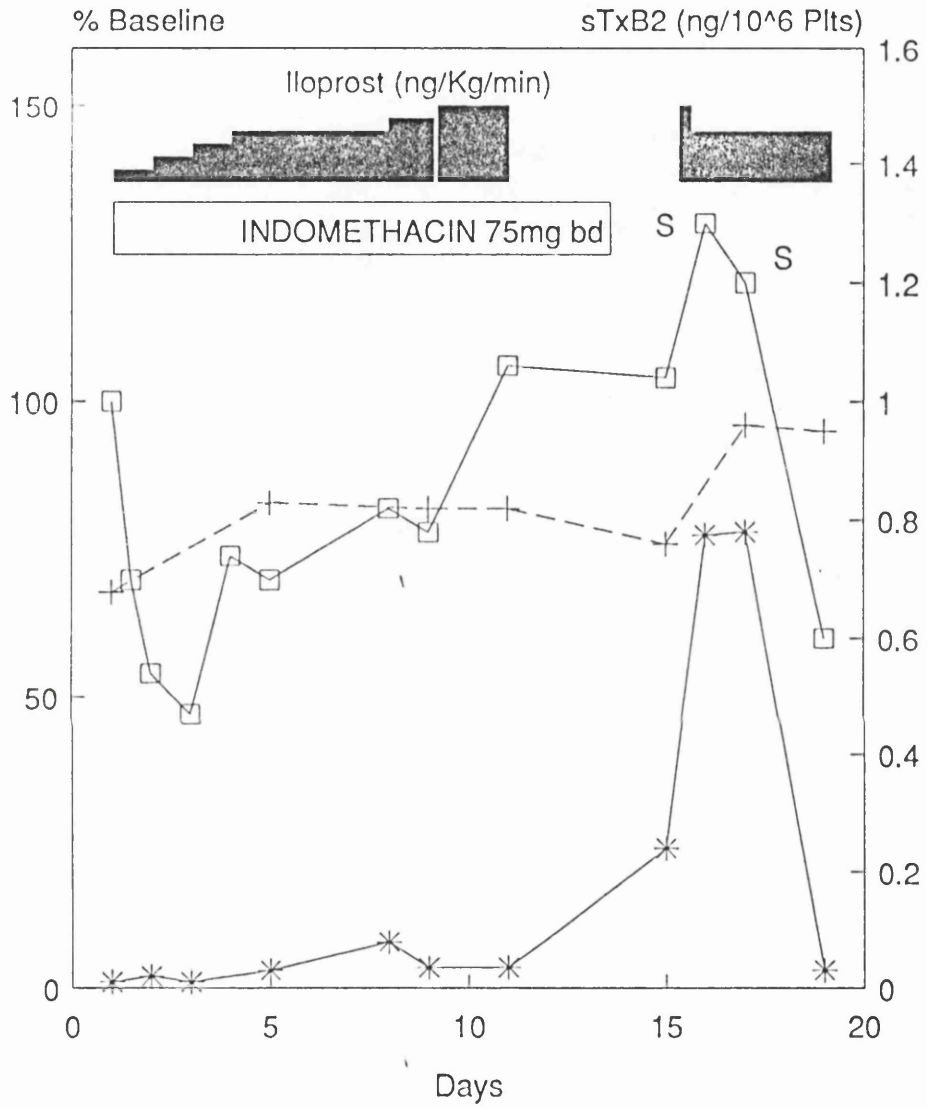

Fig. 5.5 - Platelet Function During Prolonged Continuous Infusion of Iloprost, Case 2 Mr.JT.
ADP aggregation in PRP is shown as a percentage of the baseline response (squares and solid line); in vitro inhibition of ADP aggregation in PRP was measured with 1ng/ml Iloprost (crosses and broken line); S = spontaneous aggregation; serum thromboxane B_2 was measured by RIA (asterisks and solid line).

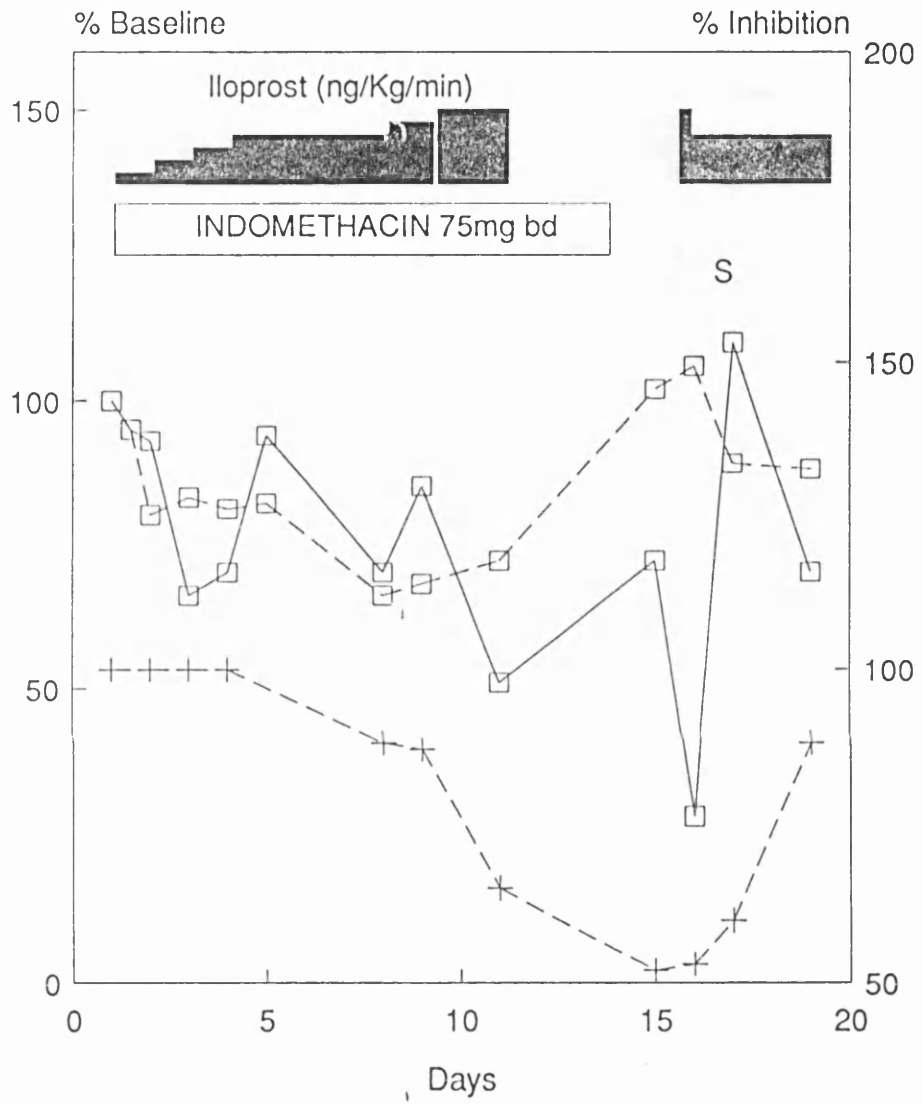

Fig. 5.6 - Whole Blood Aggregation During Prolonged Continuous Infusion of Iloprost, Case 2 Mr.JT.
Aggregation responses to 12.5uM ADP (squares and solid line) and 5ug/ml collagen (squares and broken line) are represented as a percentage of the baseline response. In vitro inhibition of collagen aggregation by 1.25ng/ml Iloprost was also measured (crosses and broken line); S = spontaneous aggregation.

with a diminution of the inhibition only towards the end of the first infusion; platelet sensitivity was again reduced at the start of the second infusion, but improved after ASA treatment. Whole blood platelet aggregation to collagen showed progressively increasing inhibition to in vitro Iloprost during days 1-3 of the first infusion, followed by a decrease in inhibition over the next 4 days, and then marked in vitro inhibition correlating with the increase in the in vivo dose of Iloprost; during the second infusion, inhibition was progressively lost until ASA was given.

5.5.4 ARTERIOPATHS AND ILOPROST INFUSIONS

On some visits, insufficient blood could be obtained from the patient for aggregation studies, and therefore all data from these visits is omitted.

Arteriopaths had a significantly decreased rate of aggregation to ADP and low dose collagen, and decreased maximum impedance to ADP in citrated whole blood, compared to the healthy control group (Tables 5.18 & 5.19).

	a		b		c		d	
	CON	**ART**	**CON**	**ART**	**CON**	**ART**	**CON**	**ART**
Mean	45.1	35.5	61.4	59.2	45.2	24.8	48.5	35.5
SD	10.4	18.7	8.3	10.5	18.6	18.5	11.2	20.5
Min	27.0	3.0	45.0	33.0	13.0	5.0	31.0	0.5
Max	62.0	71.0	73.0	75.0	77.0	68.0	75.0	74.0
p	<.05		NS		<.001		<.02	

Table 5.18. - Whole blood Platelet Aggregation (Rate) in Untreated Arteriopaths. 39 observations from 11 arteriopaths (ART) and 20 Normal Controls (CON). The pre-treatment responses were compared with healthy controls and analysed by non-parametric statistics (Wilcoxon). a = 1ug/ml Collagen; b = 5ug/ml Collagen; c = 12.5uM ADP; d = 25uM ADP.

	a		b		c		d	
	CON	ART	CON	ART	CON	ART	CON	ART
Mean	13.3	10.5	22.6	23.5	13.5	6.5	15.7	9.9
SD	4.3	5.8	4.8	6.6	7.8	4.7	6.3	6.2
Min	6.5	1.0	13.5	13.5	6.5	0.3	9.0	0.3
Max	20.0	23.5	31.5	40.0	40.0	16.5	32.0	31.5
p	NS		NS		<.001		<.005	

Table 5.19. - Whole blood Platelet Aggregation (Maximum Impedance) in Untreated Arteriopaths. 39 observations from 11 arteriopaths (ART) and 20 Normal Controls (CON). The pre-treatment responses were compared with healthy controls and analysed by non-parametric statistics (Wilcoxon). a = 1ug/ml Collagen; b = 5ug/ml Collagen; c = 12.5uM ADP; d = 25uM ADP.

In PRP, aggregation to low dose collagen was decreased (Tables 5.20 & 5.21), but with low dose ADP, aggregation was increased. Most patients showed primary aggregation to low dose ADP, but a few had secondary aggregation, which caused the mean value to be much higher than the median. This suggested that some patients had hyperactive platelets, and responded to lower ADP doses than normal.

The aggregation responses showed no significant differences after infusion of the placebo (Fig. 5.7 & 5.8). There was great variability within individuals for responses in the pre samples taken on different dates. This may have been due to normal variation, or changes in medication. Most patients were on multiple prescriptions, which could not be stopped for the study. The results could also reflect changes in clinical status and consequent modification of platelet function in the circulation; however, there did not appear to be any correlation with Iloprost infusion on a previous visit.

	a		b		c		d	
	CON	**ART**	**CON**	**ART**	**CON**	**ART**	**CON**	**ART**
Mean	73.6	46.7	77.3	81.3	65.8	75.0	80.1	82.6
SD	10.8	31.3	11.0	2.8	15.6	5.4	3.5	1.8
Min	47.0	0.0	35.0	72.0	26.0	64.0	72.0	78.0
Max	82.0	82.0	83.0	86.0	80.0	85.0	86.0	87.0
p	<0.05		NS		NS		NS	

Table 5.20. - Turbidometric Platelet Aggregation (Rate) in Untreated Arteriopaths.
The pretreatment responses of arteriopaths (ART) from each patient visit (n = 42) were analysed against responses from 19 normal controls. Data was analysed by non-parametric statistics. Key:- a = 1ug/ml Collagen; b = 4ug/ml Collagen; c = 1uM ADP; d = 5uM ADP. Min = Minimum value; Max = Maximum value.

	a		b		c		d	
	CON	**ART**	**CON**	**ART**	**CON**	**ART**	**CON**	**ART**
Mean	56.1	30.1	59.7	67.4	21.7	28.6	58.8	59.8
SD	12.4	27.0	11.7	8.9	18.0	20.9	12.8	10.7
Min	22.0	0.0	35.0	50.0	4.0	6.0	26.0	34.0
Max	72.0	75.0	76.0	95.0	63.0	74.0	75.0	92.0
p	<0.05		NS		NS		NS	

Table 5.21 - Turbidometric Platelet Aggregation (Maximum Aggregation) in Untreated Arteriopaths.
The pretreatment responses of arteriopaths (ART) from each patient visit (n = 42) were analysed against responses from 19 normal controls. Data was analysed by non-parametric statistics. Key:- a = 1ug/ml Collagen; b = 4ug/ml Collagen; c = 1uM ADP; d = 5uM ADP. Min = Minimum value; Max = Maximum value.

The infusion of 0.5 ng/Kg Iloprost caused no inhibition of aggregation in PRP or whole blood. 1ng/Kg caused mild inhibition of low doses of agonists, and 2ng/Kg caused marked inhibition of ADP and collagen aggregation in both PRP and whole blood (Fig. 5.7, 5.8, 5.9, 5.10). The degree of inhibition of platelet aggregation was generally greater in PRP than whole blood. One hour after stopping the infusion of Iloprost, ADP aggregation had returned to normal in whole blood, but not PRP, however with 2ng/Kg Iloprost, collagen aggregation in both PRP and whole blood was still decreased.

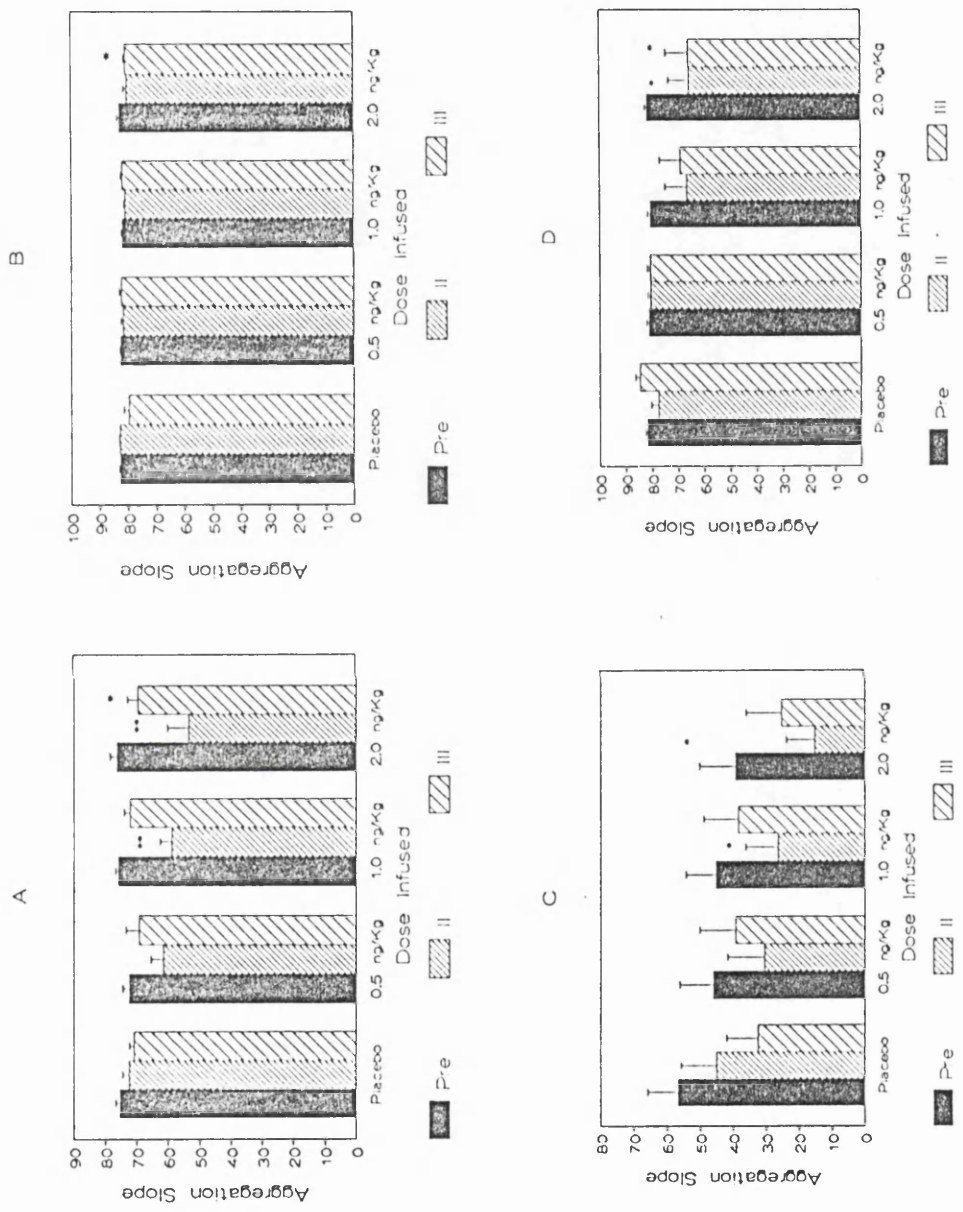

Fig. 5.7 - Platelet Aggregation Results Obtained Using PRP From Arteriopaths Receiving Iloprost or Placebo Infusions. The aggregation slope is shown using final concentrations of 1 and 5 uM ADP (A & B), or 1 and 4 ug/ml collagen (C & D).

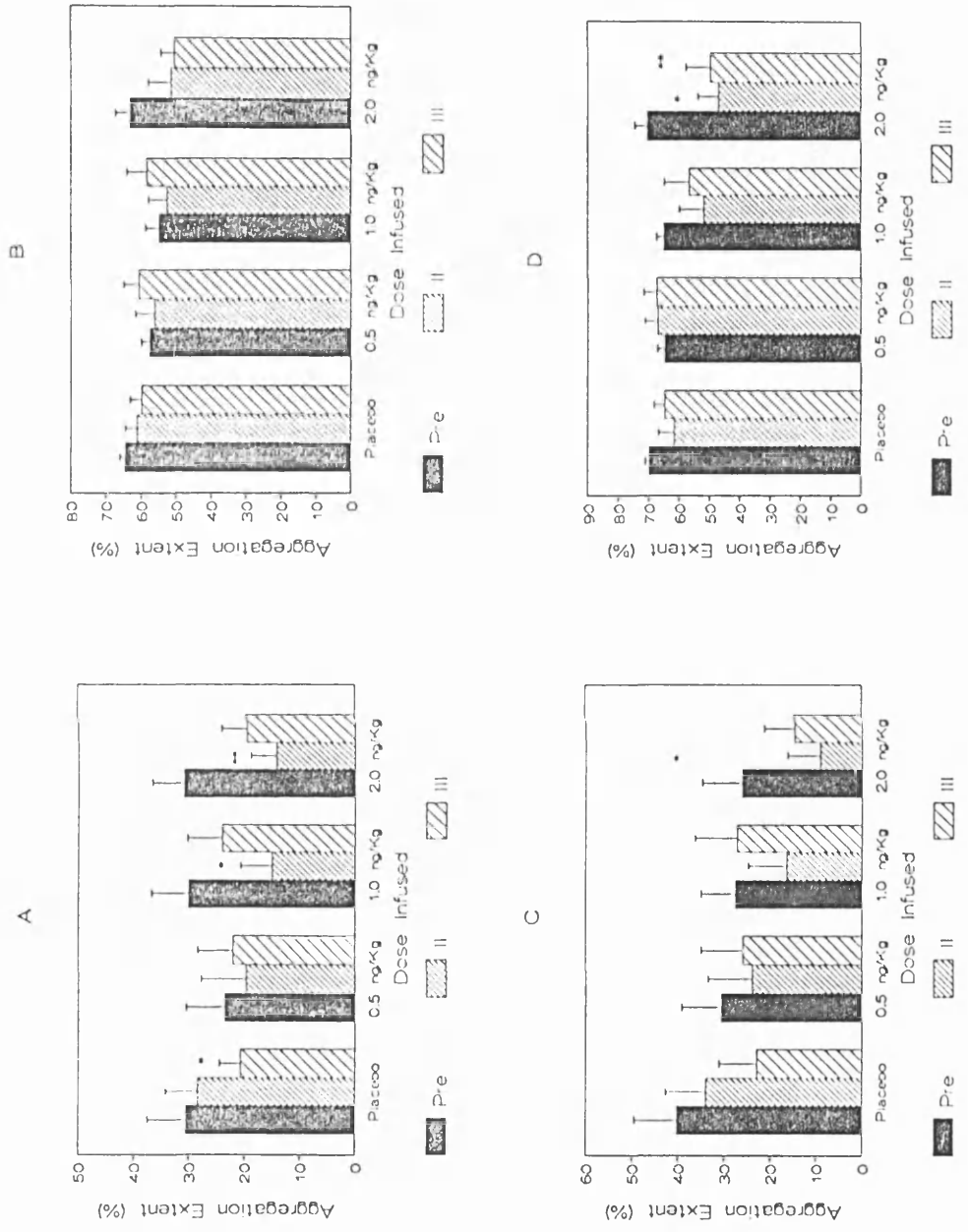

Fig. 5.8 - Platelet Aggregation Results Obtained Using PRP From Arteriopaths Receiving Iloprost or Placebo Infusions. The % aggregation (amplitude) is shown using final concentrations of 1 and 5 uM ADP (A & B), or 1 and 4 ug/ml collagen (C & D).

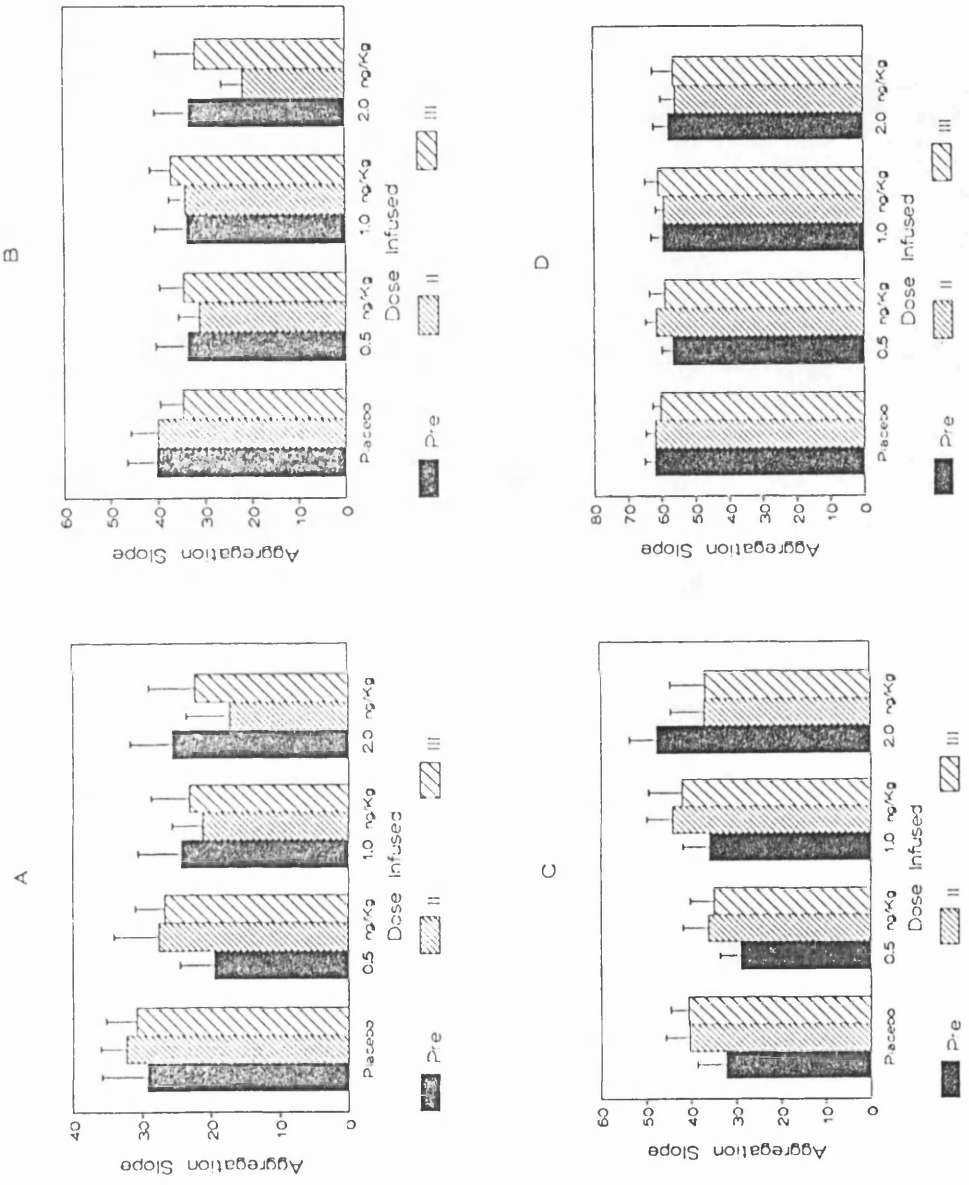

Fig. 5.9 - Whole Blood Platelet Aggregation Results in Arteriopaths Receiving Iloprost or Placebo Infusions. The aggregation slope is shown using final concentrations of 12.5 and 25 uM ADP (A & B), or 1 and 4 ug/ml collagen (C & D).

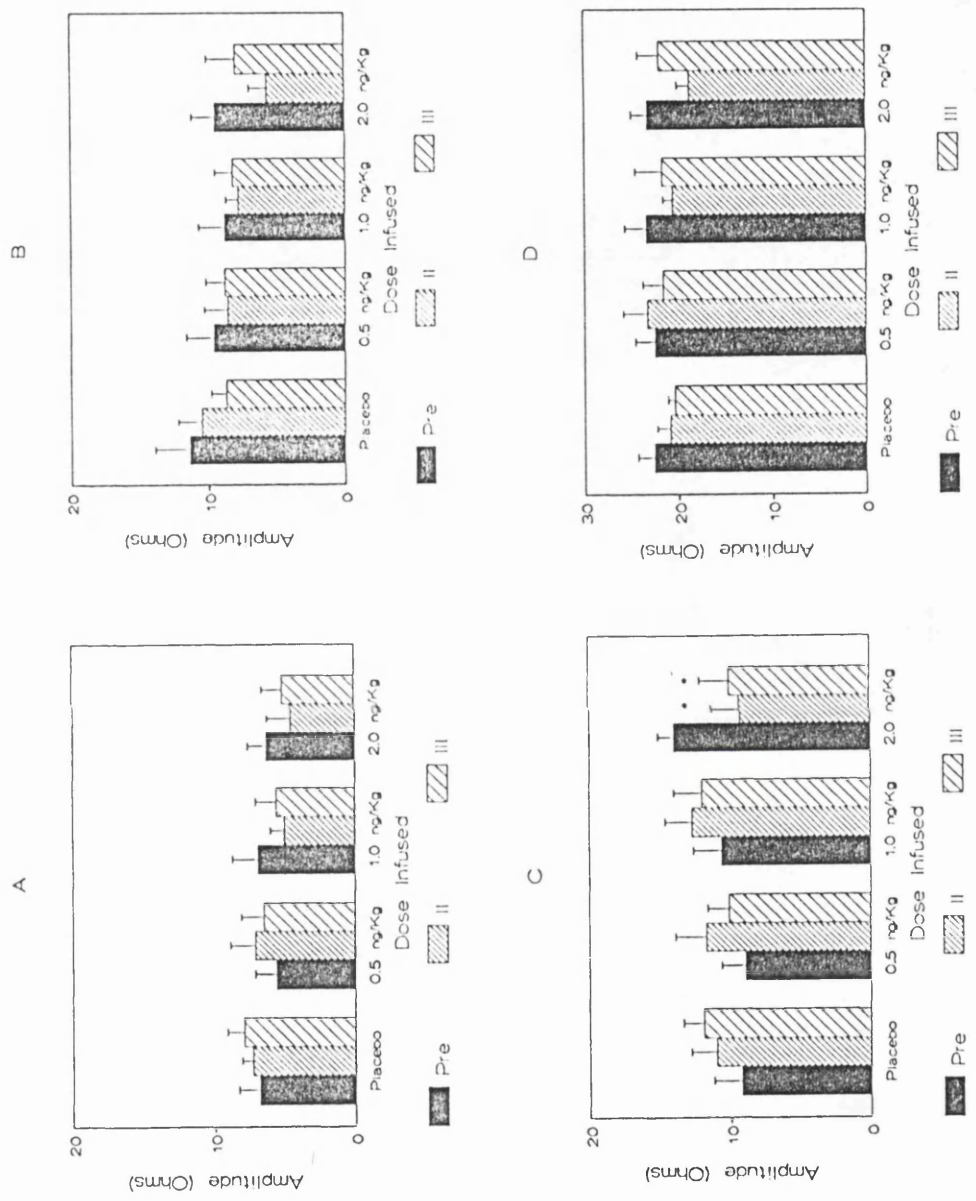

Fig. 5.10 - Whole Blood Platelet Aggregation Results in Arteriopaths Receiving Iloprost or Placebo Infusions. The maximum amplitude (Ohms) is shown using final concentrations of 12.5 and 25 uM ADP (A & B), or 1 and 4 ug/ml collagen (C & D).

Two patients showed spontaneous aggregation in PRP and whole blood on at least one separate occasion. Two further patients had spontaneous aggregation in either PRP or whole blood, but not both. The spontaneous responses disappeared when PRP was diluted to $200*10^9/l$, or whole blood was diluted to a haematocrit of 0.3 l/l. It was also abolished following in vivo infusions of Iloprost. Spontaneous aggregation was usually associated with increased aggregation in PRP, but decreased aggregation in whole blood, with both ADP and collagen low doses.

5.5.5 DISCUSSION

The first patient receiving prolonged Iloprost infusions showed initial inhibition of platelet aggregation in both PRP and whole blood, accompanied by a decrease in serum thromboxane B_2 levels. However by the sixth day, his platelets were refractory to the inhibitory effects of Iloprost and despite an increasing infusion dose over the next 4 days, his platelets became progressively hyperaggregable with the development of spontaneous aggregation and raised serum thromboxane B_2 levels. This was accompanied by diminished inhibitory effect when exogenous Iloprost was added to PRP in vitro. A similar addition of Iloprost to whole blood had little inhibitory effect at any stage during the infusion regimen. When daily five hour infusions were restarted several days later, a platelet inhibitory effect was again observed but this was also accompanied by rebound platelet hyperaggregability 1 hour after stopping the infusion. This rebound effect was abolished following the ingestion of ASA.

In the second patient there was also initial inhibition of platelet aggregation, however after the fourth infusion day, this inhibition was diminished in PRP and in whole blood. This was accompanied by diminished inhibition of aggregation after in vitro addition of Iloprost. During this first infusion, the patient continued to receive indomethacin, a reversible platelet cyclo-oxygenase inhibitor; significant rebound hyperaggregability was not seen, and serum thromboxane B_2 levels remained low. During the second continuous infusion, which was started 24 hours after stopping indomethacin therapy, ex vivo aggregation demonstrated refractoriness to the inhibitory effects of Iloprost, and a hyperaggregable response with spontaneous platelet aggregation and raised serum thromboxane B_2 levels were observed. This effect was abolished by ASA.

The observed discrepancies between the PRP turbidometric method and the whole blood impedance method with regard to sensitivity to the in vitro addition of Iloprost are probably due to the presence in whole blood of other cell types. One might speculate that these cells are already activated or stressed because of the underlying chronic disease state of the patients, so that they react differently from the blood cells of normal volunteers as seen in the earlier section. Red cells are known to bind and neutralise native prostacyclin (Willems et al, 1983; Jakubowski et al, 1983), but the effects on Iloprost are unclear; leucocytes take up and release a range of active substances including prostaglandins, leukotrienes, and hydroxy acids, as well as thromboxane A_2 (see chapters 1 and 3 above).

It seems therefore that prolonged continuous infusions of Iloprost can result in progressively decreased platelet sensitivity, and regardless of whether the infusion is then continued or stopped, rebound hyperaggregability, spontaneous aggregation, and raised serum thromboxane B_2 levels may occur. Similar phenomena have been observed during prolonged infusions of native prostacyclin (Sinzinger et al, 1981, 1983), while Blattler and colleagues (1981) noted increased B-thromboglobulin release. Spontaneous aggregation occurs with increased frequency in patients with hypercoagulable states and is associated with an increased incidence of thrombotic episodes (Preston et al, 1974). The rebound hyperaggregability during and after infusions of Iloprost can be prevented by platelet cyclo-oxygenase inhibition with aspirin or regular indomethacin therapy; however this does not affect platelet refractoriness to in vitro inhibition by the drug. Decreased sensitivity to Iloprost may be due to a re-setting of the balance between pro-aggregatory thromboxane A_2 and anti-aggregatory prostacyclin, in favour of the former; this may depend on reduced platelet cAMP production, and more intracellular calcium flux to small stimuli, resulting in platelet hyperaggregability.

In the group of arteriopaths, some patients were receiving multiple medication for their underlying disease and this may have contributed to their often weak aggregation responses. Arteriopaths are known to have vaso-occlusive episodes with poor blood flow and increased incidence of thrombotic episodes. Increased levels of circulating aggregates have been found, and Beta Thromboglobulin levels

are increased. It is likely that platelets in arteriopaths are continually being activated and undergoing submaximal release reactions, so that the platelets become exhausted of their granule contents. An alternative possibility is that the constant stimulation down-regulates their activation threshold, so that bigger stimuli must be applied to activate them. This type of phenomenon may occur due to increased intraplatelet levels of inhibitory substances such as cAMP and certain prostaglandins, decreased capacity for thromboxane A_2 production, or alteration of membrane receptors for agonists. Either of these possibilities may account for the observed decrease in aggregation obtained in arteriopaths before infusion of Iloprost. It may also be that an unsuitable control group was used since this was composed of healthy young staff members who tend to be active rather than sedentary. However, differences in aggregation though occurring with increased age, are not of the same magnitude as seen here. Although some patients were taking antiplatelet medication which could not be stopped for ethical reasons, this does not explain the increase in aggregation seen after placebo infusion. This effect is probably due to the stress of being in a hospital environment and being subjected to venepunctures.

A dose dependent inhibition of aggregation was seen with Iloprost infusion, 0.5ng/Kg being ineffective, or possibly causing hyperaggregability, 1ng/Kg causing weak inhibition, and 2ng/Kg causing stronger inhibition of aggregation. This inhibition could still be detected at 2 hours after stopping the infusion for 2ng/Kg and collagen, but not at lower

Iloprost doses or with ADP at any dose. This may reflect the mechanisms of action of the agonists and Iloprost.

These results are similar in pattern to those with normal controls receiving Iloprost infusions (Chapter 3). In these subjects there was less inhibition in whole blood than PRP, and the inhibitory effects tended to disappear after two hours of the infusion. Rebound hyperaggregability was also observed in the normal controls and the two arteriopaths receiving prolonged infusions. These facts as well as the observed increase in serum thromboxane B_2, suggest that either a change occurs in the mechanisms controlling the platelet reactivity status, or there is some external influence. The latter is more likely since platelets lack a nucleus and any synthetic capacity. In Chapter 4 it was shown that PMN's appear to potentiate non-anticoagulated whole blood aggregation responses to collagen, and it is possible that the increased serum thromboxane B_2 is neutrophil or monocyte derived. Iloprost is known to inhibit leucocyte aggregation (Belch et al, 1987) and it may influence other systems in white cells, such as eicosanoid production.

The differences between whole blood and PRP aggregation may have a bearing on the disappointing clinical results obtained with Iloprost for intermittent claudication (Yardumian, 1989; Hay et al, 1987). Platelet function studies in whole blood may be more closely related to the clinical effectiveness than the potent inhibition of platelet function seen in PRP.

5.5.6 GENERAL CONCLUSIONS

Myeloproliferative disease patients generally gave similar aggregation results in whole blood and PRP. Most patients with essential thrombocythaemia showed in vitro spontaneous aggregation in whole blood (as well as PRP), which disappeared on dilution and was blocked by a prostacyclin analogue. However, the presence of spontaneous platelet aggregation in whole blood was not correlated with platelet count, platelet volume, haematocrit, or leucocyte count. Aggregation responses to collagen and ADP were generally reduced in PRP, but varied in whole blood. Patients with polycythaemia rubra vera or secondary polycythaemia had decreased whole blood and PRP aggregation, despite dilution of whole blood to a normal haematocrit, indicating that the abnormality was related to high circulating red cell count rather than the disease. The increased red cell concentration in vivo and ex vivo, may have resulted in ADP release from the red cells, resulting in platelet refractoriness to subsequent exogenous agonists. These patients had normal responses in non-anticoagulated blood, showing that the abnormalities could be overcome in the presence of thrombin and other active substances.

Hyperaggregable platelets were not detected in patients investigated for recurrent venous thrombosis or familial thrombophilia. Platelet hyperactivity is probably not a common primary cause of unexplained recurrent venous thrombosis, although platelets are almost certainly involved in arterial disease.

Critically ill patients with MOF or ARDS frequently

showed a dramatic increase in aggregation rate and amplitude in non-anticoagulated whole blood. The aggregation responses decreased as patients started to recover. Aggregation in citrated whole blood was generally normal even if recalcified, suggesting that an activity with very short half life was involved (aggregation of non-anticoagulated whole blood was always performed immediately before citrated blood). The increased responses were not related to platelet count, as the patients were mostly thrombocytopaenic, with very poor PRP aggregation responses. Whole blood clotting times varied, being normal or very prolonged due to heparin therapy, and serum thromboxane B_2 levels were not increased. Iloprost prolonged the lag period of non-anticoagulated whole blood, but had no effect on the amplitude or rate. Aggregation in non-anticoagulated blood was decreased by colchicine, a neutrophil inhibitor, but the results could not be explained by an additive effect of neutrophil aggregation and platelet aggregation, since stimulated neutrophils only give a slow weak impedance response in whole blood. However, the increased aggregation correlated with the leucocyte and neutrophil counts, and an increased plasma neutrophil elastase level was seen, indicating neutrophil activation and degranulation. Neutrophils and platelets may release substances which activate one another, resulting in coherence of the cell types, with an apparent increase in aggregation rate. Endotoxin may also have stimulated platelets and neutrophils, the latter producing PAF-acether and leukotrienes which can further activate platelets.

There was increased activation of the kallikrein-kinin

system and consumption of inhibitors, ie. AT-III and heparin cofactor II, allowing more rapid ex vivo thrombin generation with less inhibition. Thrombin is a potent activator of polymorphs, but could not on its own explain the increased non-anticoagulated whole blood aggregation, since it did not occur in recalcified whole blood. The mechanism of the observed increase in response in critical illness is likely to be multifactorial.

Patients with chronic arterial disease had reduced aggregation responses before treatment, which were compatible with minimal in vivo activation and release, leading to an acquired storage pool defect. Iloprost infusion was less effective as judged by the whole blood method than by PRP aggregation, and the former approach may give a better indication of the clinical effectiveness of such infusions. Prolonged continuous infusions of Iloprost in arteriopaths resulted in a progressive decrease in platelet sensitivity to inhibition by Iloprost, with rebound hyperaggregability, increased serum thromboxane B_2 levels, and decreased in vitro sensitivity to the prostacyclin analogue.

postscript

The infusion of Iloprost into 11 arteriopaths was performed with informed consent following approval of the local ethical committee.

CHAPTER 6

CIGARETTE SMOKING AND PLATELET HYPERAGGREGABILITY

6.1 CIGARETTE SMOKING AND PLATELET ADHESION

6.1.1 Introduction

In a rat model, cigarette smoking has been shown to result in a depression of prostacyclin synthesis by the endothelial cells of the thoracic aorta (Pittilo et al, 1982). This was associated with increased platelet adhesion and aggregation observed in parallel morphological studies as well as hyperaggregability of the circulating platelets. It is generally accepted that platelets do not adhere to intact endothelial surfaces (Meyer & Baumgartner, 1983), however animal studies have indicated that this may not always be the case, since the platelets of rats exposed to cigarette smoke can adhere to apparently intact endothelium (Sieffert et al, 1981). The aim of this study was to see whether cigarette smoking in human volunteers caused increased platelet adhesiveness, and resulted in platelets adhering to intact endothelium using an in vitro system.

6.1.2 Materials and Methods

Volunteers - 10 non-smoking healthy male volunteers were bled before and immediately after smoking 2 medium tar cigarettes (yielding 19 mg tar and 1.5 mg nicotine), at a rate of one inhalation every 15 seconds under close observation. A further 7 non-smoking volunteers were bled for control experiments. Blood was collected into 0.106M tri-sodium citrate (ratio 9 volumes blood:1 volume citrate).

Preparation of Baumgartner Chamber - A separate rabbit was killed by intravenous injection of sodium pentabarbitone (0.5ml/kg) for use with each volunteer's blood. The aorta was dissected free from below the renal arteries to the bifurcation, transected at these points, and immediately washed in oxygenated Kreb's ringer solution within which it was subsequently maintained. The aorta was everted and cut into pieces, before carefully mounting onto the central spear of a Baumgartner chamber (see chapter 2). The endothelial surface was at all times kept exposed to oxygenated Kreb's ringer solution. The first piece of aorta from each rabbit was exposed to pre-smoking volunteer blood; the second piece was exposed to blood taken after smoking, and the final piece was in seven cases exposed to blood from a non-smoking volunteer. Blood was equillibrated for 10 minutes at 37°C prior to being circulated over the vessel at 150ml/min for 10 minutes, with oxygenation during the circulation. The aortic samples were rinsed and prepared for analysis by scanning electron microspcopy.

Haemostatic Variables - Platelet aggregation in PRP was studied with final concentrations of 0.5, 1.0, and 5.0uM ADP, 1.0 or 4.0ug/ml collagen; and 1.25mg/ml ristocetin, by a turbidometric method (see section 2).

Von Willebrand Factor antigen (VWF:Ag) levels were measured by immunoelectrophoresis on plasma before and after smoking.

6.1.3 Results

In each of the specimens, 90% of the endothelium was intact, areas of damage were only found at the ends of the specimens, in the regions where they were handled by forceps. The platelet count from 10 random fields from each specimen is given in Table 6.1. Platelets were either absent or few in number on the aortic samples exposed to blood from volunteers before smoking and on samples exposed to blood from the additional volunteer at the end of a run (Fig. 6.1). The samples exposed to blood obtained immediately after smoking showed large numbers of adhering platelets all of which had undergone shape change (Fig. 6.2); these could be observed in almost every field examined.

Volunteer	Pre-smoke	Post-smoke	Post-smoke
1	0	720	—
2	0	1140	—
3	6	940	—
4	0	480	23
5	4	654	17
6	0	832	7
7	13	906	0
8	11	560	17
9	4	839	41
10	0	663	4

Table 6.1. Platelet Quantification. Platelet counts from 10 random fields are shown. One-way analysis of variance for the pre-smoke and post-smoke samples gave $p < 1*10^{-6}$; and for the post-smoke verses post-smoke control $p < 5*10^{-6}$.

Platelet aggregation to ADP and collagen showed no consistent differences between pre- and post-smoking samples, or comparing blood before and after circulation of blood through the Baumgartner system. However, platelet agglutination to ristocetin was increased in 5 of 8 subjects studied after smoking. Von Willebrand factor antigen was also increased in 6 of 8 subjects after smoking (Table 6.2).

Fig. 6.1. Rabbit Aorta Exposed to Control Blood Samples. SEM picture shows endothelial cell surface exposed to blood from a normal human volunteer before cigarette smoking.

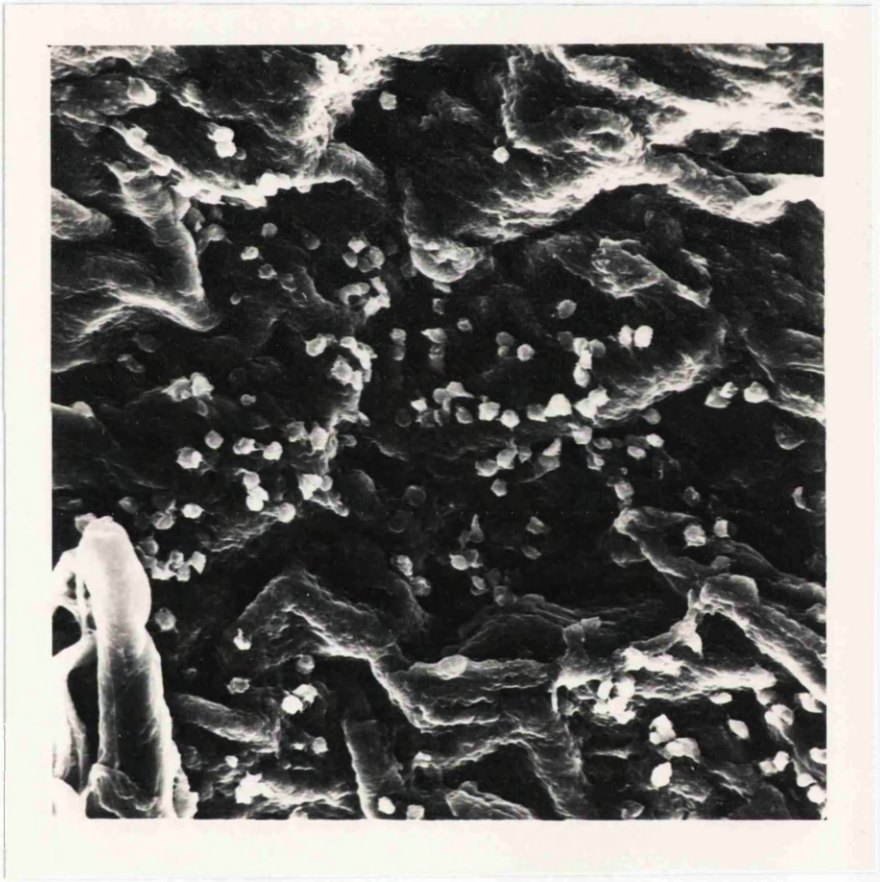

Fig. 6.2. Rabbit Aorta Exposed to Blood Collected After Cigarette Smoking. SEM picture shows large numbers of platelets adhering to the endothelial cell surface.

RIPA	VWF:Ag
0	24
0	17
-1	7
4	-8
181	18
74	103
12	32
4	0

Table 6.2 - Ristocetin Induced Platelet Agglutination (RIPA) and VWF:Ag After Smoking. Results are expressed as a percentage increase after smoking.

6.1.4 Discussion

The passage of whole anticoagulated blood over intact everted rabbit aortic endothelium in a Baumgartner chamber resulted in significant and extensive adhesion of platelets to an intact endothelial surface after cigarette smoking by non-smoking volunteers. The use of blood from a non-smoking volunteer as a control, demonstrated that the increased adherence of platelets seen with the post-smoking blood did not result from the vessel deteriorating during storage in the Kreb's buffer.

It has been demonstrated in a rat model that chronic smoking can induce morphological damage to the vascular endothelial surface and decrease endothelial cell synthesis of prostacyclin (Pittilo et al, 1982). In the rats, the platelets also became hyperaggregable after smoking; similar observations have been made with human platelets (Bierenbaum et al, 1978; Hawkins, 1972; Levine, 1973; Grignani et al, 1977; Davis & Davis, 1979; Sinzinger & Kefalides, 1982), and may account for the increased platelet adhesion to the intact endothelial surface observed in the rats (Pittilo et al, 1982). However, the studies in humans were all carried out in

regular smokers, whose response may well be different to the non-smokers studied here, due to a cumulative effect of chronic smoking. Recent human studies have shown no evidence of platelet activation after smoking (Laszlo et al, 1983) and no change in ADP, or collagen induced aggregation of PRP was seen here in the post-smoking samples.

Ristocetin induced platelet agglutination, was increased in some subjects; since platelets and endothelial cells contain VWF:Ag, the increased plasma levels observed may result from release of VWF:Ag from these sources due to the toxic effects of cigarette smoke causing direct cellular damage, or as a stress response. It has been shown that cigarette smoking increases the levels of adrenaline, vasopressin, and cortisol, (Cash et al, 1974; Ingram et al, 1977) all of which can release VWF:Ag from the vessel wall. The increase in VWF:Ag may contribute to the observed adhesion of platelets to the apparently intact endothelium after smoking. Measurement of the biological activity of this protein rather than immunological assay is necessary to try and resolve this matter.

These observations are almost certainly multifactorial in origin, and are probably influenced by a depression in prostacyclin release by endothelial cells and a physical and chemical alteration of their luminal membrane integrity and microstructure. Although post-smoking platelets were not hyperaggregable, the findings suggest that cigarette smoking results in them becoming intrinsically more adhesive.

6.2 CIGARETTE SMOKING AND HAEMOSTASIS IN HEALTHY VOLUNTEERS

6.2.1 Introduction

The preceding studies have established that cigarette smoke inhalation causes increased platelet aggregation and vessel wall damage in a rat model; increased adhesion of human platelets to rabbit aorta, and evidence of endothelial cell damage, in human blood taken immediately after smoking. These observations were extended in a further group of human volunteers in a highly controlled study of platelet aggregation in whole blood as well as PRP, von Willebrand factor, and other haemostatic parameters. The acute effects of cigarette smoking were studied in two groups of healthy volunteers, regular cigarette smokers and non-smokers.

6.2.2 Non-Smoker Volunteers

Fourteen healthy, male volunteers who were usually non-smokers were enrolled in the study (mean age 26 +/- 5.3 yrs); they had not smoked in the preceding week, and only two had smoked in the preceeding month, (less than 5 cigarettes per day on less than 3 days per week). They were asked to refrain from alcohol for 24 hours, and fast overnight before the study. All subjects were over 18 years of age and had not received non-steroidal anti-inflammatory agents, (eg. aspirin) or other drugs known to influence platelet function for at least ten days. No subject had any history of cardiovascular or pulmonary disease or any current medical condition. Each subject was asked to smoke two medium-high tar, non-filtered cigarettes in rapid succession, within 20 minutes. The

cigarettes were smoked until they had burnt down to the last 2cm, and the volunteers were supervised and encouraged to inhale the cigarette smoke. Blood samples, pulse rate, and blood pressure were taken immediately before and within 5 minutes of completing the smoking session. The parameters measured are shown in Table 6.3.

Blood Pressure	Fibrinogen
Pulse Rate	Circulating Endothelial Cell Casts
Full Blood Count	Factor VIII:C
Platelet Count	von Willebrand Factor Antigen
Platelet Aggregate Ratio	Ristocetin Cofactor (vWF:RiCof)
B-Thromboglobulin	Platelet Factor 4
Platelet Aggregation	

Table 6.3. Parameters Measured.

The differences between the pre- and post-smoking values fitted a normal distribution and were tested by Student's t-Test. There were no changes in full blood or platelet counts, platelet aggregate ratio, B-thromboglobulin, platelet factor 4, fibrinogen, or circulating endothelial cell casts. The other parameters are shown in Table 6.4.

PARAMETER	**PRE**		**POST**	
Pulse Rate	76	(13.7)	80	(13.9)
Systolic BP	122	(15.1)	119	(9.2)
FVIII:C	0.97	(0.57)	1.05	(0.43)
FVIII:RAg	0.81	(0.30)	0.90	(0.39)
FVIII:RiCof	0.84	(0.37)	0.85	(0.39)
RIPA 0.75 mg/ml	12	(11.3)	22	(17.7)
CRP	1.72	(3.64)	1.33	(2.70)

Table 6.4. The Effects of Cigarette Smoking in Healthy Non-Smoking Volunteers - Mean and (SD) of Selected Cardiovascular and Haemostatic Variables. CRP = C-Reactive protein; RIPA = ristocetin induced platelet agglutination.

A slight increase in pulse rate, and fall in systolic blood pressure were seen, but there was considerable variability in cardiovascular response between subjects (range of pre:post differences -26 to +36, and -20 to +20 respectively). The cardiovascular effects of cigarette smoking have been studied previously (Ashton et al, 1981), and in smokers, generally are an increase in heart rate and blood pressure, with decreased peripheral blood flow (Larson et al, 1961). The effects all occur almost immediately on starting smoking, and there is evidence that they are mainly due to nicotine (Herxheimer et al, 1967). However, nicotine stimulates and then blocks nerve tissue, and may initiate reflex compensatory mechanisms. The overall effects of cigarette smoking therefore depend on the individual and their current autonomic balance. There is great variability amongst individuals, and sometimes the cardiovascular effects are reversed (fall in blood pressure, bradycardia), in the non-smoker in particular, this may be accompanied by nausea, vomiting and syncope. The changes described above did not correlate with the recorded adverse events (these occurred in some but not all volunteers), which included dizziness, nausea, and coughing; one subject fainted during blood sampling. After smoking, there was an increase in vWF:Ag, FVIII:C, and platelet agglutination to low doses of ristocetin, but not of vWF:RiCof (Table 6.4). These results may reflect toxic effects of cigarette smoking on the vessel wall, or an acute stress response. The neuropharmacology of nicotine is complex (Ashton et al, 1981), but it is known to cause the release of catecholamines from the adrenal medulla,

and these compounds along with their metabolites are increased in the urine of smokers (Westfall, 1965); plasma levels of noradrenaline and adrenaline increase towards the end of smoking a cigarette, but not after sham smoking (Kashemsant et al, 1965). In addition, nicotine stimulates nerve cells in the hypothalamus, which brings about secretion of vasopressin from the anterior lobe of the posterior pituitary. Increase plasma levels of adrenergic compounds could also account for the results in this study since infusion of adrenaline, vasopressin (or its analogue, DDAVP) into healthy volunteers, causes release of von Willebrand factor from the vascular endothelium, and an increase in circulating FVIII:C (Cash et al, 1974; Ingram et al, 1977). Platelet alpha granule release is unlikely to account for the increase in vWF, since there was no correlation with increases in BTG. CRP levels fell which is inconsistent with a stress or acute phase reaction, although the time interval after smoking is probably too short for CRP levels to change due to the synthesis of new protein in an acute phase reaction. The change in CRP levels cannot be adequately explained at present, as little is known about the physiology and function of this protein.

	SLOPE		HEIGHT	
	PRE	POST	PRE	POST
ADP				
0.5uMol/l	46 (17.6)	52 * (16.9)	5 (3.7)	7 (3.6)
1.0uMol/l	69 (10.2)	71 (12.0)	18 (10.0)	23 * (12.9)
2.5uMol/l	77 (6.2)	77 (6.8)	41 (14.0)	45 (16.1)
COLLAGEN				
0.5ug/ml	42 (21.7)	48 (24.9)	20 (11.3)	26 (16.5)
1.0ug/ml	66 (20.1)	70 (14.9)	44 (17.1)	45 (20.8)
2.0ug/ml	75 (5.8)	75 (9.8)	49 (12.9)	52 (15.9)

Table 6.5. Effects of Cigarette Smoking in Non-Smoking Volunteers - Platelet Aggregation Results. Mean values are shown for selected parameters (SD in parenthesis). * = $p < 0.05$.

The only statistically significant changes observed in this small group of individuals were an increase in the slope and height of platelet aggregation responses in PRP to low doses of ADP (Table 6.5), and increases in slope and maximum impedance responses to collagen in citrated (but not in non-anticoagulated) whole blood (Table 6.6). Similar increases were seen for low doses of collagen in PRP, although these did not achieve statistical significance; the collagen lag phase at a dose of 0.5ug/ml was prolonged, but no change in the platelet response to arachidonate was seen. B-TG levels were increased in some individuals, although the levels did not correlate with increased aggregation to ADP. These results therefore suggest in vivo platelet activation, which may be offset in whole blood by the influence of activated leucocytes. When calcium ions are chelated this latter

influence disappears, and this may either be due to the loss of a very labile substance, or to the loss of activity in a calcium dependent mechanism. The changes in platelet function were accompanied by evidence of vessel wall damage or stimulation.

	LAG	SLOPE	IMPEDANCE
NON-ANTICOAGULATED WHOLE BLOOD			
Pre Smoking	1.35	52.0	9.4
	(0.20)	(10.2)	(4.8)
Post Smoking	1.48	50.6	9.9
	(0.30)	(10.8)	(3.2)
	NS	NS	NS
CITRATED WHOLE BLOOD			
Pre Smoking	1.45	31.9	8.2
	(0.54)	(10.4)	(2.8)
Post Smoking	1.11	45.1	11.3
	(0.26)	(11.0)	(3.4)
	NS	$p<0.01$	$p<0.02$

Table 6.6. The Effects of Smoking on Aggregation in Non-Anticoagulated and Citrated Blood From Non-Smokers. Mean and sd (in parenthesis) are shown for responses to 5ug/ml collagen.

The haemostatic and cardiovascular parameters studied may have been influenced by the way in which the volunteer smoked the cigarette; nicotine and cotinine levels were therefore measured as an indicator of the effectiveness of smoke inhalation, and absorption of smoke components. Peak plasma levels of about 30ng/ml nicotine are found 60-90 seconds after inhalation of cigarette smoke; there is a biphasic decay curve, with a rapid initial fall over several minutes, followed by a slower phase with a half life for clearance from blood of up to 2 hours (Feyerabend & Russel, 1980; Armitage et al, 1975). The initial degradation of nicotine to cotinine is very rapid, but the latter has a longer half-life of 10-20

hours (Curvall & Enzell, 1986). Since blood samples could not be taken from the above subjects within 5 minutes of cessation of the smoking period (for logistic reasons), peak plasma nicotine levels were not necessarily measured; and the efficiency of cigarette smoking was therefore judged from the total of nicotine plus cotinine levels. The subjects fell into two natural groups in terms of their nicotine/cotinine levels, those with total nicotine plus cotinine values of 3.1-10.6ng/ml, and those with 22.1-134.0 ng/ml. This was assumed to reflect the degree of cigarette smoke inhalation, and the haemostatic results were divided into the two arbitrary groups for analysis.

In the high nicotine group, there was a trend towards a smoking induced increase in platelet aggregation, with increases in responses to lower doses of ADP and Collagen (Tables 6.7 and 6.8). The low nicotine group surprisingly showed more marked, and in some cases statistically significant changes in platelet aggregation after smoking. Previous studies (Brinson, 1974; Brinson & Chakrabarti, 1974) have shown that nicotine has no effect on platelet aggregation to ADP at concentrations similar to the "physiological" levels found in cigarette smokers, but higher concentrations cause a decrease in ADP aggregation responses in PRP in-vitro, and inhibition of this effect by adrenaline. The results presented in this thesis may therefore reflect a balance between the inhibitory effects on platelets of nicotine absorbed from cigarette smoke, and the potentiating effects of adrenaline released from the adrenal medulla in response to nicotine and following the stress of smoking. Alternatively,

it may be that nicotine is not a good way of monitoring cigarette smoke inhalation. Another factor may be the sampling time, since the effects of cigarette smoking may not be fully exacerbated until a longer time has passed.

	NICOTINE + COTININE				
	22.1 - 134.0ng/ml		3.1 - 10.6ng/ml		
	PRE	POST	PRE	POST	
0.5 uM ADP					
Slope	37 (24.3)	45 (23.8)	51 (10.5)	55 (11.1)	+
Height	5 (5.7)	5 (3.4)	5 (2.1)	7 (3.7)	*
1.0 uM ADP					
Slope	70 (10.3)	70 (15.0)	68 (10.6)	71 (10.4)	*
Height	16 (6.9)	19 (11.7)	20 (11.9)	25 (14.0)	*
2.5 uM ADP					
Slope	80 (2.5)	80 (2.6)	75 (7.3)	75 (8.4)	
Height	40 (14.8)	42 (15.1)	42 (14.3)	48 (17.4)	

Table 6.7. Differences in Platelet Parameters Before and After Smoking in Subjects With High and Low Post-Smoking Nicotine Levels. Mean and SD (in parenthesis) are shown. * = $p<0.05$; + = $p<0.10$.

Other cigarette smoke components may be more relevant to haemostasis; Toivanen et al (1986) showed that smoking caused increased levels of serum thromboxane B_2, while nicotine inhibited in-vitro thromboxane B_2 production by platelets, and stimulated PGI_2 production by perfused lung sections of human lung. This indicated that nicotine exerted its effect below the level of cyclo-oxygenase, since this enzymatic step is necessary in the production of both compounds, and suggested that the increase in serum thromboxane B_2 in smokers serum is due to some constituent of smoke other than nicotine. However, leucocytes have also been shown to produce thromboxanes (Higgs, 1982), and the effects of cigarette smoke and nicotine

on thromboxane production by these cells has not been investigated. Activation or modification of the function of granulocytes by cigarette smoke and its compnents may explain some of the results obtained. Thromboxane B_2 production in recalcified, citrated whole blood is also more sensitive to inhibition by nicotine than production in PRP (Toivanen et al, 1986). It may be that a loss of function of certain cells occurs after blood has been standing, and if calcium ions are sequestered, even temporarily, so that the influence of nicotine or cigarette smoke on thromboxane B_2 production and platelet aggregation may vary according to the cell population present and the experimental conditions. Carbon monoxide has also been shown to inhibit platelet aggregation to adrenaline and arachidonic acid (Mansouri & Perry, 1982), but there are many other components of cigarette smoke which have not been investigated, or remain to be identified.

	NICOTINE + COTININE			
	22.1 - 134.0ng/ml		**3.1 - 10.6ng/ml**	
	PRE	**POST**	**PRE**	**POST**
Collagen				
0.5 ug/ml				
Slope	40 (23.6)	46 (25.0)	45 (22.4) **	51 (26.2)
Height	16 (10.1)	21 (15.7)	25 (12.1) *	32 (17.8)
1.0 ug/ml				
Slope	64 (29.7)	76 (6.8)	61 (24.8)	67 (17.7)
Height	47 (23.5)	50 (26.0)	41 (11.7)	41 (16.8)
2.0 ug/ml				
Slope	76 (7.6)	74 (15.0)	74 (4.4)	75 (4.2)
Height	53 (17.6)	55 (23.6)	46 (7.6)	48 (8.4)

Table 6.8. Differences in Platelet Parameters Before and After Smoking in Subjects With High and Low Post-Smoking Nicotine Levels. Mean and SD (in parenthesis) are shown. * = $p<0.05$; ** = $p<0.01$.

The increase in pulse rate, factor VIII and von Willebrand factor levels (Table 6.9) after smoking was more marked in the high nicotine group. This may reflect the neurohumoural action of nicotine as discussed above, or a direct effect on the vascular endothelium. Nicotine has been shown to have dramatic effects on the morphology and function of cultured human umbilical vein endothelial cells (Bull et al, 1988). The white cell count also showed a small but statistically significant rise after smoking, in the high nicotine group, which may also represent a stress response.

	NICOTINE + COTININE			
	22.1 - 134.0ng/ml		3.1 - 10.6ng/ml	
	PRE	POST	PRE	POST
PULSE RATE	72 *	83	79	78
	(8.9)	(15.5)	(16.3)	(13.3)
SYSTOLIC BP	121	118	122	120
	(11.8)	(13.7)	(7.6)	(17)
WBC	5.9 **	6.2	6.0	5.9
	(1.3)	(1.2)	(1.3)	(1.3)
FVIII:C	1.02	1.19	0.93	0.95
	(0.67)	(9.49)	(0.54)	(0.38)
FVIII:RAg	0.76 *	0.98	0.85	0.84
	(0.76)	(0.38)	(0.25)	(0.30)
FVIII:RiCof	0.82	0.89	0.85	0.81
	(0.35)	(0.46)	(0.41)	(0.37)
RIPA 0.75mg/ml	16	24	6.3	18.0
	(13.3)	(26.7)	(2.1)	(17.8)

Table 6.9. Factor VIII and Other Parameters in Subjects With High Nicotine Levels Before and After Smoking. Mean and SD (in parenthesis) are shown. * = $p < 0.05$; + = $p < 0.01$.

6.2.3 REGULAR SMOKERS

8 healthy, male volunteers who regularly smoked between 15 and 20 cigarettes a day were studied. The smokers normally used a variety of different brands with a range of tar and nicotine contents. During the study they smoked 2 high tar, non-filterred cigarettes in rapid succession, down to the last 2 cm, within a period of 20 minutes. They were supervised and

encouraged to inhale the cigarette smoke. Blood samples were collected on the day before the smoking study for baseline nicotine measurement, and the volunteer was instructed not to smoke from 18.00 hr until the conclusion of the study next day. Further blood samples as well as pulse rate and blood pressure were taken immediately before, and within 5 minutes of finishing the smoking session. Subjects fasted overnight, refrained from alcohol in the preceeding 24 hours, and had not taken compounds known to influence plastelet function within the preceeding 2 weeks. No subjects had any current medical condition, or history of cardiovascular or pulmonary disease.

	PRE		POST
PULSE RATE	69	*	85
	(11.3)		(7.1)
SYSTOLIC BP	116		117
	(7.8)		(5.6)
PLATELETS	280		294
	(55.5)		(69.6)
FVIII:C	0.85		1.12
	(0.30)		(0.60)
vWF:Ag	0.95		1.08
	(0.28)		(0.47)
vWF:RiCof	0.95		1.17
	(0.49)		(0.70)
B-TG	38		29
	(19.3)		(10.1)
PF4	19.5		11.3
	(10.3)		(3.2)

Table 6.10. Effects of Cigarette Smoking on Regular Smokers. Mean and SD (in parenthesis) are shown. * = $p < 0.001$.

There was a significant increase in plasma nicotine and cotinine levels after cigarette smoking (mean nicotine + cotinine = 112.8 and 139.5, range = 23.7-249.8 and 40.9-280.6, respectively for pre- and post-smoking; $p<0.01$). In general, much higher levels of nicotine/cotinine were observed both before and after smoking, than those seen in the non-smokers; it was not therefore feasible to separate the smokers into

good and bad inhalers on the basis of plasma nicotine levels. Smoking had similar effects on the cardiovascular system and factor VIII/von Willebrand factor complex in smokers and non-smokers. A statistically significant increase in pulse rate was seen, but no change in systolic blood pressure (Table 6.10); as well as an increase in platelet count, factor VIII:C, vWF:Ag, and vWF:RiCof. There was no increase in B-TG or PF4 levels after smoking, and platelet responsiveness to ADP and collagen actually decreased rather than increased in PRP (Table 6.11), achieving statistical significance for 2.5 uM ADP, and showing no significant change in whole blood (Table 6.12). Ring and colleagues (1983) were not able to show any enhancement of ADP induced primary aggregation, despite a shortening of the bleeding time, after smoking. The data on smokers is interesting when compared with the non-smokers achieving higher levels of plasma nicotine, since the latter showed much smaller changes, or no change at all in platelet aggregation after smoking. It is possible that high nicotine levels damage or inhibit platelets, whereas in the non-smoker, low levels of nicotine evoke stress responses which increase platelet aggregability. It is known that adrenaline can act in synergy with ADP and other platelet agonists, and in vivo catecholamine levels may rise after smoking.

	PRE		POST	
	SLOPE	HEIGHT	SLOPE	HEIGHT
ADP 1.0 uM	72	22	69 *	18 *
	(9.0)	(12.5)	(12.0)	(10.6)
ADP 2.5 uM	80	51	77 ***	42 ***
	(4.6)	(14.3)	(4.0)	(14.0)
ADP 5.0 uM	81	56	80	50 *
	(3.4)	(11.5)	(3.0)	(9.5)
Collagen	71	46	67	44
1 ug/ml	(9.3)	(16.2)	(17.1)	(1 5 . 6)
Collagen	78	55	76 **	49 *
2 ug/ml	(3.5)	(10.9)	(3.2)	(6.8)
Collagen	80	57	78 *	51 *
4 ug/ml	(3.5)	(12.0)	(2.5)	(5.9)

Table 6.11. Effects of Cigarette Smoking on Regular Smokers. Mean and SD shown. * = $p < 0.2$; ** $p = < 0.01$; *** = $p < 0.05$.

	LAG	SLOPE
NON-ANTICOAGULATED WHOLE BLOOD		
Pre Smoking	1.14	56.4
	(0.22)	(10.9)
Post Smoking	1.31	53.9
	(0.21)	(11.4)
	NS	NS
CITRATED WHOLE BLOOD		
Pre Smoking	1.46	53.0
	(0.35)	(6.78)
Post Smoking	1.30	51.9
	(0.30)	(4.26)
	NS	NS

Table 6.12. Whole Blood Aggregation Using Non-Anticoagulated and Citrated Blood From Smokers. The results shown are the mean values with SD in parenthesis.

A comparison of the pre-smoking values for the regular smokers and the non-smokers, showed that the former had higher vWF:Ag and vWF:RiCof levels, lower FVIII:C, and increased platelet aggregation, both in PRP and in whole blood (non-anticoagulated as well as citrated) (Tables 6.13 & 6.14). These differences did not achieve statistical significance, with the exception of the whole blood measurements ($p<0.01$), because of the small numbers in each group and wide ranges of results. In a study of pregnant women, Davis and coworkers

(1987) recently found increased platelet reactivity to ADP by whole blood impedance aggregation in the smoking group. Regular smokers may be chronically stimulated and have altered baseline values regardless of whether they have refrained from cigarette smoking for a number of hours before the study. This would explain the lack of effect of 2 cigarettes in causing increased platelet aggregability and other changes, since the subjects were already stimulated, and their platelets activated. Alternatively, it may be that regular smokers tolerated the cigarette smoke much better and were therefore less stressed; however, their factor VIII levels and pulse rate were still increased after smoking, in a similar way to the non-smokers.

	VIII:C	**vWF:Ag**	**vWF:RiCof**
NON-SMOKERS	0.97	0.81	0.84
SMOKERS	0.85	0.95	0.95

	ADP 1.0 uM		**ADP 2.5 uM**		**ADP 5.0 uM**	
	Slope	Height	Slope	Height	Slope	Height
NON-SMOKERS	69	18	77	41	78	49
SMOKERS	72	22	80	51	81	56

	Coll 1 ug/ml		**Coll 2 ug/ml**		**Coll 4 ug/ml**	
	Slope	Height	Slope	Height	Slope	Height
NON-SMOKERS	66	44	75	49	78	53
SMOKERS	71	46	78	55	80	57

Table 6.13. Comparison of The Pre-Smoking Results From Regular Smokers and Non-Smokers From The 2 Studies. (mean values are shown, for SD, etc. see tables above)

	CWB		**NWB**	
	lag	**slope**	**lag**	**slope**
NON-SMOKERS	1.5 (0.5)	32 (10.4)	1.4 (0.2)	52 (10.2)
SMOKERS	1.4 (0.4)	53 (6.8)	1.1 (0.2)	56 (10.9)
	NS	$p<0.01$	$p<0.05$	NS

Table 6.14. Comparison of The Pre-Smoking Results From Regular Smokers and Non-Smokers From The 2 Studies. Non-anticoagulated and citrated whole blood (NWB & CWB) aggregation responses to 5ug/ml collagen (mean values with SD in parenthesis).

6.2.4 CONCLUSIONS

There was a small increase in platelet aggregation by both impedance and turbidometric methods, after cigarette smoking in a group of non-smokers. Those with relatively lower serum nicotine levels had a greater increase in platelet aggregation than those with higher nicotine levels. This may be due to stimulation of the adrenal medulla by nicotine, causing the release of adrenaline (which may prime platelets, or act in synergy with ADP and collagen), offset by a toxic effect of nicotine on the platelets themselves. These changes were not seen in a group of regular smokers, who had decreased aggregation responses by the turbidometric method, and showed no change in whole blood. However increased baseline platelet aggregability was seen in smokers, and their platelets were probably already stimulated by the effects of chronic cigarette smoking, or had reset their haemostatic balance.

Non-smokers showed an increase in von Willebrand factor levels after smoking, and regular smokers had increased baseline levels. This may be due to toxic effects of cigarette smoke on the vessel wall, direct pharmacologic effects of absorbed cigarette smoke components such as nicotine, or stress mediated effects.

postscript

The studies on the effects of cigarette smoking in healthy normal smokers and non-smokers was performed with approval of the local ethical committee.

SUMMARY AND CONCLUSIONS

Platelet function in prothrombotic states has previously been extensively investigated using platelet rich plasma, but not with the natural milieu, whole blood, which is the subject of this thesis. In order to study platelets in this way a whole blood method was established using the impedance technique. Initially the method was optimised and standardised in relation to a number of variables, and the type of platelet aggregate being detected was then investigated.

The haematocrit had a pronounced effect when it was above 0.35 l/l. Haematocrit values above this level generally gave reduced impedance responses, with poor reproducibility. Dilution of whole blood with isotonic saline or autologous platelet poor plasma reduced the haematocrit, and yielded stronger, more reproducible responses. Saline dilution was easier, since it avoided delay in measuring aggregation due to plasma preparation. Although this reduced the sensitivity to plasma activators and inhibitors, only a small dilution was usually necessary and all of the formed elements remained present.

Impedance aggregation measurement was not dependent on the platelet count in normal blood, and was unaffected by mild thrombocytopaenia. A linear relationship existed between platelet count and impedance response at platelet counts below approximately $50*10^9$/l, in diluted normal blood, or blood from a patient with ITP. Certain thrombocytopaenic patients, particularly those with an haematological malignancy, did not conform to this pattern (see Chapter 3), suggesting that they had a platelet dysfunction as well as a reduced count. The

method gave better sensitivity than the traditional turbidometric technique, which may yield falsely abnormal aggregation responses at platelet counts below $150*10^9/l$.

Collagen produced similar responses in normal blood and PRP, but higher ADP concentrations were needed to induce aggregation by the impedance technique, with only a single wave of aggregation observed at all ADP doses used. The numbers of free platelets, as measured by performing serial platelet counts from a whole blood aggregation cuvette, always fell before impedance aggregation was detected. The fall in free platelets followed a similar time course to turbidometric aggregation. Similar results were observed with a range of agonists, including ADP, collagen, and U46619. Low doses of ADP (eg. 1uM) gave no impedance response, despite causing a fall in free platelets. The release reaction also preceded impedance aggregation in whole blood, and a patient with storage pool defect, with no measurable ADP/ATP release showed normal impedance aggregation to collagen. Impedance aggregation was therefore not dependent on dense granule secretion. Irreversible aggregation of platelets was required for aggregate detection, although aggregate size and the presence of adhesive proteins may also be important.

An initial decrease in impedance values was seen after reagent addition, and before a wave of aggregation (increased impedance) was seen. This phenomenon may be due to the formation of platelet aggregates, which are initially unable to adhere to the impedance electrode, but whose formation lowers the resting impedance of the blood. As the aggregates become larger and perhaps express suitable surface

characteristics, they start to adhere to the electrode, and the measured impedance value increases. Thromboxane A_2 generation may be important for aggregate detection, since in vitro addition of acetyl salicylic acid to inhibit cyclo-oxygenase, could completely block aggregation responses to high and low dose ADP, but only partially blocked aggregation induced by U46619, a precursor and mimetic of thromboxane A_2. In addition, the thromboxane receptor blocker AH23848, had a very potent inhibitory effect on impedance aggregation induced by U46619. Whole blood impedance aggregation was more sensitive to PGI_2 and its analogues than turbidometric techniques. During infusion of the prostacyclin analogue Iloprost into normal volunteers, refractoriness was detected earlier in whole blood than PRP. Hyperaggregabilty in these volunteers coincided with increased serum thromboxane B_2 levels.

Impedance aggregation studies in non-anticoagulated blood allowed the examination of platelet aggregation in conditions closer to the natural milieu. Platelet aggregation was generally more rapid in non-anticoagulated and recalcified citrated blood, than in citrated blood. This increased activity was only partially reduced by the thrombin inhibitor hirudin. When thrombin was generated after contact activation in the absence of exogenous platelet agonists, no impedance aggregation was observed, therefore the increased aggregation in native whole blood was not just due to thrombin generation. Aggregation in non-anticoagulated blood was partly inhibited by colchicine, at doses which did not affect platelet aggregation; this reagent inhibits neutrophil degranulation,

adhesion and aggregation. The addition of washed neutrophils to citrated blood caused an increase in collagen induced aggregation despite the fact that neutrophils fail to aggregate to collagen, and even strong neutrophil agonists such as FMLP require extracellular calcium ions for neutrophil aggregation to occur. It is therefore likely that neutrophils become activated either by exposure to foreign surfaces, generation of thrombin (a neutrophil activator), or by products released from activated platelets. Once activated, neutrophils may produce substances which accelerate platelet aggregation, or improve adherence of platelet aggregates to the impedance electrode. In non-anticoagulated blood, where extracellular calcium is present, neutrophils may also cohere to the forming platelet aggregate, and to the electrode. The relatively low numbers of neutrophils present means that coherence alone would not explain the results. Neither would this mechanism explain the observation that collagen induced impedance aggregation increases when washed neutrophils are added to citrated blood. However, it is possible that platelet dense granule calcium ion stores may play some role in mediating neutrophil aggregation, and the degranulation and eicosanoid production mechanisms of neutrophils are not so highly dependent on calcium ions.

A group of patients with multi-organ failure, particularly those with adult respiratory distress syndrome (ARDS), showed a dramatic increase in the rate of collagen aggregation in non-anticoagulated blood, but normal aggregation in citrated blood. This was a consistent observation despite the fact that platelet counts were often

low and reduced platelet aggregation responses were seen by turbidometric techniques, so that there was insufficient platelet mass to account for the increased aggregation. There was a correlation between the increased aggregation in non-anticoagulated blood and the leucocyte and neutrophil counts, as well as with high levels of neutrophil elastase:alpha 1-antitrypsin complexes (an indicator of neutrophil activation and release). Serum thromboxane B_2 levels were not raised, and were not therefore the cause of the increased aggregation.

Recalcification of citrated blood from multi-organ failure patients did not cause similar, increased aggregation responses. This indicated the involvement of a platelet stimulator with a relatively short half life, or some labile cellular mechanism in non-anticoagulated blood, which had disapeared by the time that the citrated whole blood was recalcified and tested. Neutrophil aggregation may have accounted for some of the observed increase in aggregation in non-anticoagulated blood, since the neutrophil count was elevated, and the aggregation responses were reduced by the addition of colchicine. However, neutrophils in whole blood stimulated with potent agonists such as FMLP, showed comparatively slow and weak responses, insufficient to account for the increased aggregation in non-anticoagulated blood samples. Leukotrienes C_4 and D_4, as well as PAF-acether are all released by polymorphs and can enhance platelet aggregation, but have a relatively short plasma half life, and are therefore potential candidates for increased platelet stimulation. Iloprost, which can inhibit both platelet and neutrophil aggregation, prolonged the lag phase in non-

anticoagulated blood, but had no effect on the amplitude or rate of aggregation.

The cause of the increased aggregation in non-anticoagulated blood in multi-organ failure may be multifactoral. Sepsis and bacterial endotoxin are known to stimulate platelets as well as neutrophils and monocytes. The patients had reduced levels of serine protease inhibitors, and increased activation of the kallikrein-kinin system. Thrombin could therefore, in theory, be generated more readily and more rapidly, and inhibited more slowly. However, recalcification of citrated whole blood in these patients did not cause an increase in impedance aggregation, and so the effect was not simply due to an increased thrombin levels. Thrombin may play an important role activating both platelets and neutrophils with further production of labile mediators and the formation of fibrin strands interlinking the various blood cell types. Although clots were not detected in the native blood during the phase in which aggregation was measured, fibrin strands could have been incorporated into the cell mass adhering to the electrode, and may have potentiated this adherence. It is pertinent that mixed platelet-neutrophil-fibrin masses have been detected by other workers in the pulmonary vasculature of ARDS patients at post-mortem.

Patients with myeloproliferative disease generally had similar aggregation responses in whole blood and PRP. Most patients with essential thrombocythaemia showed spontaneous aggregation by both methods unless the blood/PRP was diluted or prostacyclin analogue was added. In whole blood the presence of spontaneous aggregation did not correlate with

platelet count, mean platelet volume, haematocrit, or leucocyte count, whereas in PRP spontaneous aggregation was more likely to occur at higher platelet counts. ADP and collagen aggregation were often reduced in PRP from essential thrombocythaemia patients, but the whole blood responses were more variable.

In polycythaemia rubra vera and in secondary polycythaemia, whole blood and PRP responses to ADP and collagen were reduced, indicating that the abnormality was related to the high circulating red cell count rather than the disease itself. Non-anticoagulated blood responses were normal supporting the idea that the phenomenon was an artifact of sample collection. This decreased aggregation may be due to ADP released from the closely packed red cells causing platelet refractoriness.

No hyperaggregability was detected in patients with recurrent venous thrombosis or familial thrombophilia; hyperactive platelets may be a rare primary cause of unexplained recurrent venous thrombosis. However platelets are known to be important in arterial disease, and a group of chronic arteriopaths showed reduced aggregation compatible with in vivo platelet activation and release, and an acquired storage pool-like defect. Iloprost infusion appeared less effective when monitored by whole blood compared to PRP aggregation. Whole blood aggregation may give a better indication of the clinical effectiveness of such infusions. Prolonged continuous infusion of Iloprost in arteriopaths patients resulted in progressively decreased platelet sensitivity to the inhibitor, rebound hyperaggregability,

spontaneous aggregation and a massive increase in serum thromboxane B_2 levels, with decreased in vitro sensitivity to Iloprost.

Cigarette smoking is regarded as a major risk factor for vascular disease and atherosclerotic/thromboembolic events, and provides a model for short term prothrombotic states in otherwise healthy subjects. Inhalation of cigarette smoke by subjects who are normally non-smokers, induced the adhesion of their platelets to apparently intact endothelial cell surfaces of rabbit aorta segments. Increased ristocetin induced platelet aggregation and von Willebrand factor antigen levels were observed and could contribute to the increased adhesion, although they may simply be the result of a stress response because of the unpleasant nature of cigarette smoke. Cigarette smoking caused a small increase in platelet aggregation to collagen and low dose ADP in PRP, and to collagen in citrated whole blood, but not non-anticoagulated blood. Those with lower serum nicotine levels had the most increased aggregation. This may be due to a balance between direct, toxic effects of nicotine on platelets, and stimulation of the adrenal medulla to produce adrenaline, which can prime platelets or act synergystically with ADP or collagen to potentiate aggregation.

In regular smokers a small decrease in aggregation responses was seen, although their baseline, pre-smoking samples showed stronger aggregation responses as compared to non-smokers. Similar patterns of results were obtained for von Willebrand factor, with non-smokers showing a post-smoking increase, and regular smokers having higher baseline levels.

These results may be due to toxic or pharmacologic effects of inhaled cigarette smoke components on the vessel wall or platelets.

Whole blood aggregation offers a global measurement of haemostasis, in which the only missing factors are rheological parameters. The method used was shown to detect a different type of platelet aggregate as compared to other methods. Neutrophils, and other leucocytes influenced the response, either by coherence or by the release of biologically active substances. These interactions of leucocytes with platelets may play a major role in the pathogenesis of thrombosis in certain disease states such as multi-organ failure. Such patients showed excessive aggregation respnses in non-anticoagulated blood, which appeared to result from the interplay between platelets, neutrophils, thrombin, and fibrin formation. This shows that some prothrombotic states may only be identified by whole blood methods. Anti-platelet drugs gave different results depending on the platelet aggregation method used; measurements in whole blood appeared to be most suitable for certain anti-platelet drugs. Platelet function studies may also be useful in determining the requirement for platelet concentrate infusions in patients with thrombocytopaenia, and prevent the unnecessary use of blood products.

APPENDIX I - REFERENCES

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APPENDIX II - ABBREVIATIONS

ADP - Adenosine 5′-Diphosphate
ATP - Adenosine 5′-Triphosphate
AML - Acute myeloid Leukaemia
ARDS - Adult Respiratory Distress Syndrome
ARF - Acute Renal Failure
ASA - Acetyl Salicylic Acid
CGL - Chronic Granulocytic Leukaemia
CML - Chronic Myeloid Leukaemia
CWB - Citrated Whole Blood
EC_{50} - Effective Concentration 50%
ET - Essential Thrombocythaemia
FMLP - f-Met-Leu-Phe (Bacterial Cell Wall Peptide)
HCt - Haematocrit
ITP - Idiopathic Thrombocytopaenia
MI - Myocardial Infarct
MOF - Multi-Organ Failure
MPV - Mean Platelet Volume
NWB - Native (Non-Anticoagulated) Whole Blood
PGI_2 - Prostaglandin I_2
PNH - Paroxysmal Nocturnal Haemoglobinuria
PPP - Platelet Poor Plasma
PRP - Platelet Rich Plasma
PRV - Polycythaemia Rubra Vera
SLE - Systemic Lupus Erythematosus
TTP - Thrombotic Thrombocytopaenic Purpura
TXB_2 - Thromboxane B_2
UF-100 - Ultraflo 100 Cell Counter
WB - Whole Blood