"STUDIES ON HUMAN BLOOD PLATELETS"

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Abbreviations

A23187 . . . . . Calcium Ionophore

AD . . . . . Abolition Dose

ADP . . . . . Adenosine Diphosphate

ATP . . . . . Adenosine Triphosphate

AT-III . . . . . Antithrombin III

ß-TG . . . . . ß-Thromboglobulin

C . . . . . Citrate

cAMP . . . . . Cyclic Adenosine Monophosphate

CCB . . . . . Calcium Channel Blocker

CPD . . . . . Citrate-Phosphate-Dextrose Anticoagulant

CPDA . . . . . Citrate-Phosphate-Dextrose-Adenine Anticoagulant

Crbc . . . . . "Control", Gluteraldehyde-fixed erythrocytes

DMSO . . . . . Dimethylsulphoxide

DVT . . . . . Deep Vein Thrombosis

E or EDTA . . . . . Ethylenediamine Tetraacetic acid

FPA . . . . . Fibrinopeptide A

G . . . . . GTP-binding protein

GBM . . . . . Glomerular Basement Membrane

GP . . . . . Glycoprotein

GTP . . . . . Guanosine Triphosphate

H . . . . . Hirudin

HDL . . . . . High Density Lipoprotein

HHT . . . . . 12-Hydroxy-5,8,10-heptadecatrienoic acid

HLA . . . . . Human Leukocyte-Associated Antigen

I . . . . . Insulin

IDDM . . . . . Insulin Dependent Diabetes Mellitus

IgG . . . . . Immunoglobulin G

ITP . . . . . Idiopathic Thrombocytopenic Purpura

i.u. . . . . International units

Ka . . . . . Association constant

Kd . . . . . Dissociation constant

LDL . . . . . Low Density Lipoprotein

Lp(a) . . . . . Lipoprotein (a)

LPS . . . . . Lipopolysaccharide

MDA . . . . . Malondialdehyde

MPV . . . . . Mean Platelet Volume

N . . . . . Nifedipine

N/A . . . . . Not applicable

NAG . . . . . N-Acetyl-Glucosamine

NIDDM . . . . . Non Insulin Dependent Diabetes Mellitus

Nrbc . . . . . Neuraminidase-treated, Gluteraldehyde-fixed erythrocytes

OD . . . . . Optical Density

ΔOD . . . . . Change in Optical Density

PAEF . . . . . Platelet Aggregation Enhancing Factor

PC . . . . . Platelet Concentrate

PDGF . . . . . Platelet-Derived Growth Factor

PEP . . . . . Phospho(enol)pyruvate

PF3 . . . . . Platelet Factor 3

PGE_1 or P . . . . . Prostaglandin E_1

$PGF_{1\alpha}$ . . . . . Prostaglandin $F_{1\alpha}$

PGG_2 . . . . . Prostaglandin G_2

PGH_2 . . . . . Prostaglandin H_2

PGI_2 . . . . . Prostaglandin I_2, Prostacyclin

PK . . . . . Pyruvate Kinase

PLA_2 . . . . . Phospholipase A_2

PLC . . . . . Phospholipase C

Plt. . . . . Platelet

PPP . . . . . Platelet Poor Plasma

PRP . . . . . Platelet Rich Plasma

RBC . . . . . Red Blood Cell

sd . . . . . Standard deviation

SPA . . . . . Spontaneous Platelet Aggregation

STA . . . . . *Solanum tuberosum* agglutinin

TBA . . . . . Thiobarbituric acid

TMP . . . . . Tetramethoxypropane

Trbc . . . . . Trypsin-treated, Gluteraldehyde-fixed erythrocytes

TxA_2 . . . . . Thromboxane A_2

TxB_2 . . . . . Thromboxane B_2

V . . . . . Verapamil

WBC . . . . . White Blood Cell

SUMMARY

This project was undertaken to increase the understanding of platelet function, with paticular emphasis on abnormal platelets in diabetes mellitus, and improving the quality of platelet concentrates for transfusion.

{1} Potential platelet antagonists, including PGE_1, verapamil, insulin and hirudin, were added to platelet concentrates in an attempt to improve the recovery and shelf-life of the concentrate. Each "preservative" caused some improvement in platelet concentrate quality, as measured by functional tests, and radio-immunoassay for the platelet activation marker, ß-thromboglobulin. The best results were obtained with the addition of PGE_1, which facilitated recovery of all samples to which it was added, suggesting a cheap way of ensuring consistently good platelet concentrates.

{2} Various investigations were carried out regarding abnormal platelet function in diabetes mellitus. No significant differences were found in the responses of platelets from diabetics and age-matched controls to the calcium-channel blocking drug, Verapamil, *in vitro*. Similarly, the capacity of diabetic platelets to produce malondialdehyde, a by-product of thromboxane A_2 synthesis, was not significantly different from control platelets.

The use of insulin in aggregometry studies showed, surprisingly, that insulin could have a pro-aggregatory effect on platelets from diabetics, but not those from healthy controls. In addition, evidence for the existence of a platelet aggregation-enhancing factor in the plasma of diabetics and older controls was obtained.

{3} Extensive tests to investigate the nature of spontaneous platelet aggregation (SPA) in whole blood have established the existence of two types of SPA, (i) ADP-dependent, and (ii) ADP-independent. The results obtained suggest a major role for erythrocytes in the development of inappropriate platelet aggregation.

INTRODUCTION

1.1: Platelets: Background.

Platelets were first identified in human blood smears by four independent researchers in 1842. Various explanations as to their origins and function were put forward. Zimmerman in 1846 reported that he believed the "*elementarblaschen*" were red cell precursors[1]. Shultze, 20 years later put forward the idea that the particles were fragmentation products of white cells that were able to come together to form a granular mass[1].

By the 1870's it was clear that the particles were a distinct shaped element of the blood, but the hypothesis that they were red cell precursors persisted and they were termed "*hematoblasts*" by Hayem[1]. During the later years of the century it was established that the particles were formed in the bone marrow by giant cells named "*megakaryocytes*" and that the fragments released by these cells were important in coagulation and clot retraction. Hence they were renamed "*thrombocytes*" by Deckhuzyen in 1901[1].

The ability of platelets to form aggregates and release vasoconstrictor substances was soon established, but the fundamental role of platelets only really came to be understood in the 1950's when the release mechanism of platelets was identified[1,2]. Since then much has been learned about the physiological and biochemical details of platelet function and about what can go wrong if platelets fail to do their job properly.

1.2 Platelets: Morphology.

Platelets are not true cells, rather they are anucleated fragments of the giant bone-marrow cells, megakryocytes. When fully mature, megkaryocytes break up to form several thousand platelets which are released into the circulation. The platelet is a roughly discoid object, with an average volume of 5 to 7.5 fl (figure 1.1).

The lack of a nucleus means that platelets have no DNA and very little ability to synthesise proteins. Hence, once released into the blood they age slowly and die within about

10 days[3]. Human blood typically contains 2-4 x 10^8 platelets / ml, but this can vary greatly in certain disease states[3].

The discoid shape of the platelet is maintained by a ring of microtubules which form a framework around the inner perimeter of the platelet. This framework, or cytoskeleton, consists of actino-myosin filaments similar to those seen in muscle fibres. These contractile proteins give the platelet its elasticity, and are essential in platelet shape change, release reaction and clot retraction[4] (see section 1.4).

The platelet plasma membrane is covered by a 15 - 20 nm thick coat, the glycocalyx[3,4] that is rich in glycopoteins and glycolipids. The two most prominent glycoproteins are termed GP-IIb and GP-IIIa, with approximately 50,000 copies of each per platelet[4]. GP-IIb is a dimer with subunits of mass 132 kD and 23 kD, and GP-IIIa is a single polypeptide of mass 95 kD with approximately 27 disulphide bonds[5]. Platelet stimulation leads to conformational changes resulting in the formation of GP-IIb/GP-IIIa complexes, which are thought to serve as receptors for fibrinogen, von Willebrand's factor and fibronectin. The second most prominent glycoprotein is GP-Ib with about 25,000 copies per platelet. It consists of two disulphide-linked subunits of mass 143 kD and 22 kD and is a potential receptor for von Willebrand's factor and thrombin[4]. GP-Ib is thought to be important in clot retraction since it becomes linked to the platelet cytoskeleton via actin-binding-protein on platelet activation[4] (see section 1.4). The other major platelet glycoproteins are GP-IIa, a protein of approximately 138 kD which becomes expressed on stimulation of the platelet by thrombin, increasing from 800 sites to 9600 sites per platelet[4], and GP-V, a 82 kD single-chain polypeptide that is the only known thrombin-substrate in the platelet plasma membrane. Treatment of platelets with thrombin results in the release of a 69.5 kD hydrolytic fragment of this glycoprotein[4]. The functions of GP-IIa and GP-V have yet to be ascertained.

The plasma membrane itself has a typical trilaminar appearance but unlike the membranes of other blood cells it is invaginated at numerous points to form the surface-connected-canalicular system, a series of channels throughout the platelet cytoplasm that serve as outlets for platelet secretion[3,4].

Close associations have been described between the surface-connected-canalicular system and the dense tubular system. The dense tubular system originates from the endoplasmic reticulum of the parent megakaryocyte and consists of narrow, membrane-limited tubules, 40-60 nm in diameter[4]. This system contains enzyme activities such as peroxidase and glucose-6-phosphatase[4]. It also possesses Ca^{2+}-stimulated ATPase and adenylate cyclase activities and is thought to be involved in the regulation of intracellular Ca^{2+} transport.

A variety of formed organelles and particulate elements are embedded in the cytoplasmic matrix of the platelet. In addition to common organelles such as mitochondria, lysosomes, microperoxisomes and glycogen stores, platelets possess specialised storage organelles known as alpha-granules and dense-bodies. Platelet mitochondria are simple in structure and few in number[3]. In addition to their normal metabolic functions, platelet mitochondria function as calcium stores similar to smooth-muscle mitochondria, releasing Ca^{2+} into the cytoplasm on platelet activation[4]. Lysosomes are small vesicles of 175-250 nm diameter that contain various enzymes that break up proteins and complex polysaccharides[4]. Lysosomal enzymes are released into the cytoplasm during platelet secretion, but their function is unclear. Microperoxisomes originate in megakaryocytes and contain the enzyme catalase which degrades toxic hydrogen peroxide. They are relatively few in number in platelets[4]. The glycogen stores take the form of granules, consisting of a collection of large molecules of the polymer with the enzymes responsible for its degradation to glucose bound to the surface of the granule[2,3,4] .

Of the platelet-specific organelles, by far the most numerous are the alpha-granules, forming the major proportion of platelet organelles. They are spherical or oval, 300-500 nm in diameter and contain a mixture of proteins including fibrinogen, platelet factor 4 (PF4), beta-thromboglobulin (β-TG), fibronectin, albumin, platelet-derived-growth-factor (PDGF) and possibly GP-IIb and GP-IIIa[2,4]. Fibrinogen and fibronectin are important platelet adhesive proteins, PF4 counteracts the anticoagulant effects of heparin[4], and PDGF is a potent cell-growth stimulant[4] .The function of β-TG is uncertain, but it may act as a granule packing protein[4].

The second type of platelet granule is the dense body. They number up to about 15 granules per platelet, with a mean of 5.4 per platelet, and are 200-300 nm in diameter. The dense bodies contain a highly concentrated mixture of the amine serotonin, calcium, ADP and ATP[2,4]. Dense bodies are not present in megakaryocytes, but precursor vesicles exist there which mature into dense bodies. Serotonin is taken up from the plasma by the platelets[4]. Dense bodies are the primary secretory organelles of the platelet and on platelet activation, release their contents into the plasma.

1.3 Platelet function

Blood platelets are essential for normal haemostasis. They play two distinct roles in the cessation of haemorrhage, the haemostatic function and the thromboplastic function. The former is accomplished by the physical blocking of openings in blood vessels by masses of platelets known as thrombi, and the thromboplastic function involves the participation of chemical constituents of platelets in blood coagulation.

Intact blood vessels have a continuous thin lining of endothelial cells that prevent the platelets coming into contact with the blood-vessel wall itself[7]. When a blood vessel is cut, the endothelial lining is disturbed and the fibrous matrix of the vessel wall is exposed. A major constituent of the matrix is collagen which serves as the primary stimulus for platelet activation, described in detail in the following section. Platelets in the blood flowing through the wound adhere to the collagen, setting in motion a complex cascade of platelet aggregation events and within a minute a haemostatic plug builds up, preventing any further blood loss.

The occurrence of platelet activation can, under different circumstances, lead to serious pathological consequences. Abnormal or inappropriate platelet aggregation can lead to the formation of circulating thrombi or microaggregates potentially capable of occluding circulation and initiating a heart attack or stroke.

1.4. Platelets: Biochemistry of activation.

There are several stimuli for platelet activation. One is the exposure of collagen fibres at sites of vascular injury, another is the release of pro-aggregatory agents from disrupted endothelial cells. Platelet agonists, together with their source are listed in table 1.1.

The *in vivo* agonists listed are very different chemically, each has its own paticular receptor. The links between receptor and effector mechanisms are similar but not identical.

Collagen is the most abundant protein in mammals, constituting a quarter of the total. It is present in nearly all organs and serves a major structural role. At least eight types of collagen have been identified in humans, differing in the nature of the triple-helical tropocollagen molecules that make up the collagen fibres[6]. Six different collagens are known to be present in blood vessels, namely types I, III, IV, V, VI and VIII[7]. Following injury to the blood vessel wall, collagen, present in the sub-endothelial matrix, is exposed and brought into contact with circulating blood. Platelets begin to adhere to the collagen, principally types I and III, the two most common types in blood vessels[8]. This is the first step in the cessation of bleeding. It has been suggested that platelets possess separate collagen receptors, one mediating adhesion, and a second mediating aggregation. Saito *et al*[9] have tentatively identified a surface-bound, zymogen form of platelet factor XIII as the platelet's collagen-aggregation receptor. The initial adhesion receptor may be platelet GP-Ib, or the GP-IIb/GP-IIIa complex, both of which are able to bind von Willebrand's factor, which in turn forms the adhesive 'glue' between the platelet and collagen.

Platelet-collagen interactions set into motion a series of complex and poorly understood events within the platelet that result in the change of shape of the platelet from discoid to a 'spiny sphere' and the secretion of platelet granule contents. Amongst the granule contents released are further platelet agonists, including ADP, serotonin and PAF. Historically, ADP is the oldest of the agonists, having been recognised as a platelet activator since the initial observations of Hellem in 1960[11]. ADP plays two roles that together produce platelet activation[10]. At low concentrations, (0.1 - 0.5μM) ADP causes shape change, followed by a reversible aggregation. At higher concentrations, irreversible, secondary aggregation with concomitant granule

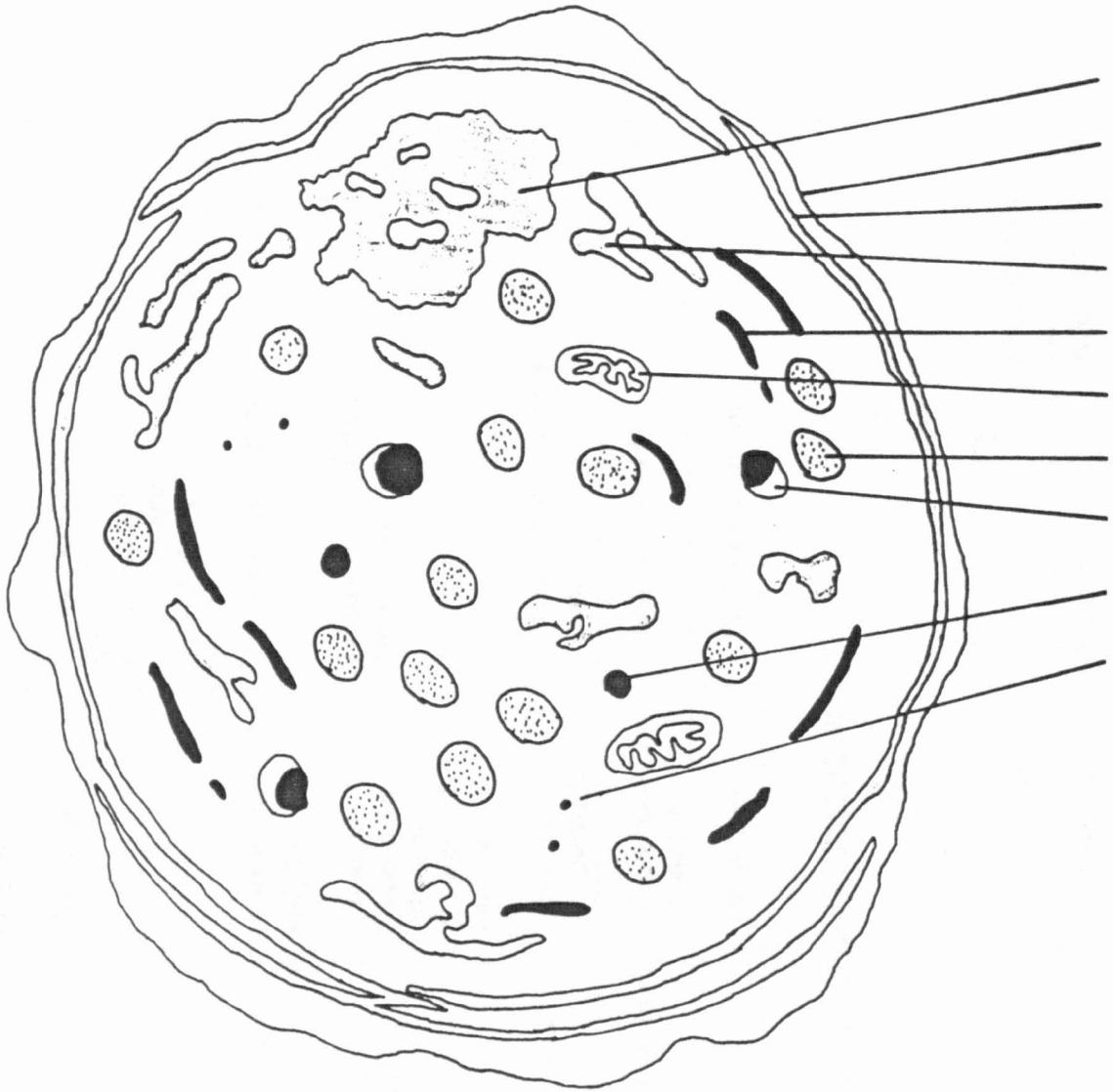


FIGURE 1.1: Platelet Morphology

AGONIST	NATURE	SOURCE	STRENGTH OF AGONIST
Collagen	Fibrous Protein	Vascular sub-endothelium	Strong
ADP	Nucleotide	(i) Platelet dense granules (ii) Lysed red cells (iii) Disrupted Endothelial cells	Weak
Thrombin	Proteolytic enzyme	Plasma Prothrombin	Strong
Platelet-activating -factor	1-o-alkyl-2-acetyl-sn -glyceryl-3 phosphoryl choline	Leukocytes, platelets, blood vessel wall(?)	Weak
Serotonin	5-hydroxy-tryptamine	Platelet dense granules, plasma	Weak
Epinephrine	Catecholamine hormone	Adrenal medulla	Very weak
TxA_2	Prostanoid	Platelet membrane	Strong

Table 1.1. *In vivo* Platelet Agonists.

AGENT	NATURE	SOURCE	MECHANISM
Prostacylin	Prostanoid	Vascular endothelium	Adenylate cyclase activation
Antithrombin III	Polypeptide	Liver	Complexes thrombin
Ecto-nucleotidases		Vascular endothelium	ADP --------> ATP

Table 1.2. *In vivo* Platelet aggregation inhibitors.

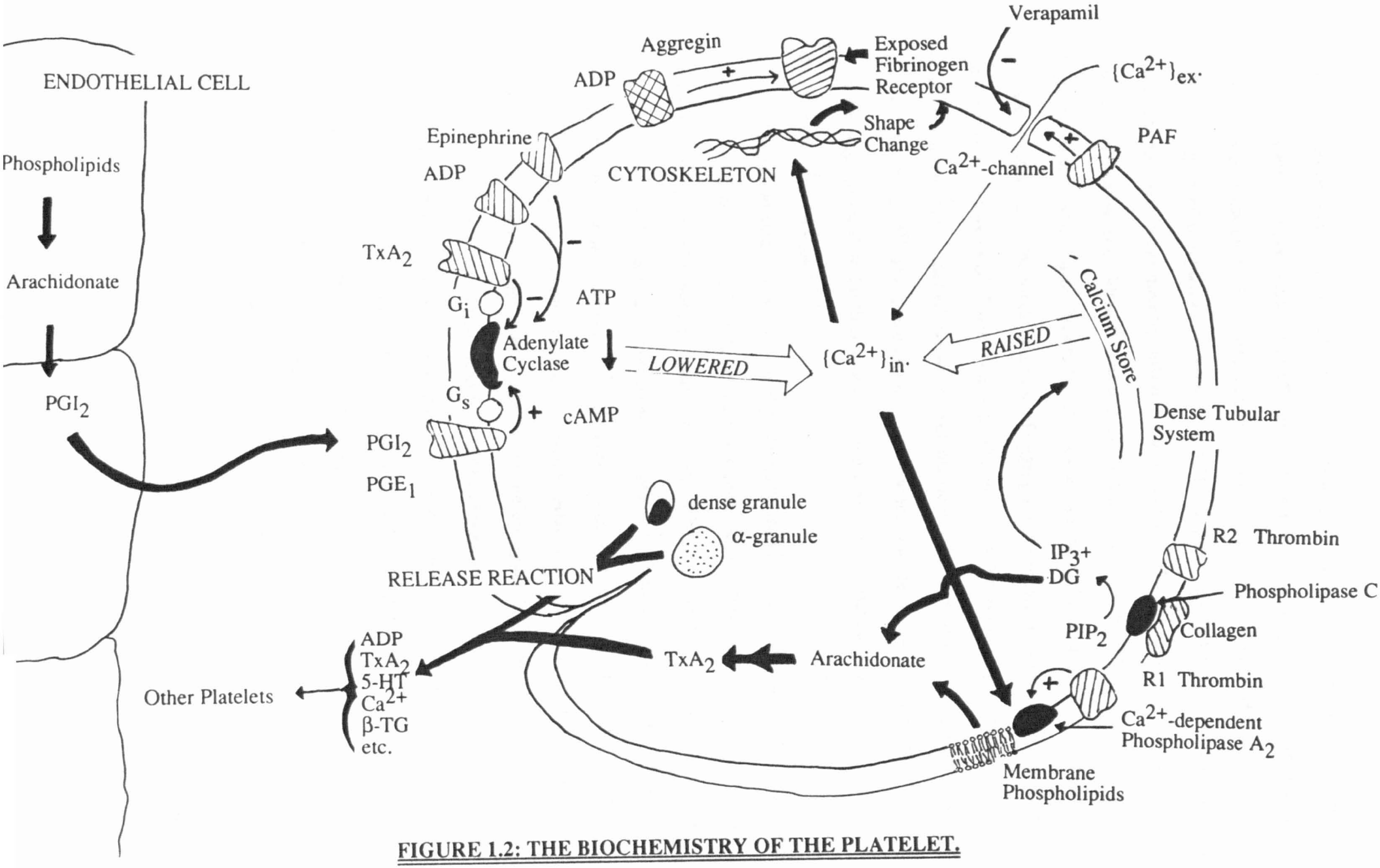


FIGURE 1.2: THE BIOCHEMISTRY OF THE PLATELET.

release is observed (see figure 1.2). The mechanism by which ADP causes platelet activation is by an inhibition of adenylate cyclase[4]. This causes a decrease in cytosolic cAMP, leading to increases in cytosolic Ca^{2+}. This mechanism is discussed in detail later. However, recent work has suggested that platelets possess two ADP receptors, one linked to adenylate cyclase, and a second that is more directly linked to shape change and aggregation[10]. The 'shape change' receptor has been identified as a single, 100kD polypeptide, termed aggregin[10]. Aggregin bears certain simlarities to GP-IIIa, a component of the fibrinogen receptor, but the two are not identical. The mode of action of aggregin is hypothesised to be as an inhibitor of the association between GP-IIb and GP-IIIa. On binding ADP, aggregin undergoes a conformational change that allows the two components of the fibrinogen receptor to associate and bind fibrinogen[10]. The shape change phenomenon may be mediated via a direct link between aggregin and/or the fibrinogen receptor and actinomyosin in the platelet cytoskeleton[10] (see figure 1.3).

The initial stages of activation of platelets by thrombin may also proceed via aggregin (figure 1.3). Thrombin is a 33.7kD active serine protease, synthesised as a 66kD zymogen called prothrombin[6]. Cleavage of prothrombin by activated coagulation factor X during the cogulation cascade leads to the generation of thrombin[3]. Thrombin appears to activate platelets by multiple mechanisms[10,12]. At very low concentrations (<1nM) its action may depend on secretion of secondary agonists such as ADP, but unlike collagen, TxA_2 and epinephrine, thrombin is also able to stimulate platelets by an ADP-independant mechanism[10]. Two types of thrombin receptor have been identfied in human platelets[10], the first (R1) is mediated by low concentrations of thrombin and is coupled to the inhibition of adenylate cyclase and actvation of phospholipase A_2. R1 receptors are few in number (50/platelet), of high affinity (Kd=0.3nM) and probably represent the receptor for ADP-dependent actions. The second receptor type (R2) are intermediate in number (1700/platelet), with a moderate affinity (Kd=11nM) and require higher thrombin concentrations (>2nM). This receptor is linked to phospholipase C and hence to protein phosphorylation and IP_3-dependent Ca^{2+} mobilisation[10]. The mechanisms for these activations are described later.

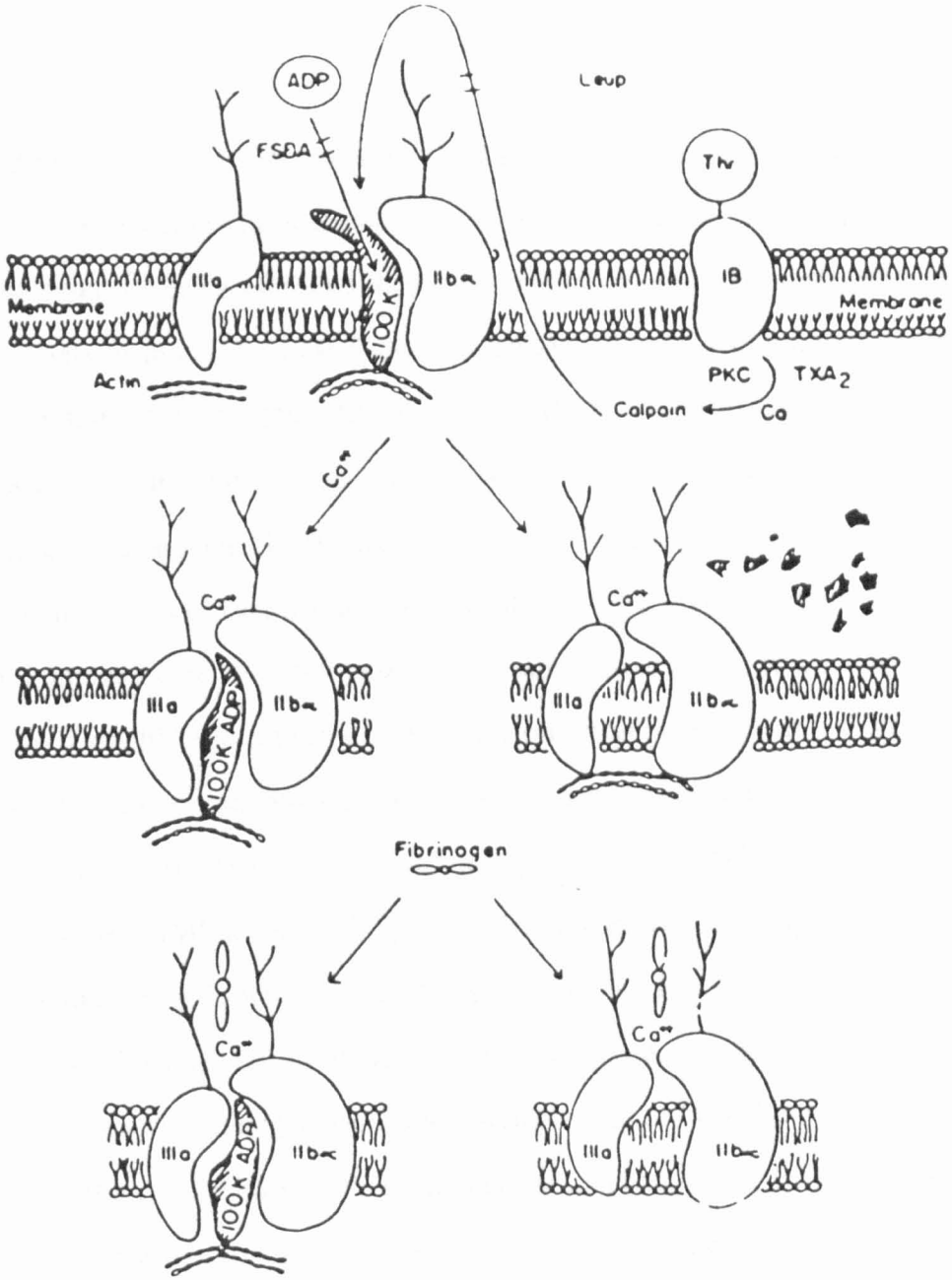


FIGURE 1.3: Aggregin-modulated ADP and Thrombin induced platelet aggregation (source, ref.10)

Leup. = Leupeptin, inhibits Calpain 100k = Aggregin

In addition to the thrombin receptors, platelets also possess thrombin substrates on their surface. The first substrate identified was GP-V, a single chain, 82kD polypeptide[4]. However, no evidence has been found to suggest that thrombin-induced cleavage of GP-V activates platelets. A more likely candidate is aggregin. Thrombin is believed to cleave aggregin by an indirect mechanism. On binding to GP-Ib, thrombin causes activation of protein kinase C which in turn activates the intracellular protease, calpain. Calpain then cleaves aggregin, leaving the GP-IIb and GP-IIIa free to form their complex[10].

Thromboxane A_2 (TxA_2) is a product of the platelet's cyclooxygenase pathway (see figure 1.4). Stimulation of phospholipase C or phospholipase A_2 by primary agonists leads to the liberation of arachidonic acid from membrane phospholipids, which is then converted to TxA_2. TxA_2 is then released into the plasma, where it acts as an autocoid, activating other platelets. The mechanism of TxA_2 stimulation of platelets is considered to be via the lowering of cytosol cAMP levels[4,10]. Specific TxA_2 receptors are coupled to adenylate cyclase via an inhibitory G-protein (G_i). This stops the conversion of ATP to cAMP by the enzyme, thus inactivating a cAMP-dependent protein kinase. This kinase is thought to be responsible for phosphorylating a 24kD, membrane-associated Ca^{2+} pump, enabling it to take Ca^{2+} from the cytosol into the dense tubular system (see figure 1.2)[4]. The outcome is that cytosolic Ca^{2+} levels rise, causing shape change, the release reaction and aggregation. A second protein phosphorylated by the cAMP-dependent protein kinase is the myosin light chain kinase[4]. This phosphorylation decreases the activity of the myosin kinase, preventing shape change. Hence, lowering cAMP causes shape change by a second mechanism.

Platelet activating factor (PAF) is a product of stimulated cells, paticularly polymorphonuclear leukocytes. The compound can mediate autotoxic reactions such as anaphylaxis and shock. Levels of this potentially lethal substance are regulated by serum and cellular acetylhydrolases which convert PAF to an inactive metabolite[4]. Platelets possess between 150-300 PAF receptors with a dissociation constant of Kd=1.36nM[4]. PAF appears to occur via the phospholipase C dependent release of IP_3 and DG independent of ADP release and TxA_2 formation[4,13]. This suggests that PAF causes an IP_3-dependent opening of Ca^{2+}-channels in the

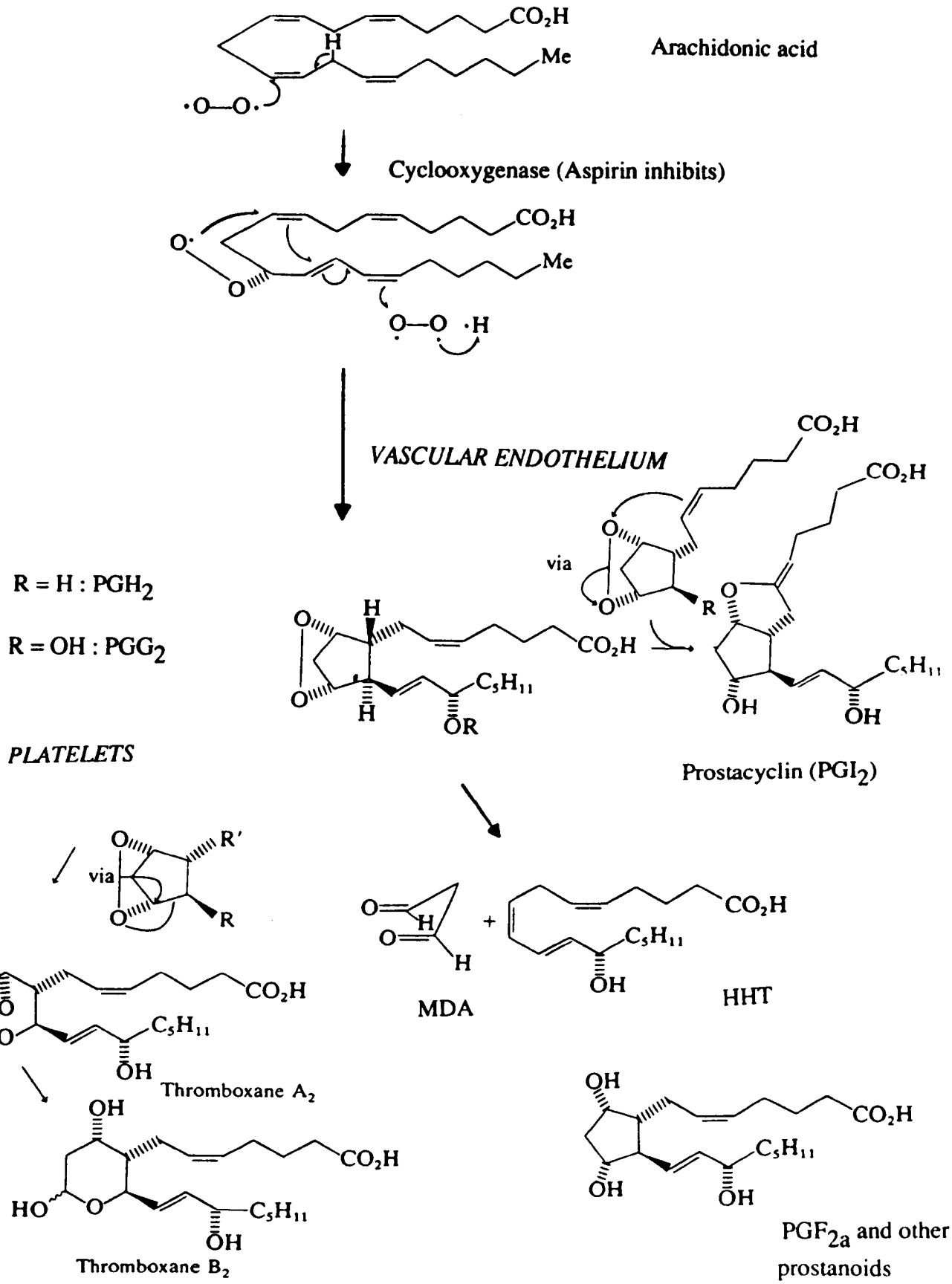
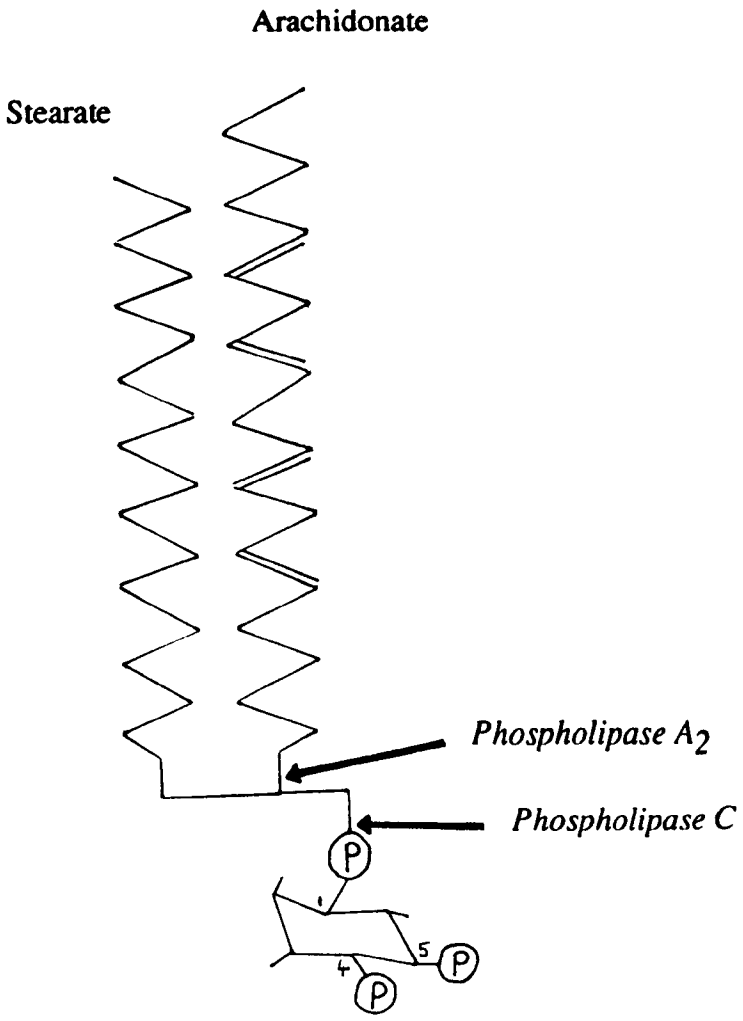


FIGURE 1.4: The Biosynthesis of Thromboxane A_2 and Prostacyclin

platelet membrane or dense tubular system, mediating aggregation by directly raising Ca^{2+} levels[13].

Epinephrine is a very weak platelet agonist[14]. Its principal effect is to potentiate the effects of other agonists *in vivo*[15]. Normal plasma contains approximately 2mM Ca^{2+}, which appears to prevent epinephrine associating with platelet α_2-adrenergic receptors[15]. However, anticoagulated blood, with its reduced Ca^{2+}, is able to sustain epinephrine-induced platelet aggregation. Platelets possess approximately 300 α_2-adrenergic receptors each[14]. They are coupled to adenylate cyclase via the inhibitory G_i protein and cause activation in much the same way as TxA_2[16]. In addition, epinephrine *in vitro* appears to be a very strong stimulant of TxA_2 synthesis, suggesting a link to phospholipase activities[16].

There are various mechanisms of coupling stimulus to response in platelets, that involve different "second messengers", formed in response to receptor occupation. These mediate the biochemical and morphological responses to agonists. One of the major second messengers in platelets is Ca^{2+}. Resting platelets have an internal calcium concentration of <0.1μM, compared to a plasma concentration of 2mM[17]. On activation, cytosolic Ca^{2+} levels rise rapidly , with associated thresholds for the various stages of platelet activation; shape change occurs at 0.8μM and full aggregation at 2μM cytosolic Ca^{2+}.[17] The Ca^{2+} comes from two sources, the plasma and from within the platelet itself. The major proportion of platelet Ca^{2+}, some 60%, is stored in the dense granules, but this Ca^{2+}, in common with that found in the mitochondria, has no role as a second messenger[4]. The internally released Ca^{2+} appears to come from the inner surface of the plasma membrane and the dense tubular system, from where it is released by IP_3. Plasma Ca^{2+} enters the platelet via ligand operated Ca^{2+} channels that appear to be dependent on ADP. Thrombin, ADP and PAF produce only small (5-10mV) depolarisations of the platelet membrane, suggesting that these agonists promote Ca^{2+} influx via receptor-operated channels, not voltage-gated channels[18]. Much of the platelet cytosolic Ca^{2+} is bound to calmodulin, a low molecular-weight, calcium-binding protein that mediates the activation of myosin light-chain kinase[4]. Calmodulin is a subunit of this kinase which becomes activated when cytosolic Ca^{2+} levels are raised. Ca^{2+} is also required for full activation of platelet protein



Phosphatidylinositol-4,5,diphosphate

FIGURE 1.5 : Sites of Action of Platelet Phospholipases

kinase C, phopholipase C and phospholipase A_2, but calmodulin does not appear to be directly involved in these activations[4].

Platelet membranes possess two major phospholipase activities, phospholipase C[19] and phospholipase A_2[4]. Both hydrolyse inositol lipids which make up 5% of the total platelet phospholipid. The major inositol lipid is phosphatidylinositol (PIP), mainly in the form phophatidylinositol-4,phosphate (PI-4,P) although the phosphatidylinositol-4,5,diphosphate form (PI-4,5,P_2) is also present (see figure 1.5). The major fatty acids found are stearic and arachionic acid at positions 1 and 2 of glycerol, respectively[4]. The phospholipases cleave PIP_2 at the given positions to give the products shown below:

PIP_2 $\xrightarrow{\text{Phospholipase C}}$ IP_3 + DG

PIP_2 $\xrightarrow{\text{Phospholipase A2}}$ Lysophospholipid + Arachidonate

IP_3 is membrane insoluble, and passes into the cytosol where it is essential for the release of calcium from intracellular stores. DG, in contrast is membrane soluble and serves a two-fold purpose[20]. Primarily it is the activator of protein kinase C, but it can also serve as a substrate for phospholipase A_2, yielding arachidonate. Phospholipase C is activated directly by certain agonists through a G-protein link between the receptor and phosholipase C[19]. By contrast, phospholipase A_2 does not seem to be directly linked to a receptor-agonist complex, but rather is activated as a consequence of raised Ca^{2+} levels[4].

Arachidonate is the substrate for an enzyme cascade leading to the formation of TxA_2 (figure 1.4). In the presence of O_2, arachidonate is converted to prostaglandin G_2 by membrane-bound cyclooxygenase, a single 72kD polypeptide chain containing two distinct enzymatic activities, oxygenase and peroxidase[21]. The enzyme is self-destructive. It is rapidly and irreversibly inactivated by its products. Prostaglandin G_2 is rapidly peroxidised to prostaglandin H_2, which in turn is converted to TxA_2 by thromboxane synthetase[21].

The third major pathway involved in platelet activation uses cyclic AMP. Platelet cAMP levels are controlled by two enzymes, adenylate cyclase and cAMP-

phosphodiesterase[22]. Adenylate cyclase prouces cAMP from ATP, and phosphodiesterase converts it to AMP. In resting platelets, cAMP controls cAMP-dependent protein kinases which in the presence of cAMP phosphorylate a 24kD protein believed to form part of the membrane-associated Ca^{2+} pump, and the myosin light-chain kinase[4]. These phosphorylations inhibit Ca^{2+} influx and shape-change, effectively preventing platelet aggregation. However, adenylate cyclase is also linked to receptors for agonists such as TxA_2 and epinephrine via an inhibitory G-protein[23]. On binding these agonists, adenylate cyclase is inhibited, cAMP levels fall and the activity of cAMP-dependent protein kinases diminish, allowing Ca^{2+} influx and myosin light chain phosphorylation.

The phosphorylations caused by the kinases described leads to disassembly of the platelet microtubules and their subsequent reassociation. The myosin light chain is only 10% phosphorylated in resting platelets, but on platelet activation it becomes 100% phosphorylated within 30 seconds, causing association with actin[4]. This has a number of physical effects on the platelet. The platelets change shape, and actinomyosin filaments become associated with surface GP-Ib and GP-IIb/GP-IIIa[4]. Platelet granules migrate to the centre of the platelet, presumably due to links between these organelles and the actinomyosin filaments. Ultimately they release their contents into the surface-connected-canalicular system. Binding of fibrinogen and other adhesive proteins by the surface glycoproteins forms a physical link between platelet cytoskeletons and the fibrin network of clotting blood, via actin-binding protein, the receptors and adhesive proteins. This link is thought to play an important role in subsequent clot-retraction processes[4].

In addition to the complex platelet activation mechanisms discussed here it is important that aggregation should have a counter-regulatory inhibition mechanism, otherwise the smallest incidence of platelet activation would lead to a chain reaction of aggregation involving the entire circulation. A list of *In vivo* anti-aggregatory agents is given in table 1.2.

Under normal conditions, endothelial cells present a non-thrombogenic surface to flowing blood[7]. A major role has been proposed for the endothelial glycocalyx. This consists of anionic glycoproteins and glycosaminoglycans that electrostatically repulse blood cells. In

addition, the vascular endothelium produces various enzymes with an ectonucleotidase activity that convert pro-aggregatory ADP to non-aggregatory ATP[7].

The major anti-aggregatory function of the vascular endothelium is to produce prostacyclin (PGI_2). PGI_2 is produced by a pathway analogous to that which produces TxA_2 in platelets (figure 1.4). The initial steps are the same as for TxA_2 production, but vascular endothelial cells use the enzyme prostacyclin synthetase to produce PGI_2 from PGH_2[21]. PGI_2 is the most potent inhibitor of platelet function known[4]. It interacts with platelet membrane receptors of two types, a high affinity (Kd=12.1nM) type with 93 sites per platelet, and a low affinity (Kd=0.9μM) type of 2700 receptors per platelet[4]. Both receptors are coupled via a stimulatory G-protein, G_s, to the same adenylate cyclase system as the TxA_2 receptor. In contrast to TxA_2[23], PGI_2 activates adenylate cyclase, increasing cytosolic cAMP levels and preventing aggregation (figure 1.2). Endothelial cells constantly produce low amounts of PGI_2, but to produce significant amounts of PGI_2, endothelial cells must be stimulated by clotting factors or aggregation agonists such as factor X_a, thrombin and ADP[7]. In addition, endothelial cells are able to use platelet-derived endoperoxides for their own PGI_2 synthesis, suggesting that the antithrombogenic activity of enothelial cells is amplified by activated platelets, causing a feedback inhibition of platelet aggregation[7]. Only at sites of endothelial damage, where the capacity to produce PGI_2 is destroyed and adhesive fibres are exposed, should any major degree of platelet aggregation occur. The effects of both PGI_2 and TxA_2 are extremely localised as each is rapidly metabolised to a non-active analogue, 6-keto-$PGF_{1\alpha}$ and TxB_2 respectively.

Numerous proteins which bind and prevent the action of thrombin are present in plasma and on the enothelial surface. Antithrombin III is the principal plasma protein responsible for the inactivation of thrombin[3,7], but other plasma proteins such as α_2-macroglobulin and α_1-proteolytic inhibitor and endothelial proteins including thrombomodulin are also able to bind and inactivate thrombin[7].

1.5: Diet and Platelet Function.

Much recent attention has been focused on the use of dietary fish oil supplements, and their beneficial effect in the prevention of cardiovascular disease[24]. Dietary fish oils contain large amounts of ω-3 polyunsaturated fatty acids, including linolenic acid (18 carbon, 3 double bonds) which is processed via the cyclooxygenase pathway to give PGI_3 and TxA_3, analogues of PGI_2 and TxA_2 produced naturally from arachidonic acid (20 carbon, 4 double bond). In comparison to TxA_2, TxA_3 is a very weak platelet agonist, but the anti-aggregatory effect of PGI_3, compared to PGI_2 is reduced by a relatively small factor. Hence the balance favours platelet disaggregation, preventing inappropriate platelet activation.

In contrast, platelet hypersensitivity appears to be enhanced by the incorporation of cholesterol into platelet membranes[25]. This event leads to the increased aggregation seen in platelets from sufferers of familial hypercholesterolemia[26].

Aspirin is a potent inhibitor of platelet cyclooxygenase, preventing TxA_2 formation. The effect of aspirin on endothelial cyclooxygenase is comparatively small at low dose, hence the beneficial effects of low dose aspirin on cardiovascular patients[21].

Evidence has been put forward to suggest a direct effect of exercise on platelet activation. Platelets from marathon runners 24 hours after a race show significantly greater PGI_2 inhibition than non-runners[27]. This effect is not due to an increase in PGI_2 receptors, but seems to be caused by a desensitisation by epinephrine of G_i, leaving more catalytic units of adenylate cyclase able to be stimulated by G_s[27].

Other known inhibitors of platelet function include; alcohol, vitamin E[28], several antibiotics, indomethacin and dipyridamole.

1.6; Diabetes mellitus; Background.

Diabetes mellitus affects 1-2% of the European population. It is not a single disease, but a collection of disorders with different treatments and pathologies, related by the presence of high concentrations of glucose in the blood (hyperglycaemia) due to a deficiency in the production or action of the hormone, insulin.

The earliest description of the condition appears to be on the Elbers papyrus, dating from 1550 BC[29], which outlines a treatment for polyuria. The term 'diabetes' refers to the excessive urination seen in the disease, and 'mellitus' comes from the latin, meaning 'sweetened with honey'. The Greek, Aretaeus in the second century AD, described the condition thus[29,30];

"Diabetes mellitus is .....a melting down of the flesh and limbs into urine .....one cannot stop them from drinking or making water...."

This clinical description has been expanded over the years, but not surpassed. Aretaeus believed the disease to be triggered by the bite of a reptile, the Dipsas[32], but the Indian, Susruta, writing in the sixth century, was closer to the truth claiming that diabetes mellitus was[29];

"A disease from which the rich principally suffer .....by their own overindulgence in rice and flour.....".

From the time of Galen, approximately 200 AD, the kidneys were believed to be the cause of diabetes mellitus[29] and this theory persisted until 1679, when Thomas Willis stated that[29];

"We believe it to be more an affection of the blood rather than the kidneys caused by immoderate drinking.....or by sadness and long grief".

The first indication of the role of the pancreas came three years later, when Brumner noted that pancreatectomy in dogs led to polyuria[32]. However, most physicians still believed

the condition to be caused by the kidneys. Matthew Dobson, in the 1770's made several advances in the understanding of the disease. He evaporated the urine of a diabetic to give a 'white cake' which[29];

"smelled sweet like brown sugar and tasted the same".

He also noted the opaqueness of diabetic blood plasma, probably the first record of hyperlipidaemia together with hyperglycaemia[29];

"The saccharine matter was not formed in the kidney

but existed primarily in the serum".

Dobson was also the first to distinguish between type-1 and type-2 diabetes mellitus, noting that the disease could[29];

".....terminate fatally in less than 5 weeks. In

others it becomes a chronic complaint".

A missed opportunity came in 1788 when Thomas Cawley noted that the pancreas of a diabetic was full of 'calculi'. Unfortunately, Cawley regarded this as an incidental finding and continued to blame the kidneys[29].

John Rollo in 1796 prescribed the first dietary intervention in diabetes mellitus. He advised a diabetic patient to have a diet of 'animal food', which incidently was very low in carbohydrate content[29,30]. As the patient was overweight, and probably a type-2 diabetic, he not surprisingly responded to the treatment, reinforcing Rollo's view that the stomach was the primary affected organ in the condition.

The next major advance came in 1848 when Claude Bernard showed sugar to be formed endogenously by the liver, proposing that diabetes was due to faulty nerve control of the liver itself, resulting in too much sugar production[29,32].

Paul Langerhans, in 1869, became the first to describe the pancreatic 'islets' that are now named after him[32]. Laguesse, in 1893, observed granules in the islets and suggested that they constituted an internal secretion[29]. Four years later, the classical depancreatised dog experiments of Minkowski and von Mering took place, leading to the development of diabetes in the animals and strong evidence for the role of the pancreas in the condition[29,30,32].

These observations led to the first attempts to produce an anti-diabetic pancreatic extract. De Witt in 1906, had some success with islet tissue from cats that was glycolytic *in vitro*[29]. In the same year, Zuelzer injected a pancreatic extract into a comatose, 50-year old diabetic, leading to an improvement that unfortunately was not sustained[29]. This extract, termed Acomatol by Zuelzer, was further purified and by 1911 had been shown to decrease acetone and sugar levels, but the side effects of the treatment were severe. Also in 1911, E. Scott prepared his own version of the extract by extracting the pancreas in alcohol[29]. This extract worked well on dogs, leading Scott to conclude that previous extracts had not been protected against oxidation or digestion by enzymes. Further improvements came with the work of Kleiner in 1919 and Paulescu in 1921[29]. Kleiner's extract reduced blood glucose by up to 50% in 16 diabetic dogs. The only complication was a mild fever. Paulescu was able to lower glucose in both diabetic and normal dogs wiyh his extract known as Pancreine[29]. However, when used on patients, it caused irritation at the injection site leading Paulescu to attempt further purification.

In the meantime, in Toronto, Banting and Best had begun their own experiments in the laboratory of Professor MacLeod, using the techniques developed by Scott[29-32].Early results with experimental diabetes were good, but the first use of their extract, Isletin, on a patient, Leonard Thompson, was disappointing. Further purification of the extract, with a research biochemist named Collip, eventually led to retreatment of the same patient on the 23rd of January, 1922 with great success. Shortly afterwards, the patent for the extract was given to the University of Toronto, and the drug company Eli Lilley took up the licence to produce the drug, now known as Insulin.

1.7: Diabetes Mellitus: Pathogenesis and Treatment.

The two types of diabetes mellitus have long been recognised. Lancereaux in 1880 termed them *diabete maigre* (thin diabetes) and *diabete gras* (fat diabetes)[30]. The two types are now generally refered to as insulin-dependent diabetes mellitus (IDDM), or type-1

diabetes, and non-insulin-dependent diabetes mellitus (NIDDM), or type-2 diabetes respectively. The differences between the two conditions are summarised in table 1.3.

Insulin is a protein hormone (molecular weight 6kD) which is synthesised by the β-cells of the pancreatic Islets of Langerhans. It plays a major role in regulating the metabolism of carbohydrates, fats and proteins. The way insulin lowers blood glucose is particularly important and well understood.

High levels of glucose, such as occur after a meal, cause the pancreas to secrete insulin which inhibits glucose output from the liver and stimulates glucose uptake by muscle and adipose tissue, where it is converted to the storage compound, glycogen. If blood glucose levels become too low, the body produces counter-regulatory hormones which oppose the action of insulin and thus prevent prolonged hypoglycaemia[6]. Epinephrine, released by the adrenal medulla, stimulates glycogen breakdown in muscle and glucagon, a polypeptide secreted by the pancreatic α-cells causes increased glycogen breakdown in the liver. The equilibrium set up by these hormones allows the maintainance of blood glucose levels within a narrow range. In healthy people the range is about 3.5-6.5 mM, but in diabetics, concentrations in the range 1.0-30.0mM have been observed[35].

Before the onset of effective treatment for diabetes, the lack of control of blood sugar levels was the direct cause of death in many diabetics[34-36]. Glucose is the major energy source for brain cells. Abnormally low levels of glucose starve the brain of energy and a state of hypoglycaemic coma ensues if the condition is prolonged[36]. On the other hand, hyperglycaemia causes unpleasant short-term symptoms such as frequent urination and thirst, and in the long-term may be a major factor in the development of tissue damage in the blood vessels, eyes, kidneys, nerves and elsewhere (see section 1.8). In addition, prolonged hyperglycaemia can also lead to coma. Because of the large quantities of sodium and water excreted to maintain the osmolarity of the blood, a fall in plasma volume and reduced cardiac output ensues. The outcome is that too little blood reaches the brain and, once again, coma follows[35].

IDDM results from a decreased production of insulin by the pancreas. These patients have a greatly reduced β-cell mass due to destruction of these cells by any of a number of

causes. Viral infections have been implicated as a major triggering factor for IDDM. It is known that an encephalomyocarditis virus[36] can infect the β-cells and other viruses implicated from epidemiological studies include; Coxsackievirus B, cytomegalovirus and those causing mumps, rubella, glandular fever and chicken pox[36]. However, not all people infected with these viruses become diabetic, suggesting a role for genetic factors.

There is also strong evidence implicating autoimmunity as a cause for IDDM. Firstly, pathological studies of islet tissue from patients with IDDM have shown inflammatory lesions, characteristic of an immunologically mediated process[36]. Secondly, antibodies to islet cells have been found in diabetics at the time of onset of the disease[36] and thirdly, lymphocytes from diabetics are cytolytic towards cultured human β-cells[36].The third major trigger for IDDM is thought to be environmental toxins. However, as with viral attack, not all people exposed to potentially harmful chemicals become diabetic, again pointing to genetic factors.

Heredity plays a complex and poorly understood part in the pathogenesis of diabetes. Diabetes tends to run in families, but less than 20% of patients have first-degree relations with a history of diabetes and studies with twins have shown that IDDM is concordant in only about 50% of cases[36]. Diabetics are known to have an increased frequency of certain major histocompatibility antigens[35]. These HLA (Human Leukocyte Antigens) are glycoproteins, present on all nucleated cells that determine the function of cytotoxic T-lymphocytes. Current thinking is that a tendency to develop diabetes is inherited, but that additional environmental triggers are necessary for the development of the disease.

VARIABLE	IDDM	NIDDM
Age	usually<40	usually>40
Weight	normal	60-90% obese
Insulin need	dependent(ketosis without insulin)	may require insulin to control hyperglycaemia
Diet	adequate kilocalories to maintain weight	restrict kilocalories
Plasma glucose	variable	stable
Plasma insulin	low or absent	low, normal or high
Insulin receptors	normal	low or normal
HLA	high incidence of antigens B8, B15, Dw3/DR3, Dw4/DR4	no correlation
Islet cell auto-antibodies	50-80% positive at diagnosis	antibodies less than 5% positive at diagnosis
Associated auto-immune disease	present	absent
Vascular complications	present	present
Incidence	10% of diabetes	90% of diabetes

Table 1.3 Comparison of IDDM and NIDDM.

In contrast to IDDM, which can be controlled by insulin infusions, NIDDM patients have little use for such supplements. These patients possess an intact β-cell mass but they show resistance to the action of insulin at the cellular level due to a decrease in the number of insulin receptors on target cells or to defective receptors[35].NIDDM is itself a group of diseases and other factors that may lead to similar consequences are; impaired glucose-stimulated insulin secretion from the pancreas, non-immunological insulin-antagonists in the circulation and increased hepatic glucose production[30,35]. Again, the exact causes of these types of diabetes are unknown, but NIDDM tends to develop in older, overweight patients. A genetic link is suggested by the observation that identical twins with NIDDM are concordant in 90% of cases. This genetic effect shows no correlation with HLA antigens[30].

Treatment of NIDDM takes the form of low-carbohydrate diet, often supplemented by oral hypoglycaemic agents, drugs which release insulin from the pancreas and augment its action at cellular receptors. Occasionaly, NIDDM patients are transfered to insulin when diet and oral agents fail to produce suffi-ent lowering of blood glucose.

1.8: Diabetes Mellitus: Microvascular Complications.

Prior to the advent of effective treatments for diabetes, diabetic coma was the major cause of mortality for diabetics[34]. By the 1920's, following the purification of insulin, its place as the leading cause of death was taken by cardio-renal vascular disease (figure 1.6)[34]. As the development of insulins with prolonged action progressed and the life-expectancy of diabetics increased, diabetic vascular disease has become more and more of a problem, with the vas c ular complications having a longer period of time to fully exert its effects. Owing to the various types of vascular disease seen in diabetes, diabetics are twice as likely to suffer a heart attack or stroke than the general population, five times more prone to gangrene, 17 times more prone to kidney failure and 25 times more likely to suffer blindness[37]. Indeed, diabetic retinopathy is the most common cause of blindness in people under the age of 65 in England and Wales[36].

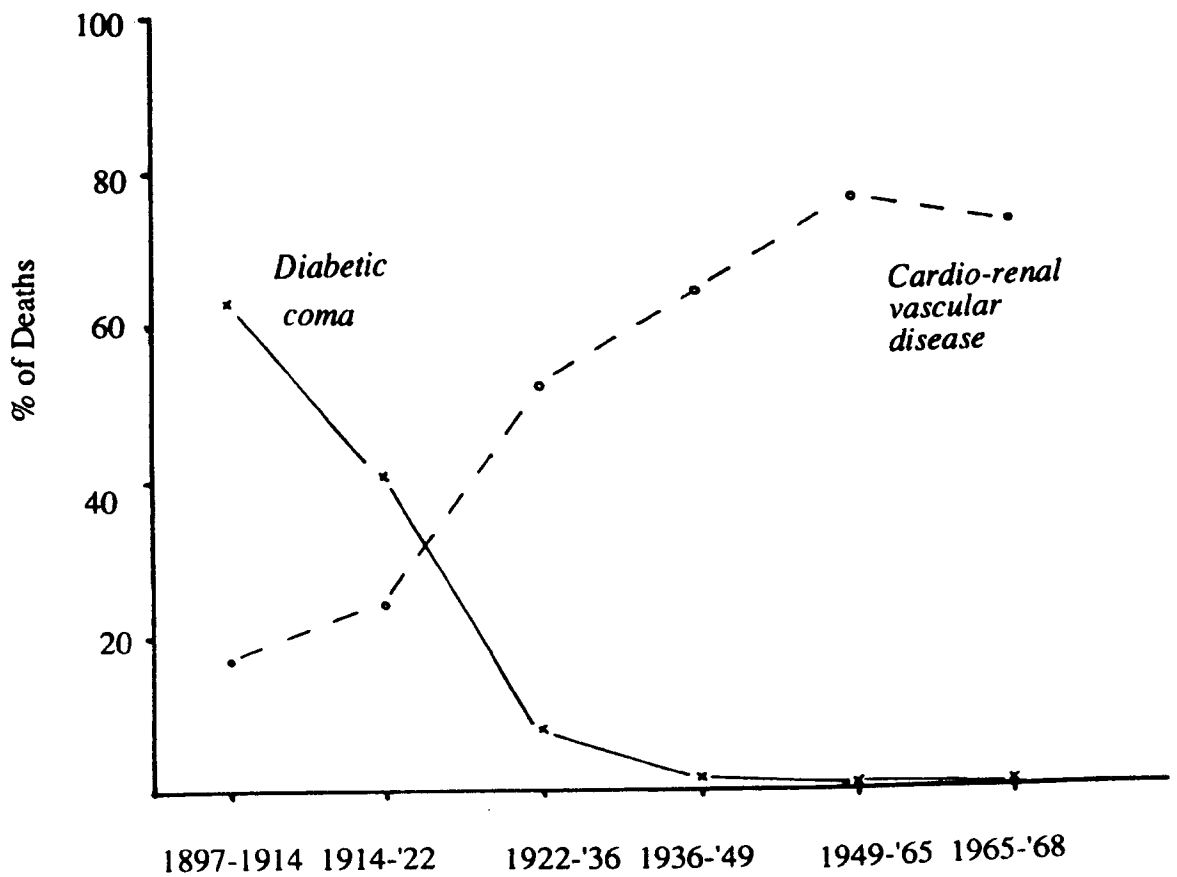


FIGURE 1.6 : Causes of death at the Joslin Clinic, 1897-1968[34].

1.8.1: Diabetic Kidney Disease: Nephropathy.

As many as 45% of IDDM patients will develop some degree of kidney disease[38]. The healthy kidney acts as an excellent filtration system. The passage of molecules through the glomerular basement membrane is dependent upon size and electrical charge. Larger molecules will not pass through the pores, and negatively-charged molecules are less readily filtered than positively-charged ones. Damage to the glomerular filtration barrier can greatly alter its function, causing leakage of plasma protein and even erythrocytes into the urine. In healthy people, up to a gram of protein leaks through the glomeruli every 24 hours, but over 95% of this is reabsorbed[38].

Glomerular filtration rate is elevated in the first few weeks after the development of diabetes. Early in the course of diabetes, excretion of urinary proteins spanning a molecular weight range of 44-160kD is significantly increased[38,39]. The principle proteins excreted are albumin, a negatively-charged 69kD molecule, and immunoglobin G (IgG), a neutral molecule of 160kD[38]. These are characteristic of glomerular filtration malfunction, rather than tubular reabsorptive capacity, since the excretion of α_2-microglobulin, a sensitive indicator of the latter process is normal[41]. Initial microproteinuria appears to be caused by an increased filtration pressure across the glomerulus, accompanied by a decrease in the level of negatively-charged sialic acid residues in the glomerular capillary walls[38,39].

As nephropathy progresses, glomerular filtration drops further as the number and size of pores in the glomerular membrane decreases, introducing a size-selectivity defect[39]. The increased glomerular permeability is accompanied by a thickening of the glomerular basement membrane (GBM)[38-41]. Progressive glomerular capillary occlusion due to continual thickening over many years ultimately leads to chronic renal failure.

Numerous theories have been advanced to try to explain the development of nephropathy in diabetics. Only certain tissues require insulin for the intracellular transport of glucose. The kidney does not, and so tends to equilibriate with the extracellular glucose concentration[40]. Thus, hyperglycaemia causes increased glycosylation of GBM proteins, interfering with polypeptide packing and/or hydroxy-lysine derived cross-link formation[39].

This may result in an increased pore size. The excessive accumulation of GBM material seen in diabetes could represent increased synthesis, decreased degradation, or a combination of the two processes[38]. Alternatively, there could be a relative overproduction of certain basement membrane components.

1.8.2: Diabetic Eye Disease: Retinopathy.

Diabetic retinopathy is characterised by a number of clinical manifestations which, together, tend to cover the light-sensitive cells of the retina. Clinically, diabetic retinopathy can be subdivided into[38];

(i) background retinopathy,

(ii) maculopathy,

(iii) proliferative retinopathy.

Background retinopathy occurs in diabetics of any age and with any duration of diabetes. The condition involves retinal capillary haemorrhages, localised capillary dilations (microaneurysms), hard exudates and 'cotton-wool spots'[38,39]. The exudates are most commonly found in the fibre layer of Henle, and consist of extracellular lipids, macrophages and amorphous extracellular fluid. Cotton-wool spots are white lesions made up of the distended stumps of ganglion-cell axons in the retinal nerve-fibre layer[38].

The term 'maculopathy' denotes actual visual loss due to increasingly serious oedema in the form of microaneurysms, haemorrhages and exudates in the region of the *macula lutea*, the yellow spot at which the retina has its highest visual acuity[38].

The essential feature of proliferative retinopathy is extraretinal neovascularisation. New blood vessels proliferate in response to localised regions of hypoxia caused by capillary closure, haemorrhage and the decreased oxygen affinity of diabetic erythrocytes[39]. Ultimately, neovascularisation can extend into the vitreous gel, causing detatchment of the vitreous gel from the retina. The detatched retina may become mis-shapen and, if there is associated haemorrhage, sub-retinal fluid may become blood-stained. Once the macula

becomes detatched, vision is profoundly affected and there may be a slow progression to complete blindness owing to atrophy of the detatched retina[38].

1.8.3: Diabetic Nerve Disease: Neuropathy.

Diabetic neuropathy is characterised by a variety of morphological changes associated with decreased sensory and motor conduction velocities. The most frequent symptoms of diabetic peripheral neuropathy are numbness and paraesthesias. Potentially more serious is autonomic neuropathy. All tissues which receive autonomic innervation may be affected, these include the cardiovascular, alimentary and genito-urinary systems, sweating mechanisms and the pupils[38]. Cardiovascular neuropathy can lead to hypotension and loss of heart-rate variability. The commonest symptom of autonomic neuropathy is diarrhoea, but other rarer gastrointestinal disorders such as gastroparesis, oesophageal and gall bladder dysfunction have also been reported in diabetic neuropathy[38]. Autonomic neuropathy is the major reason for the increased incidence of impotence in diabetic males. With increasing age and duration of diabetes, the early symptoms of hypoglycaemia may be lost, and patients then suddenly lose consciousness without warning. This too may be a result of impaired peripheral and autonomic conductance[38].

Structural studies of peripheral nerves from diabetics characteristicly show segmental demyelination and shrinkage of axons and Schwann cells. In addition to these changes, abnormalities in neuronal vasculature also occur in diabetes[39]. Marked thickening of basement membranes surrounding intraneural and perineural vessels has been described in company with hyperplasia of endothelial cells in small vessels, occasionaly occluding the vessel lumen[37]. Abnormalities in the microvaculature of peripheral nerves may reduce blood flow through small vessels, producing regional ischaemia and microinfarcts that could contribute to neuronal dysfunction.

1.8.4: Other Diabetic Complications.

In addition to the virtually diabetes-specific, microangiopathic complications already described, large blood vessel disease (macroangiopathy) is also accelerated in diabetes[39]. Epidemiological data appears to show no excess risk from diabetes independent of hypertension, cigarette smoking and hyperlipidemia[38].

Cataract, opacity of the eye lens, occurs earlier in diabetics than in the general population due to the formation of high molecular weight lens protein aggregates[39]. Non-enzymatic glycosylation of lens proteins facilitates sulphydryl oxidation and the formation of inter-protein disulphide bonds. The lens, like other body tissues that develop diabetic complications, does not require insulin for glucose transport. Hence, hyperglycaemia would increase the concentration of glucose in the lens.

1.9: Is there a Common Mechanism for the Development of Different Diabetic Complications ?

The retina, nerves, blood vessels and renal glomeruli of diabetics all contain abnormally high levels of the sugar alcohol, sorbitol[39,40]. Under normal circumstances, glucose is utilised by glycolysis. However, in the presence of very high levels of glucose, the pathway becomes saturated, and the excess glucose is metabolised by the polyol pathway (figure 1.7)[40]. This leads to a build-up of sorbitol and fructose in diabetic tissues such as nerves, the retina, lens and renal glomerulus where glucose uptake is not insulin-dependent, and high amounts of aldose reductase are present.

Increased polyol pathway activity leads to an accumulation of sorbitol in the cell membrane, which affects the integrity of the membrane and the function of Na^+/K^+ ATPase trans-membrane pumps[40]. In nerve tissue, this slows conductance, in the kidney it increases the glomerular filtration rate and in the retina it affects the integrity of the blood-retinal barrier. The build-up of sorbitol can be slowed by the use of specific aldose reductase inhibitors.

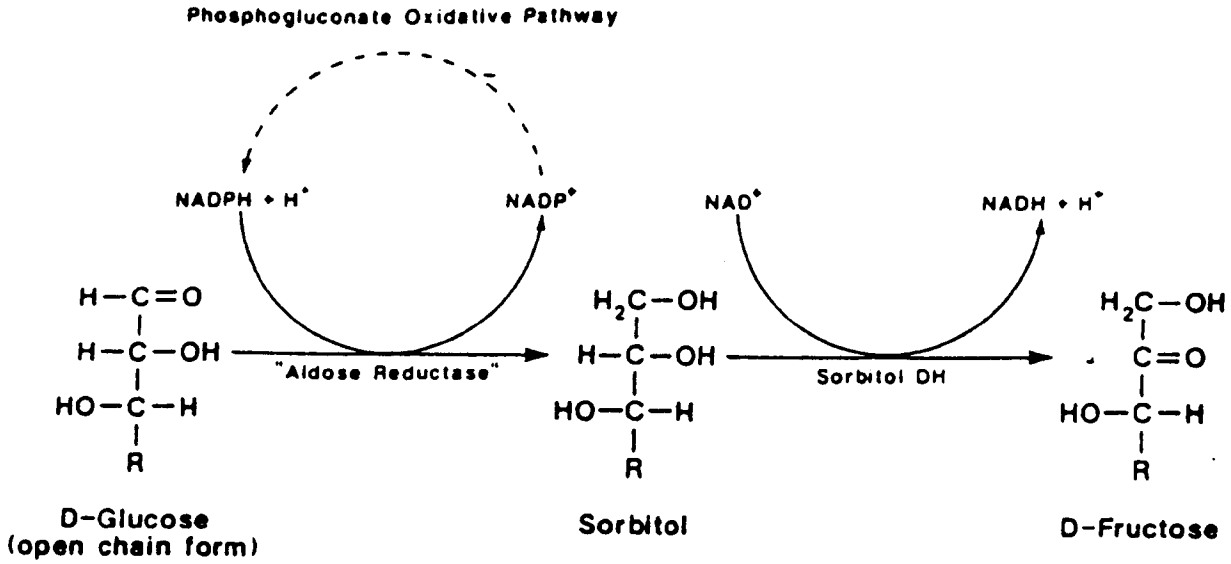


FIGURE 1.7(a) : The Polyol Pathway.

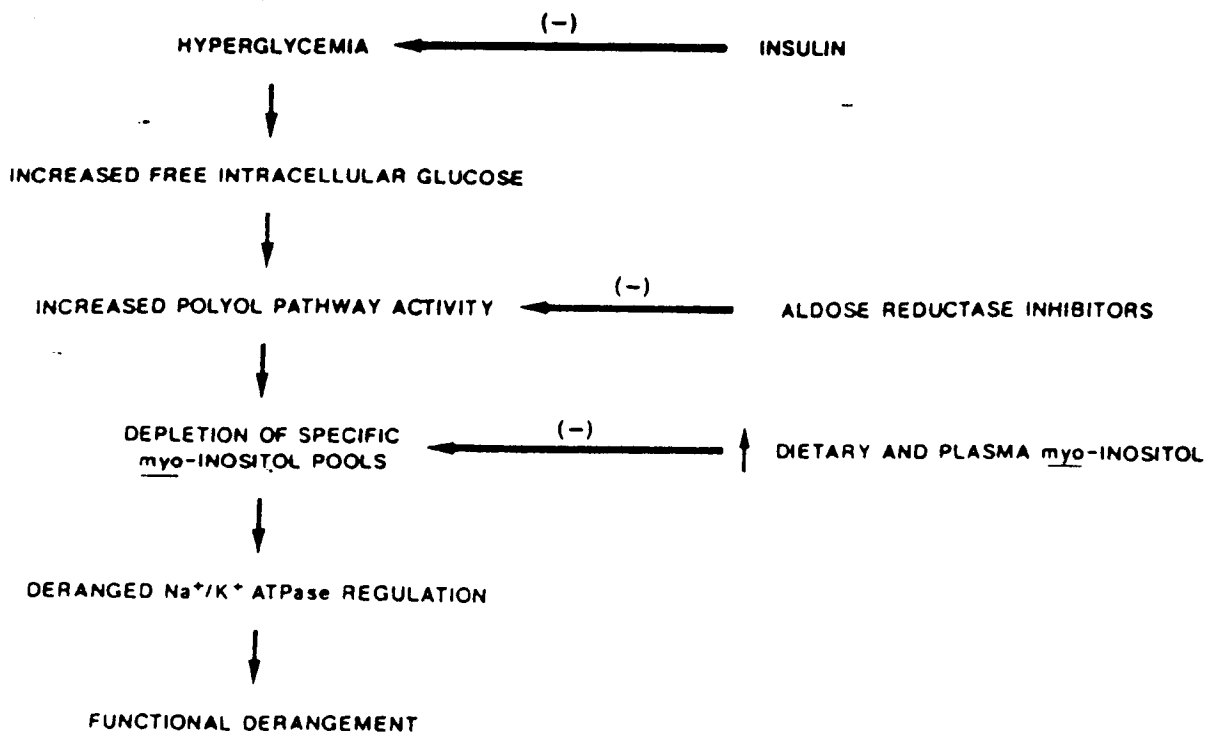


FIGURE 1.7(b) : The sequence by which hyperglycaemia causes functional alterations in susceptible sites.

Capilary basement membrane thickening is the hallmark of diabetic microangiopathy. This phenomenon is less severe with good metabolic control and the disorder appears to be a consequence of insulin deficiency rather than a seperately inherited entity. After five years of clinical diabetes, basement membranes are 25-30% thicker than in normal subjects (Osterby *et al* 1986)[41]. Non-enzymatic glycosylation of basement membrane collagen and laminin is postulated to play a major role in the thickening of basement membrane in conjunction with increased deposition of new membrane material[39].

Non-enzymatic glycosylation (figure 1.8) also affects a multitude of other proteins[42]. Typically, the life-span of glycosylated proteins is reduced. More specific effects include the inhibition of hepatic uptake of glycoproteins by glycosylated albumin[42]; the resistance of glycosylated fibrin to digestion[42]; the inhibition of endothelial cell production of PGI_2 by glucose[43]; a decreased uptake of glycosylated LDL by cells[44]; the increased O_2 affinity of glycosylated haemoglobin (HbA_{1c}) and decreased red cell deformability due to glycosylation of membrane proteins[42]. All these factors have the potential to affect the development of microvascular disease.

1.10: The Role of Platelets.

As with other elements of the blood, platelets are prone to non-enzymatic glycosylation in diabetes. However, there appears to be no significant correlation between degree of platelet aggregation and platelet glycosylation[44].

Various functional and biochemical abnormalities have been reported in platelets obtained from diabetics, including alterations in adhesion[37], increased sensitivity to aggregation agonists and increased TxA_2 production[46,50]. However, extensive research in this field has provided inconsistent results and conclusions (review: Ostermann and van de Loo,1986)[45]. Consensus results are that diabetic platelets show increased adhesion and aggregation *in vitro*, with the tendency towards abnormality increasing with age and degree of microvascular disease. Spontaneous platelet aggregation (SPA) *in vitro* is seen in many diabetics but is rare in healthy, young individuals[47]. The degree of SPA varies greatly between donors, and also appears to fluctuate with time in individuals.

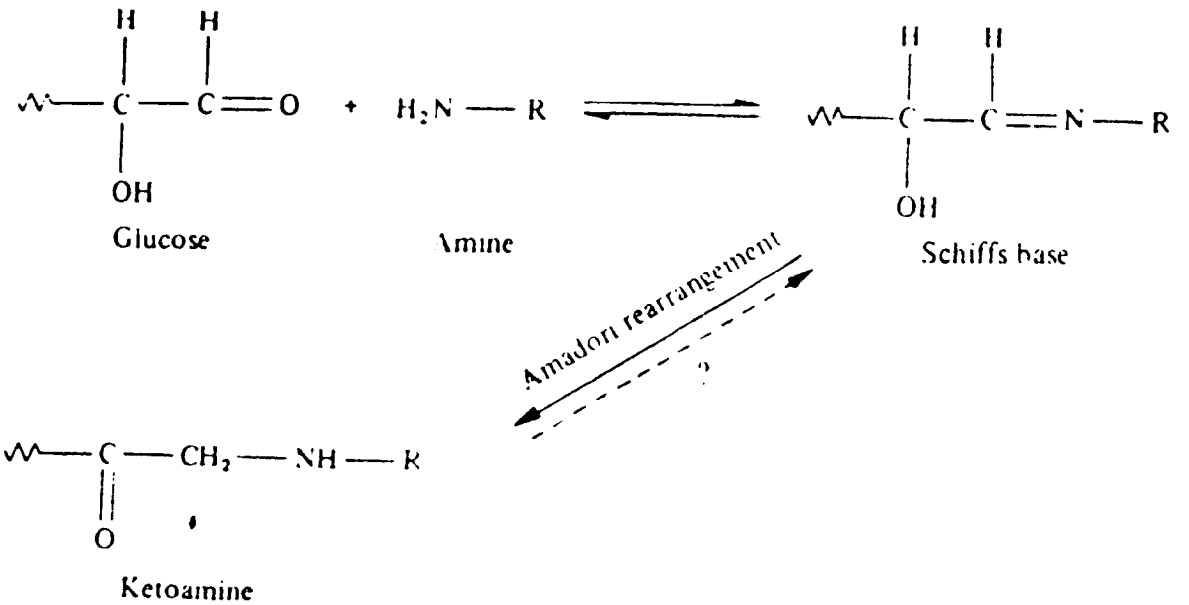


FIGURE 1.8 : The formation of glycosylated proteins

Strong evidence that SPA occurs *in vivo* in diabetics is discussed in several studies and inferred from raised levels of the platelet-activation markers, β-thromboglobulin[48,49], TxB_2[50] and malondialdehyde (MDA)[51] in diabetic blood plasma.

Small platelet microaggregates may occlude the lumen of fine capillaries already reduced in diameter by capillary basement membrane thickening. These blockages would cause haemorrhage and localised hypoxia in the tissue served by the capillaries. Numerous observations have been made and theories advanced as to why inappropriate platelet activation should occur in diabetes. In addition to making abnormally high levels of TxA_2, the response of diabetic platelets to the anti-aggregatory PGI_2 is frequently reduced[45], tipping the delicate haemostatic balance towards aggregation. Platelets from diabetics have also been shown to have an increased permeability to Ca^{2+}, rendering them more prone to activation[52].

Platelet hyperactivity may be related to the quality of diabetic control. Poorly controlled diabetics tend to progress more rapidly towards microangiopathy, although this is by no means always the case. Evidence suggests that subcutaneous insulin infusion retards platelet aggregation more effectively *ex vivo* compared to platelets from donors using optimised conventional insulin therapy with its widely fluctuating insulin and glucose levels[53].

Chapter 2

PLATELET CONCENTRATE STABILISATION

2.1.1: Introduction

Severe thrombocytopenia, or low platelet count, can cause bleeding from small blood vessels, symptomatically seen as purpura and mucosal haemorrhage. Thrombocytopenia also carries the risk of bleeding from a major blood vessel, which could prove fatal.

There are many causes for thrombocytopenia, but they can be divided into four groups[3];

(i) Thrombocytopenia due to diminished or defective platelet production such as in (a) amegakaryocytic thrombocytopenia or aplastic anaemia, or (b) in the wake of certain viral infections.

(ii) Thrombocytopenia due to enhanced platelet destruction, caused by severe burns, drug hypersensitivitys or idiopathic thrombocytopenic purpura (ITP).

(iii) Thrombocytopenia due to sequestration of platelets by the spleen, as occurs in hypersplenism or hypothermia.

(iv) Thrombocytopenia due to platelet loss after haemorrhage, multiple blood transfusions, or extracorporeal perfusion.

In general, platelet transfusion therapy is only of use in cases of defective or diminished platelet production, or platelet loss[54]. Platelets transfused into patients with thrombocytopenia due to sequestration or enhanced platelet destruction will suffer the same fate as the patient's own platelets. In addition, circulating platelets are young and metabolically active, so the risk of haemorrhage is small compared to other

thrombocytopenias. In these cases, platelet transfusion is only employed in the event of severe, life-threatening haemorrhage[54].

Platelet transfusion therapy can be used to prevent haemorrhagic death in patients with thrombocytopenia due to the failure of platelet production by the bone-marrow. Thus, transfusions are frequently used during intensive chemothrapy for acute leukaemia and for sufferers of aplastic anaemia[3,54]. In the latter condition, thrombocytopenia is more persistant and multiple platelet transfusions are necessary. This introduces the risk of platelet antibody effects. Hence, transfusions are used sparingly, and where possible with HLA-compatible platelets[54].

Multiple whole blood transfusions can cause dilutional thromocytopenia due to a replacement of viable platelets with non-viable platelets transfused in with the stored blood samples[3]. For this reason, platelets are routinely transfused after each 10-12 units of whole blood. Similarly, extra-corporeal perfusion can cause a 50% drop in platelet number in patients undergoing surgery assisted by perfusion. This is due to platelet damage and loss within the perfusion apparatus itself[3].

2.1.2: Difficulties and Solutions

In order to transfuse a large enough number of platelets to prevent haemorrhage, it is necessary to pool platelets from several donors and to concentrate the platelet-rich-plasma (PRP) so obtained four-fold.

The high-speed centrifugation and pelleting of platelets involved in the preparation of platelet concentrates invariably leads to some degree of platelet activation and aggregation. Evidence of this is the presence of platelet activation markers in the plasma of both stored and fresh platelet concentrates. β-thromboglobulin (β-TG)[68,69], a marker of the release reaction, and TxB_2[69], the degradation product of the pro-aggregatory TxA_2, synthesised and released by activated platelets, are both found at abnormally high concentrations even in apparently "good" quality concentrates. The cytoplasmic enzyme, lactate dehydrogenase[55] is also found in platelet concentrates, demonstrating that platelet lysis, as well as conventional

activation mechanisms, plays a role in the "storage lesion" of platelet concentrates. Abundant amounts of platelet factor 3 (PF3)[56] are found in stored concentrates. This factor possesses prothrombinase activity, thus increasing the likelihood of thrombin generation in the concentrate. Further evidence for the role of thrombin in platelet damage is given by the presence of fibrinopeptide A (FP-A), a peptide cleaved from fibrinogen by thrombin, in the plasma of blood products collected for transfusion[57].

Numerous studies have been undertaken to attempt to improve the quality and extend the shelf-life of platelet concentrates. These have included varying storage medium[58], nutrients[59,60], storage temperature[61,62] and even the type of container[59].

Polyolefin plastic bags are now preferred to polyvinylchloride bags for the storage of concentrates, due to the enhanced gas permeability of the former, which prevents plasma becoming anoxic[59]. Storage at room temperature with gentle agitation gives a better preservation of platelet morphology and increased survival time than cold storage or storage at body temperature[62]. Progress has also been made in the development of non-plasma media for storing platelet concentrates[58] and in adding platelet aggregation inhibitors to PRP prior to concentrate preparation[68-70].

Despite these improvements, platelets in stored concentrates rapidly lose their ability to aggregate in the presence of physiological agonists *in vitro*, although transfused platelets do seem to recover functionality to some degree[59,60], presumably due to replenishment of platelet metabolites from the patient's plasma.

To meet demand for platelet concentrates, some centres prepare concentrates from over 50% of the units of whole blood obtained. Fewer platelet concentrates would be needed if activation and loss of function associated with preparation and storage of platelets could be reduced. Thus, it makes good clinical and economic sense to find ways in which platelet survival *in vitro* can be extended, or to improve the quality of platelet concentrates so that fewer transfusions are needed to achieve haemostasis.

To this end, I have studied the effects of various anti-aggregatory reagents, either individually or in combinations, on the preparation and survival of platelet concentrates.

2.2; Methods.

2.2.1: Preparation and Storage

450ml whole blood was collected, with informed consent, from healthy adults into plastic bags containing CPDA-1 anticoagulant ('Biopack'-Biotest Pharma Ltd.). Platelet-rich plasma (PRP) was separated from whole blood by centrifugation at 1000g for 7 minutes at room temperature, as soon as possible after phlebotomy. PRP from four or five ABO-matched donors was pooled and separated into roughly equal aliquots of 250-300ml. Sterile sampling site couplers (Fenwall Laboratories, USA) were attatched to the PRP bags for addion of 'preservatives' and withdrawal of samples. Sterile platelet antagonists were added at this point using sterile syringes and needles.

Platelet concentrates (PC) were prepared by centifuging PRP at 3500g for 7 minutes so that all but 70-80ml of plasma was transferred to a second container, leaving pelleted platelets.

The PC were left to stand for 30 minutes, then gently agitated for 15 minutes before being placed in an incubator shaker (LH Engineering Ltd.) at 22°C, 10rpm.

Initial preparative work was performed at the Regional Blood Transfusion Centre, Queen Elizabeth Hospital, Birmingham by Dr. Richard Daw and staff.

2.2.2: Assays

Samples of PC were taken at various times using sterile syringes and needles. Samples thus obtained were tested for functionality, platelet number, pH and, where possible, for released β-thromboglobulin (β-TG).

Functionality was tested using a specially adapted MSE "Spectroplus" spectrophotometer, fitted with a cuvette holder with a magnetic stirrer base. 0.25ml of PC was diluted with 0.75ml of autologous platelet poor plasma (PPP) to give a platelet count

similar to that seen in PRP. All cuvettes and magnetic stirrers were siliconised using 'Sigmacote' (Sigma Ltd.) prior to use to prevent interaction between their surfaces and platelets. Platelet activation, on the addition of agonist was measured by the decrease in optical density of the stirred sample at 600nm and 30°C.A stirring speed of 500rpm was used (see appendix 1, Platelet Aggregometry).

Platelet counts were performed on either the Technicon H*1 or Technicon H-6000 automated haematology analyzers at the Coventry and Warwickshire Hospital.

pH was measured using an EIL 7030 pH meter and electrode (Kent Industrial Measurements Ltd.)

The Amersham radioimmunoassay for β-TG was used to test for the release of the protein from platelet dense granules. 1ml samples of PC were taken at various times and PGE_1, a more stable analogue of prostacylin, was added to a final concentration of 1μM, more than sufficient to prevent further platelet activation during sample preparation. The samples were centrifuged at 10,000g for 3 minutes in a MSE Microcentaur. 0.5ml aliquots of the resultant platelet free plasma were removed and frozen at -20°C. All samples collected during the PC stability study were then assayed by comparison with a β-TG standard curve using the Amersham kit and recommended assay protocol.

The assay protocol was as follows;

	β-TG Standards (ng/ml)					Unknowns (Sample no.)	
	10	20	50	100	225	1	2etc.
Tube Number	1,2	3,4	5,6	7,8	9,10	11,12	13,14
Standard Solutions	50	50	50	50	50	-	-
Unknown Solutions	-	-	-	-	-	50	50
^{125}I-labelled β-TG	200	200	200	200	200	200	200
Anti-β-TG Serum	200	200	200	200	200	200	200

Table 2.1; Contents of β-TG assay tubes (all volumes in microlitres.)

All tubes were vortex mixed and set aside to incubate at room temperature for one hour.

0·5 ml of the kit ammonium sulphate solution was added to each tube, and the contents mixed and centrifuged at 1000-1500g in an Eppendorf 5415c microcentrifuge.

The supernatant liquids were decanted and the tubes gently inverted so as not to disturb the precipitate, and placed so that they drained onto paper tissue for 5 minutes.

The amount of radioactivity present in each tube was assessed using a gamma counter.

2.2.3: Reagents

Prostaglandin E_1 (Sigma)

Stored as 0.5mM stock solution in 50% Ethanol at -20°C. Diluted to an appropriate concentration in saline prior to use and filter-sterilised.

Verapamil Hydrochloride (Sigma)

60mM solution in saline, prepared and filter sterilised just prior to use.

Nifedipine (Sigma)

60mM solution in dimethylsulphoxide, prepared just prior to use.

Insulin (Novo)

Monotard MC insulin-zinc suspension, 100iu/ml.

Hirudin (Sigma)

5u/ml in sterile saline

Adenosine diphosphate reagent (Sigma)

Special platelet aggregation reagent, reconstituted with water to give a buffered 0.2mM solution of ADP.

Adrenaline (Epinephrine) reagent (Sigma)

Special platelet aggregation reagent, reconstituted with water to give a buffered 0.1mM solution of adrenaline.

β-Thromboglobulin Radioimmunoassay kit (Amersham International plc.)

Adenine hydrochloride (Sigma)

24mg/ml in sterile saline.

2.3; Results.

The reason for presenting the results of experiments separately in the following section is that although each experiment is entirely self-consistent, the quality of the initial PC in terms of free platelet number and aggregation potential varied tremendously.

2.3.1; A study of the effects of PGE_1 and Verapamil on PC preparation and stabilisation

PGE_1 is a cheaper, more stable analogue of the physiological platelet aggregation antagonist, prostacyclin (PGI_2). It interacts with the prostacyclin receptor, causing activation of adenylate cyclase, raising platelet cAMP concentrations and thus rendering them less susceptible to activation and aggregation[4].

Verapamil inhibits platelet aggregation by blocking Ca^{2+} influx from plasma and release from internal stores. These effects maintain a low internal Ca^{2+} concentration, even

in the presence of platelet agonists[78-83]. *In vitro*, verapamil also appears to antagonise platelet α_2-adrenergic receptors, thus directly inhibiting epinephrine-induced aggregation[82].

PGE_1 and verapamil have been shown to synergisticly inhibit platelet aggregation[78]. Therefore in this initial study, the separate effects of PGE_1, verapamil and a combination of the two were investigated when they were added to PRP just prior to the preparation of the PC.

2.3.1(a): Experiment 1

PRP was prepared and divided into three equal aliquots and treated with "preservatives" to give the following PC preparations:

(1) PC ; Control platelet concentrate.

(2) PC+PGE_1 ; Platelet concentrate containing 60nM PGE_1.

(3) PC+PGE_1+V ; Platelet concentrate containing 60nM PGE_1 and 200 M verapamil.

Samples were taken 2 hours after preparation, and at 3 and 6 days, and assayed for pH, functionality and platelet number.

Results

DAY	PC	PC+PGE_1	PC+PGE_1+V	PPP
0	7.6	7.6	7.6	7.6
3	7.7	7.6	7.6	7.8
6	7.6	7.7	7.6	8.1

Table 2.2 ; Variation of pH with time ; Experiment 2.3.1(a)

DAY	PC	$PC+PGE_1$	$PC+PGE_1+V$
1	0.125	0.050	0.0 575
3	0.050	0.033	0.0725
6	0.0275	0.0275	0.0425

Table 2.3 ; Aggregation in the presence of 10μM ADP (Δ OD); Experiment 2.3.1(a)

DAY	PC	$PC+PGE_1$	$PC+PGE_1+V$
0	705	832	1050
3	876	778	985
6	861	711	885

Table 2.4 ; Platelet count, ($x10^9/dm^3$) ; Experiment 2.3.1(a)

These results are expressed graphically in figures 2.1-2.2.

Excellent resuspension of the platelet pellet was observed in all three samples, and platelet numbers were maintained well over the 7 days of the study.

By far the greatest degree of aggregation was observed in the control PC at day 1. However, the PC's response decreased by approximately 75% over the duration of the tests. In contrast, the two treated PC showed relatively little initial aggregation, despite similar platelet numbers, but were able to maintain this level of functionality throughout the period of testing. This suggests that the aggregation of the $PC+PGE_1$ and $PC+PGE_1+V$ samples is being inhibited by the platelet antagonists present on the first day of study.

FIGURE 2.1 : Platelet Count ; Experiment 2.3.1(a)

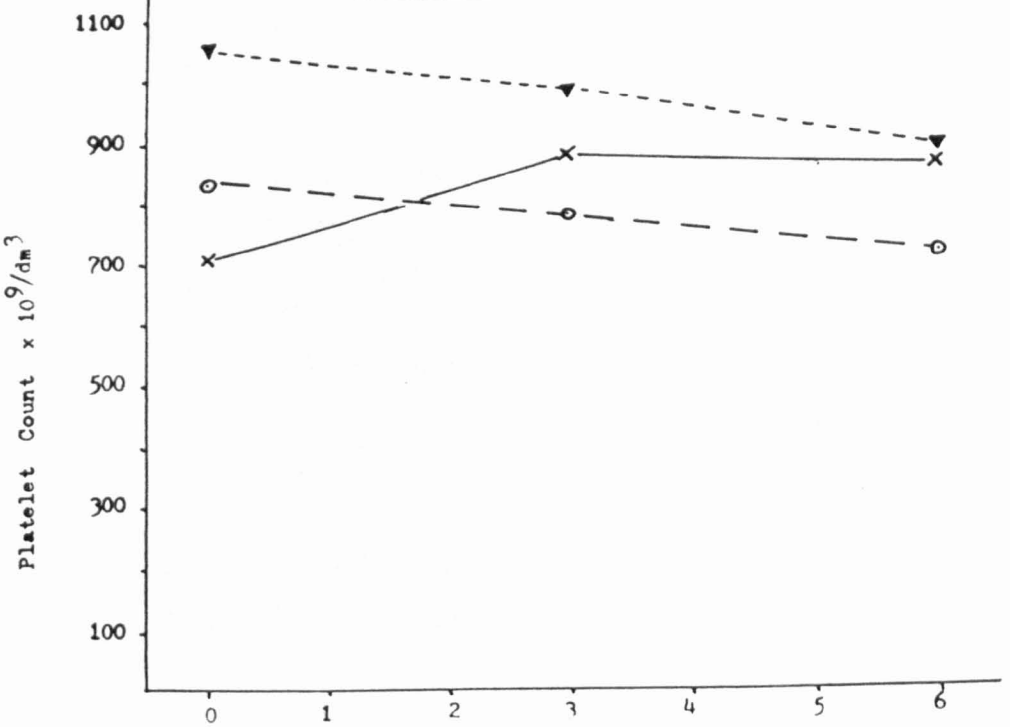
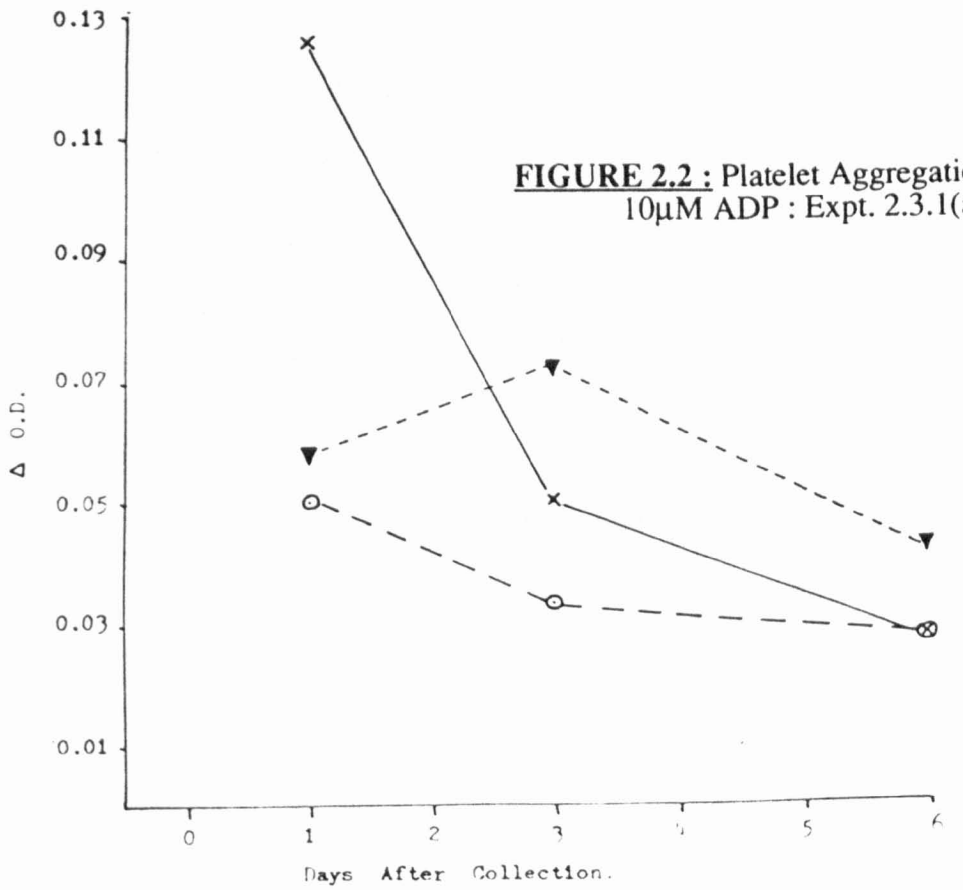


FIGURE 2.2 : Platelet Aggregation with 10μM ADP : Expt. 2.3.1(a)



KEY: PC+PGE_1+V = ▼- - -▼

PC+PGE_1 = ⊙— —⊙

PC = ×——×

2.3.1(b) ; Experiment 2

PRP was divided into three equal aliquots and treated with 'preservatives' to give the following platelet concentrates;

(1) PC ; Control platelet concentrate,

(2) PC+V ; Platelet concentrate including 200μM verapamil,

(3) PC+PGE_1+V ; Platelet concentrate including 60nM PGE_1 and 200μM verapamil.

Samples were taken after 2 hours, 1, 3, 4, 5 and 6 days and tested for functionality, pH, platelet number and released β-TG.

Results

DAY	PC	PC+V	PC+PGE_1+V	PPP
0	7.5	7.6	7.55	7.5
1	7.6	7.6	7.6	7.6
4	7.8	7.65	7.7	7.9
5	7.8	7.8	7.65	7.9
6	7.8	7.65	7.7	8.0

Table 2.5 ; Variation of pH with time ; Experiment 2.3.1(b)

DAY	PC	PC+V	PC+PGE_1+V
0	431	876	1025
1	888	964	1011
4	850	968	1025
5	753	880	950
6	651	799	905

Table 2.6 ; Platelet number ($x10^9/dm^3$) ; Experiment 2.3.1(b)

DAY	PC	PC+V	PC+PGE_1+V
0	0	0.055	0.025
1	0	0.037	0
3	0	0.032	0
4	0	0.013	0.007
5	0	0	0
6	0	0	0

Table 2.7 ; Platelet aggregation in presence of 2μM ADP (ΔOD) ; Experiment 2.3.1(b)

DAY	PC	PC+V	PC+PGE_1+V
0	0.032	0.175	0.150
1	0.050	0.160	0.060
3	0.037	0.125	0.110
4	0.025	0.090	0.090
5	0.015	0.055	0.074
6	0.006	0.040	0.072

Table 2.8 ; Platelet aggregation in presence of 10μM ADP (ΔOD) ; Experiment 2.3.1(b)

DAY	PC	PC+V	PC+PGE_1+V
0	>8000	>6000	920
1	>15,000	10,800	4800
4	23,100	18,300	14,700
5	27,000	20,500	14,500
6	-	24,000	15,600

Table 2.9 ; Released β-TG, (ng/ml PC) ; Experiment 2.3.1(b)

These results are expressed graphically in figures 2.3-2.6.

FIGURE 2.3 : Platelet Count ; Experiment 2.3.1(b)

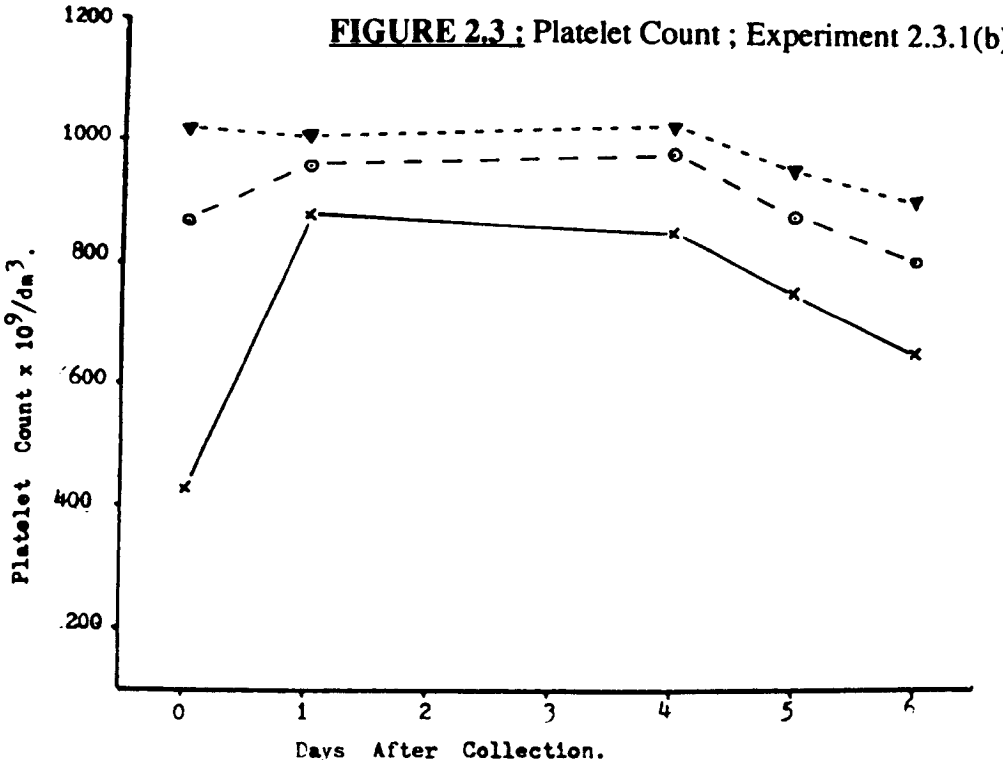
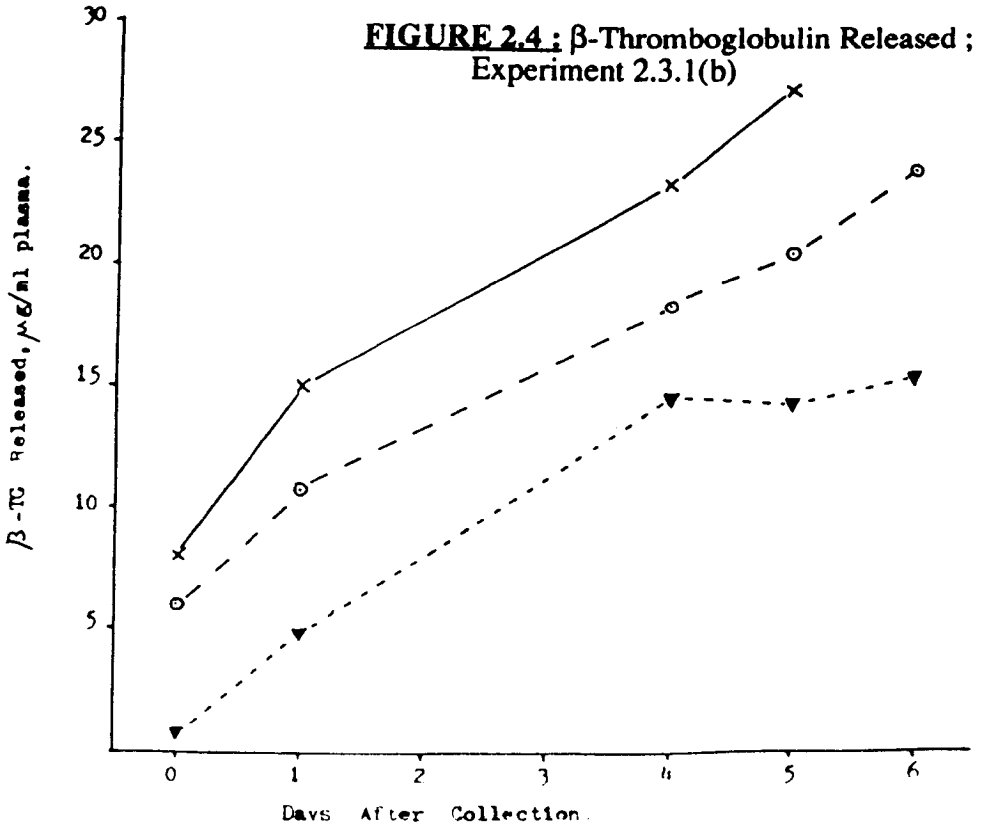


FIGURE 2.4 : β-Thromboglobulin Released ; Experiment 2.3.1(b)



KEY: PC+PGE_1+V = ▼- - -▼

PC+V = ⊙— —⊙

PC = ×——×

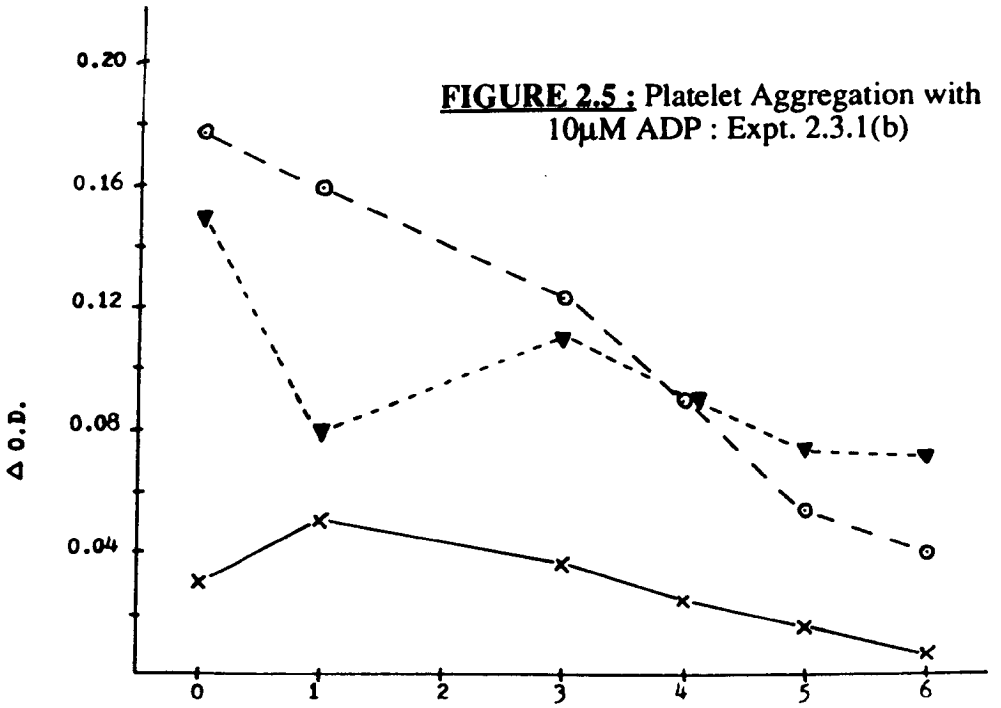


FIGURE 2.5 : Platelet Aggregation with 10μM ADP : Expt. 2.3.1(b)

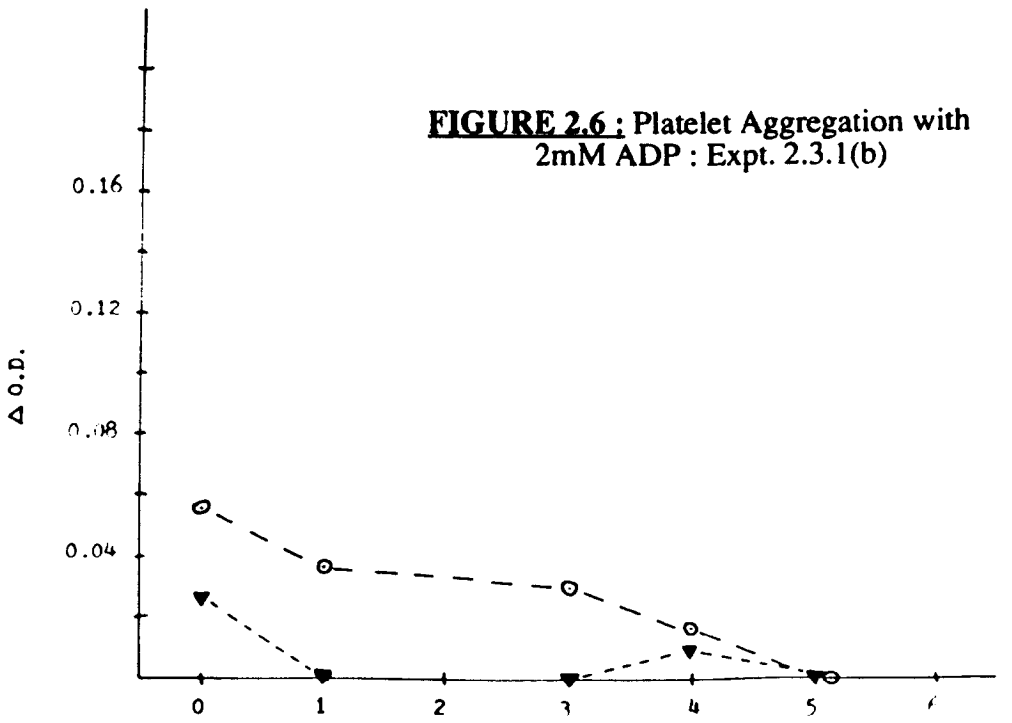


FIGURE 2.6 : Platelet Aggregation with 2mM ADP : Expt. 2.3.1(b)

KEY: $PC+PGE_1+V$ = ▼----▼

PC+V = ⊙– –⊙

PC = ×——×

In contast to the first experiment, only the treated concentrates showed excellent, rapid resuspension. The control PC achieved approximately 45% resuspension in the 2 hours following preparation, recovering to almost 90% at 24 hours. All three samples show a progressive loss of discrete platelets (approximately 10%) over days 4-6.

The relatively poor resuspension of the untreated PC is mirrored in the results of the functionality tests. The treated samples show a much higher degree of aggregation after 2 hours and, although platelet functionality in PC recovers as platelet number increases, the quality of the PC+V and PC+PGE_1+V samples is always greater. The sample treated with both verapamil and PGE_1 again shows an inhibition of aggregation up to day 3. This must be due to the presence of PGE_1, possibly acting synergistically with verapamil, since verapamil alone does not exert this effect.

The β-TG assay clearly demonstrates the benefit of PGE_1 in reducing platelet damage during concentrate preparation. Whereas both the PC and PC+V samples show a large release of β-TG into plasma in the day 0, t=2 hours sample (>300x normal plasma levels), the P+PGE_1+V sample shows a comparatively small release (>35x normal plasma level).

2.3.1(c) : Experiment 3

This study was a repeat of experiment 1, except that more time points were obtained, and β-TG was assayed.

Results

DAY	PC	PC+PGE_1	PC+PGE_1+V	PPP
0	7.6	7.65	7.65	7.6
1	7.55	7.65	7.7	7.6
3	7.6	7.6	7.7	7.75
5	7.65	7.7	7.65	7.95
6	7.6	7.6	7.7	8.0

Table 2.10 ; Variation of pH with time ; Experiment 2.3.1(c)

DAY	PC	PC+PGE_1	PC+PGE_1+V
0	1012	1173	1540
1	1203	1109	1402
2	1271	1187	1551
3	1256	1181	1555
6	1223	1060	1560
7	1068	1062	1378

Table 2.11 : Platelet number ($x10^9/dm^3$) ; Experiment 2.3.1(c)

DAY	PC	PC+PGE_1	PC+PGE_1+V
0	0.140	0.090	0.075
1	0.062	0.010	0.010
2	0.045	0.010	0.035
3	0.030	0.025	0.040
4	0.016	0.014	0
5	0.015	0.012	0
6	0.008	0.025	0

Table 2.12 : Platelet aggregation in presence of 2μM ADP. (ΔOD) ; Experiment 2.3.1(c)

DAY	PC	PC+PGE_1	PC+PGE_1+V
0	0.295	0.204	0.225
1	0.240	0.175	0.165
2	0.200	0.160	0.205
3	0.180	0.185	0.275
4	0.140	0.190	0.160
5	0.116	0.145	0.025
6	0.075	0.160	0
7	0.009	0.080	0

Table 2.13 : Platelet aggregation in presence of 10μM ADP. (ΔOD) ; Experiment 2.3.1(c)

DAY	PC	PC+PGE_1	PC+PGE_1+V
0	15,500	2100	1100
1	22,200	7650	11,400
2	25,750	7750	8000
3	20,400	14,100	9300
6	28,200	27,000	21,000

Table 2.14 : Released β-TG. (ng/ml PC) : Experiment 2.3.1(c)

These results are expressed graphically in figures 2.7-2.10.

This repeat of experiment 1 gave very similar results to the earlier test. Again, all three samples showed excellent resuspension and maintainance of platelet number. Equally, the inhibition of aggregation over the first few days is once again evident in the treated samples.

Samples PC+PGE_1 and PC+PGE_1+V did not show the massive initial release of β-TG observed in the control sample, untreated PC, confiming the results of experiment 2.3.1(b). This demonstrates that even though the PC platelets resuspended very well, they had undergone considerable, reversible activation during the preparative procedures.

The behaviour of the PC+PGE_1+V sample in functionality tests is most peculiar. Initial inhibition of aggregation disappears by day 3, at which point aggregation potential appears to be maximal, but this is followed by a complete loss of activity within the next 48 hours. This may be due to accidental bacterial contamination of the concentrate during sampling.

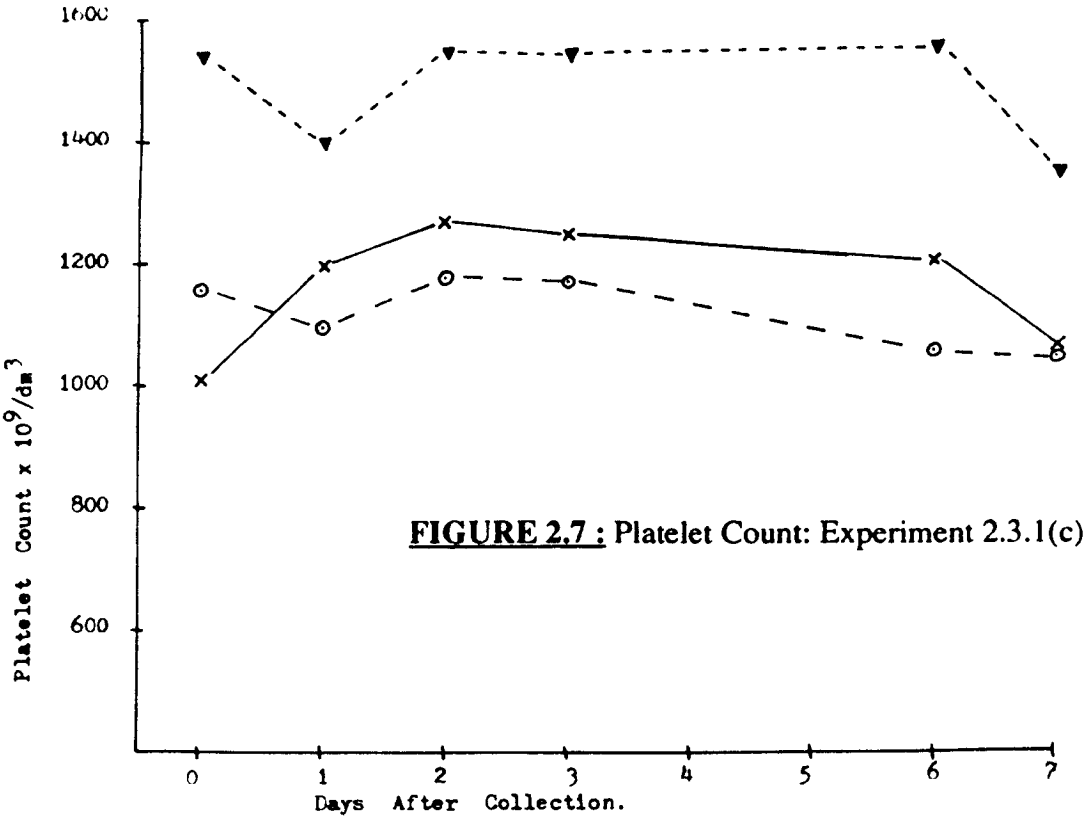


FIGURE 2.7 : Platelet Count: Experiment 2.3.1(c)

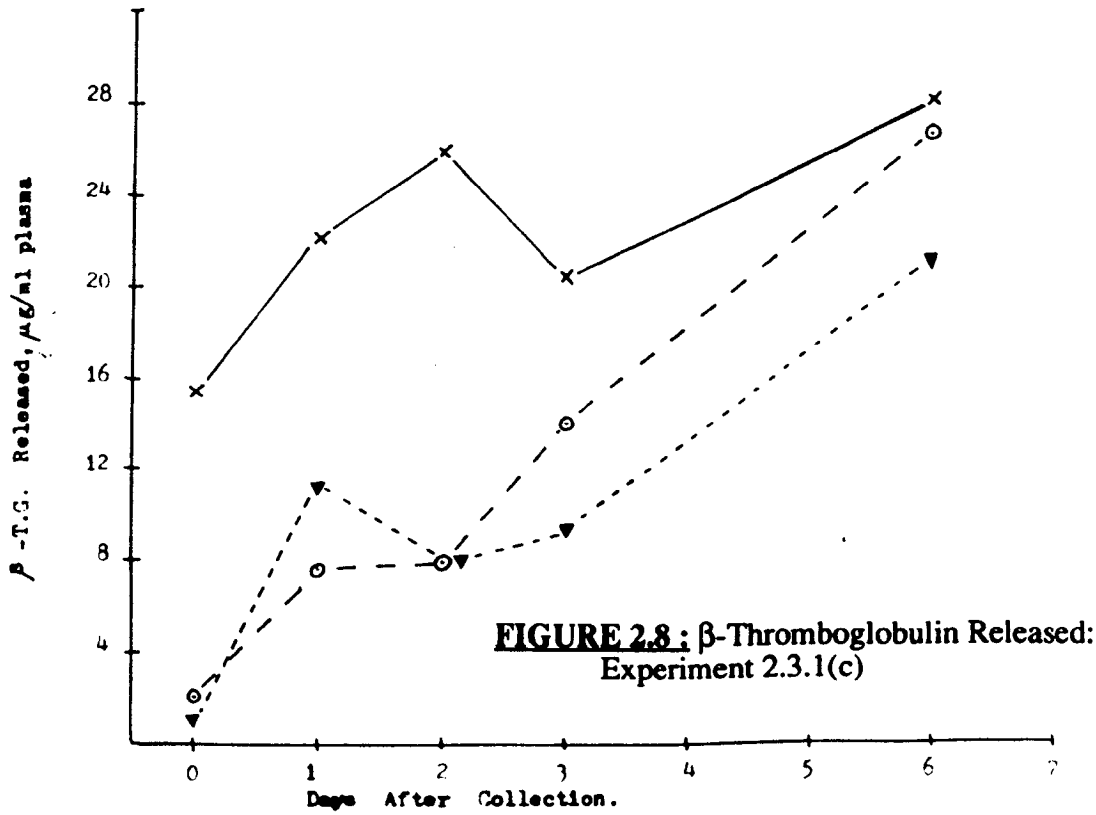


FIGURE 2.8 : β-Thromboglobulin Released: Experiment 2.3.1(c)

KEY: $PC+PGE_1+V$ = ▼- - -▼
$PC+PGE_1$ = ⊙— —⊙
PC = ×——×

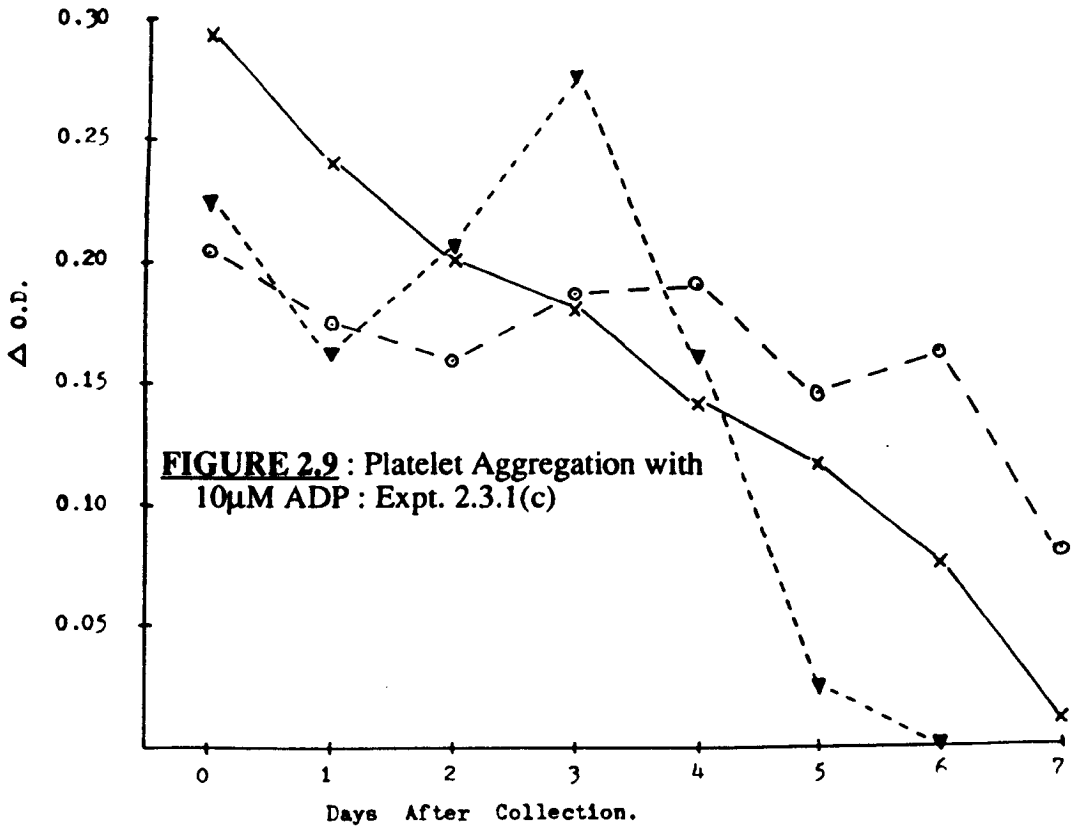


FIGURE 2.9 : Platelet Aggregation with 10μM ADP : Expt. 2.3.1(c)

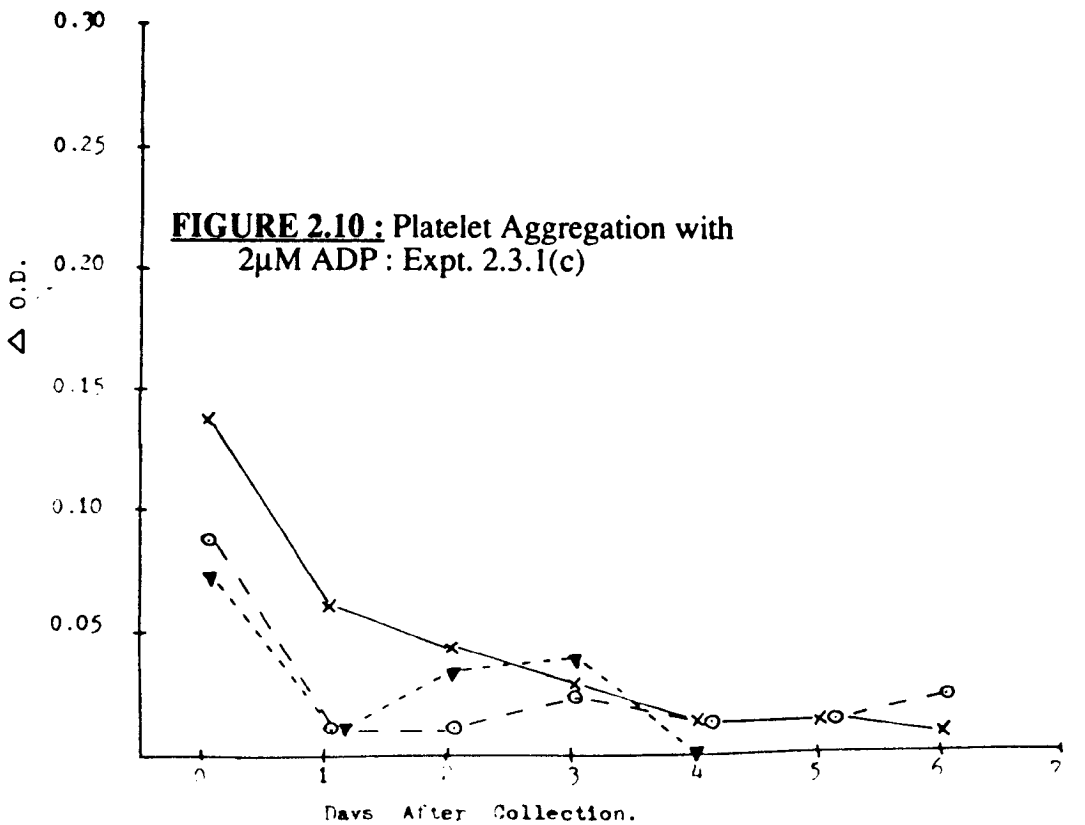


FIGURE 2.10 : Platelet Aggregation with 2μM ADP : Expt. 2.3.1(c)

KEY: $PC+PGE_1+V$ = ▼- - -▼
$PC+PGE_1$ = ⊙ — —⊙
PC = ×——×

2.3.2 : The effect of a lower dose of PGE_1 and a comparison between the calcium-channel-blockers, Verapamil and Nifedipine.

Owing to the apparent inhibition of platelet aggregation at days 0-2 in the previous experiments, it was decided to reduce the concentration of PGE_1 in the concentrates in order to try and minimise the inhibition of aggregation in treated samples in comparison with any control PC that give good resuspension.

In addition, a second Ca^{2+}-mobilisation inhibitor, nifedipine, was investigated. Nifedipine, under the trade name Adalat, is one of the most common drugs prescribed for the treatment of hypertension[35].

Experiment 4

PRP was split into four aliquots and treated to give the following PC samples;

(i) PC : Control platelet concentrate.

(ii) PC+PGE_1 : Platelet concentrate including 15nM PGE_1.

(iii) PC+PGE_1+V : Platelet concentrate including 15nM PGE_1 and 200μM verapamil.

(iv) PC+PGE_1+N : Platelet concentrate including 15nM PGE_1 and 200μM nifedipine.

Samples were taken after 2 hours, 1, 3, 4, 5 and 6 days and tested for pH, platelet count and functionality.

Results

DAY	PC	PC+PGE_1	PC+PGE_1+V	PC+PGE_1+N	PPP
0	7.55	7.60	7.60	7.65	7.50
1	7.60	7.65	7.60	7.65	7.60
3	7.60	7.65	7.60	7.65	7.60
4	7.55	7.65	7.70	7.70	7.80
5	7.60	7.70	7.70	7.75	7.85
6	7.65	7.65	7.65	7.80	7.90

Table 2.15 : Variation of pH with time : Experiment 2.3.2.

DAY	PC	PC+PGE_1	PC+PGE_1+V	PC+PGE_1+N
0	175	885	911	1235
1	661	875	830	1214
4	597	904	820	1130
5	572	809	771	826
6	526	747	728	791

Table 2.16 : Platelet number ($x10^9/dm^3$) : Experiment 2.3.2.

DAY	PC	PC+PGE_1	PC+PGE_1+V	PC+PGE_1+N
0	0	0.070	0.110	0.020
1	0.022	0.050	0.050	0
3	0.002	0.020	0.012	0
4	0.005	0.012	0.012	0
5	0	0.012	0.027	0

Table 2.17 : Platelet aggregation in presence of 2μM ADP (ΔOD): Experiment 2.3.2.

DAY	PC	PC+PGE_1	PC+PGE_1+V	PC+PGE_1+N
0	0.015	0.220	0.220	0.080
1	0.070	0.160	0.163	0.035
3	0.040	0.080	0.085	0.010
4	0.027	0.078	0.080	0
5	0.025	0.095	0.115	0
6	0.015	0.050	0.075	0

Table 2.18 : Platelet aggregation in presence of 10μM ADP (ΔOD): Experiment 2.3.2.

These results are expressed graphically in figures 2.11-2.13.

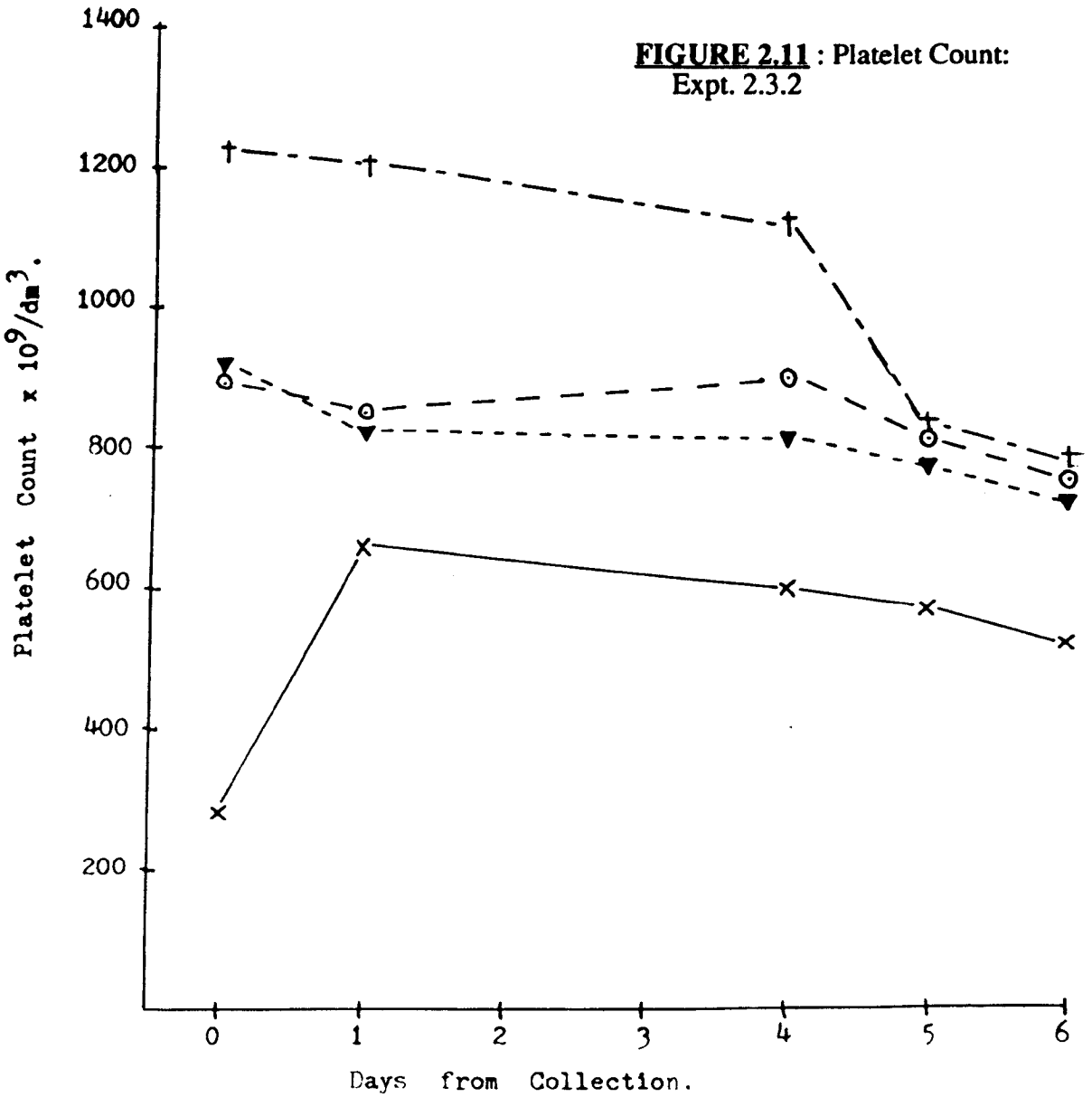


FIGURE 2.11 : Platelet Count: Expt. 2.3.2

KEY: $PC+PGE_1+V$ = ▼ - - - ▼
$PC+PGE_1+N$ = † — · — †
$PC+PGE_1$ = ⊙ — — ⊙
PC = × —— ×

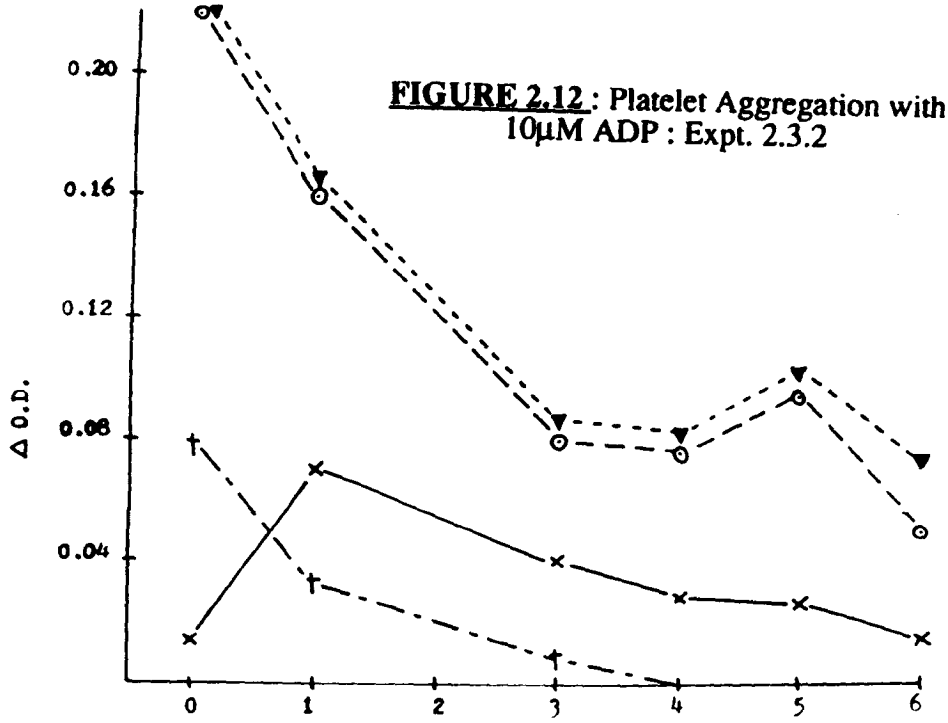


FIGURE 2.12 : Platelet Aggregation with 10μM ADP : Expt. 2.3.2

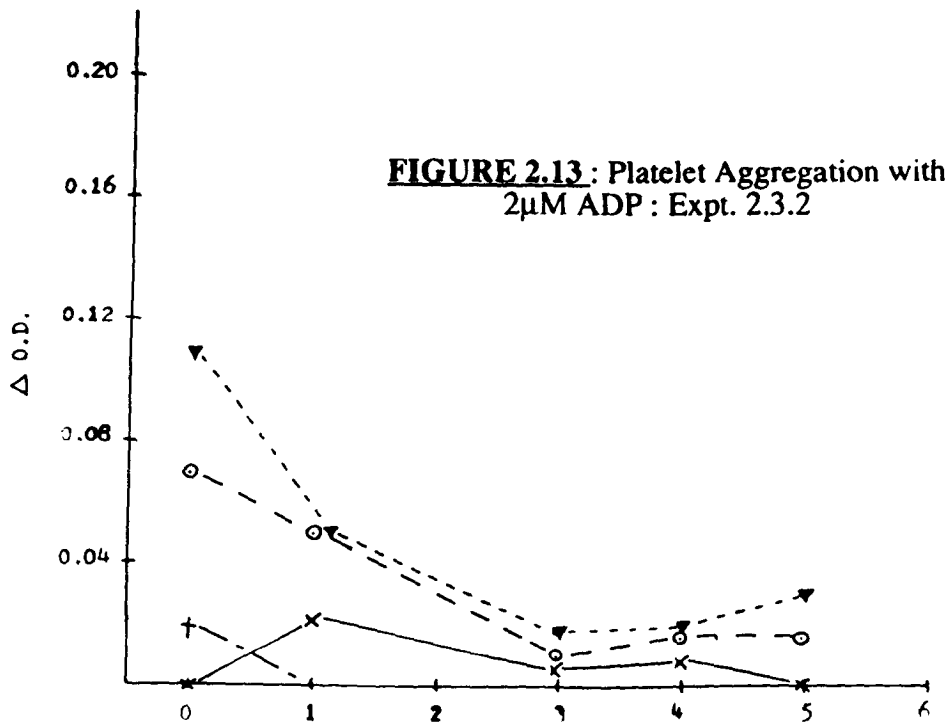


FIGURE 2.13 : Platelet Aggregation with 2μM ADP : Expt. 2.3.2

KEY: $PC+PGE_1+V$ = ▼- - - -▼
$PC+PGE_1+N$ = †— · —†
$PC+PGE_1$ = ⊙ — —⊙
PC = ×———×

The quality of platelet concentrate preparation in this experiment was similar to that achieved in experiment 2, and the control PC showed very similar patterns of resuspension and aggregation.

The lower dose of PGE_1 employed in this experiment appears to be sufficient to give rapid resuspension, and the inhibition of aggregation observed in experiments 1-3 over the first few days is not evident. Verapamil has no additional benefit compared to PGE_1 alone, since both aggregation and platelet numbers remained virtually identical.

The most interesting results were obtained from sample PC+PGE_1+N. A much higher initial platelet count was observed in this sample, but the response to ADP was greatly reduced. Both effects may be due to a combination of too high a dose of nifedipine, and the effects of dimethylsulphoxide (DMSO), used as a solvent for the drug. DMSO has been shown to inhibit platelet aggregation both *in vivo* and *in vitro* at the levels employed in this experiment[63] (0.33% v/v in PC, 0.08% in aggregometry cuvette). However the increase in platelet number compared to the other treated samples indicates that 15nM PGE_1, even in the presence of verapamil, is not sufficient to resuspend all the platelets.

2.2.3 : The effect of Insulin on PC preparation and stabilisation

Platelet concentrates, even in the presence of PGE_1, use up virtually all of their glycogen stores within 4-6 days, independent of whether glucose is present in the storage medium or not[59,70]. Therefore, platelets in stored PCs must rely on their internal glycogen stores rather than external glucose as the fuel for metabolism and ATP production, and when this glycogen is consumed, they probably become non-functional.

Platelets are known to possess insulin receptors[103-106] and a glycogen synthase enzyme which can exist in two forms, the active I-form, and the D-form, which is inactive under normal physiological conditions[3]. Insulin converts the inactive to the active form. Hence, insulin was added to the PC to test the hypothesis that inclusion of insulin in the preparation of concentrates would prevent loss of glycogen stores and promote utilisation of external glucose.

Experiment 5

PRP was split into four aliquots and treated to give the following platelet concentrate samples;

(i) PC ; Control platelet concentrate.

(ii) PC+PGE_1 ; Platelet concentrate including 60nM PGE_1.

(iii) PC+I ; Platelet concentrate including 0.3iu/ml Insulin.

(iv) PC+PGE_1+I ; Platelet concentrate including 60nM PGE_1 and 0.3iu/ml Insulin.

Samples were taken after 2 hours and at 1, 2, 3, 4 and 6 days after collection and tested for pH, platelet count and aggregation. Samples were also layered onto Percoll density gradients for ultra-centrifugation (see appendix 2 for method).

Results

The pH of all samples was 7.55 to 7.65 over the 7 days of testing. PPP was frozen and thawed just prior to use as a dilutant in functionality tests(pH = 7.60).

DAY	PC	PC+PGE_1	PC+I	PC+PGE_1+I
0	115	842	103	867
1	241	812	240	850
6	363	728	717	741

Table 2.19 : Platelet count ($x10^9/dm^3$) ; Experiment 2.3.3.

DAY	PC	PC+PGE_1	PC+I	PC+PGE_1+I
0	0	0.130	0	0.142
1	0	0.055	0	0.045
2	0	0.035	0	0.045
3	0	0.035	0	0.040
4	0	0.035	0	0.065
6	0	0.030	0	0.047

Table 2.20 : Platelet aggregation in presence of 10μM ADP (ΔOD) ; Experiment 2.3.3.

These results are expressed graphically in figures 2.14-2.15. See figure 2.16 for buoyant-density centrifugation resuts.

The quality of the initial, untreated concentrate preparation in this instance was very poor. The two samples prepared in the absense of PGE_1 both showed only about 13% resuspension after two hours. Although the presence of insulin did seem to aid resuspension in the long-term, neither sample showed any measurable response to ADP at any time during the experiment.

Comparing PC+PGE_1 and PC+PGE_1+I samples, little additional benefit was observed in aggregometry experiments by having insulin present in addition to PGE_1. However, the density gradient centrifugation did provide some interesting observations. At day 1, the PC+PGE_1+I sample gave the characteristic multiple banding seen in normal PRP. When investigated again on day 3, the intensity of the bands had decreased, although their positions were similar, and a small number of low-density platelet aggregates were seen. The pattern of bands and aggregates from the PC+PGE_1 sample showed a major loss of the higher density bands, but no increase in platelet aggregates, despite the number of discrete platelets being similar to that in the PC+PGE_1+I concentrate. The control and insulin-treated PC both gave high numbers of relatively large platelet aggregates, as would be expected owing to their poor resuspension. Despite the faintness of the bands in these two samples, many more were evident in the PC+I sample than in the control PC. The observation of a decrease in platelet density with storage time is in agreement with the discontinuous density-gradient work of Bolin *et al*[64] who reported that the density changes were related to an alteration in platelet shape from discoid to spherical, and to an increase in size with storage time.

Insulin appears to prevent this decrease in density suggesting that perhaps the platelets become lighter due to utilisation of glycogen stores.

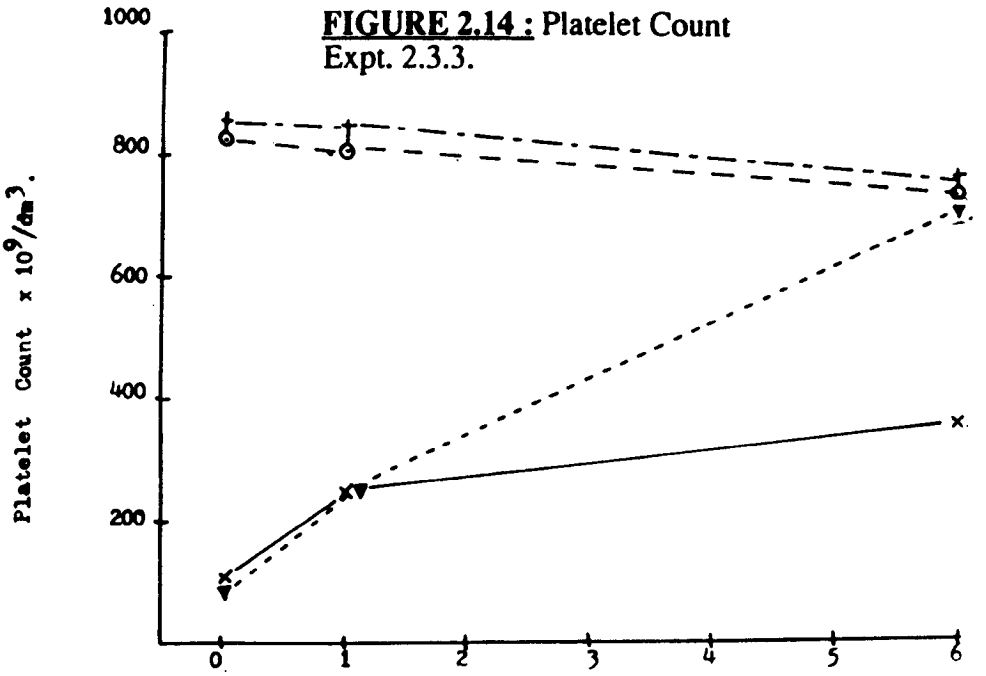


FIGURE 2.14 : Platelet Count Expt. 2.3.3.

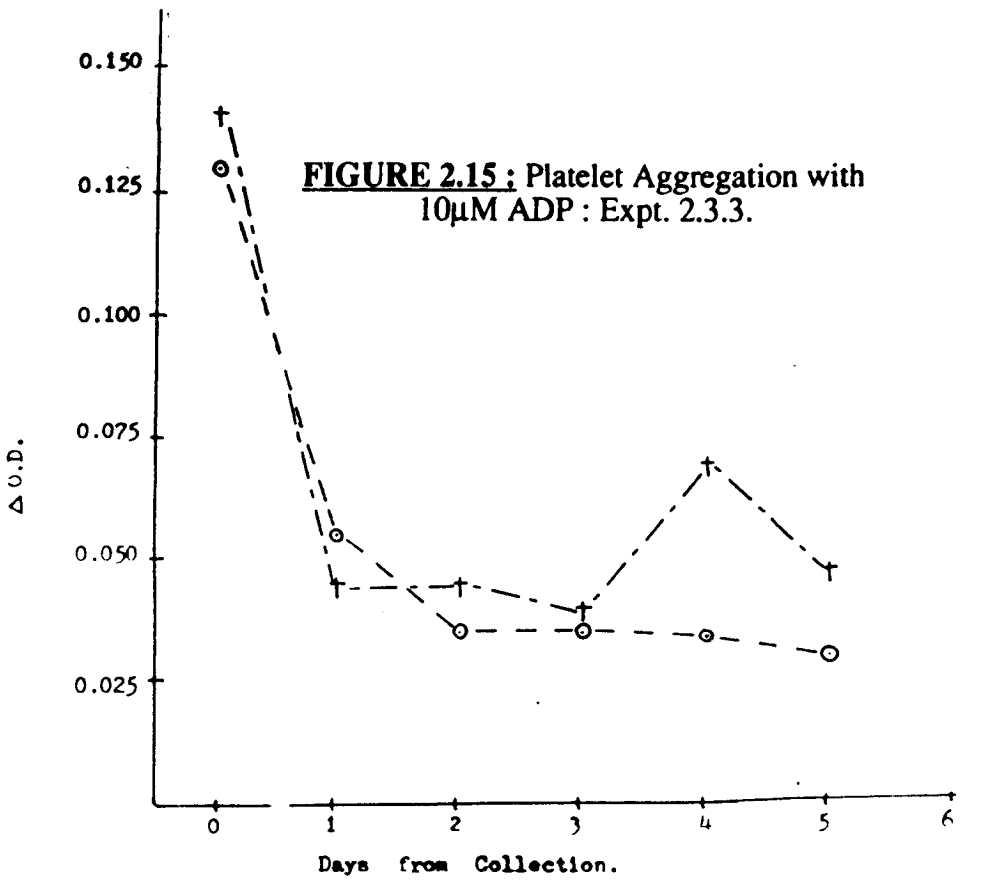


FIGURE 2.15 : Platelet Aggregation with 10μM ADP : Expt. 2.3.3.

KEY: PC+PGE_1+I = †—·—†
PC+PGE_1 = ⊙— —⊙
PC+I = ▼- - - -▼
PC = ×—— ×

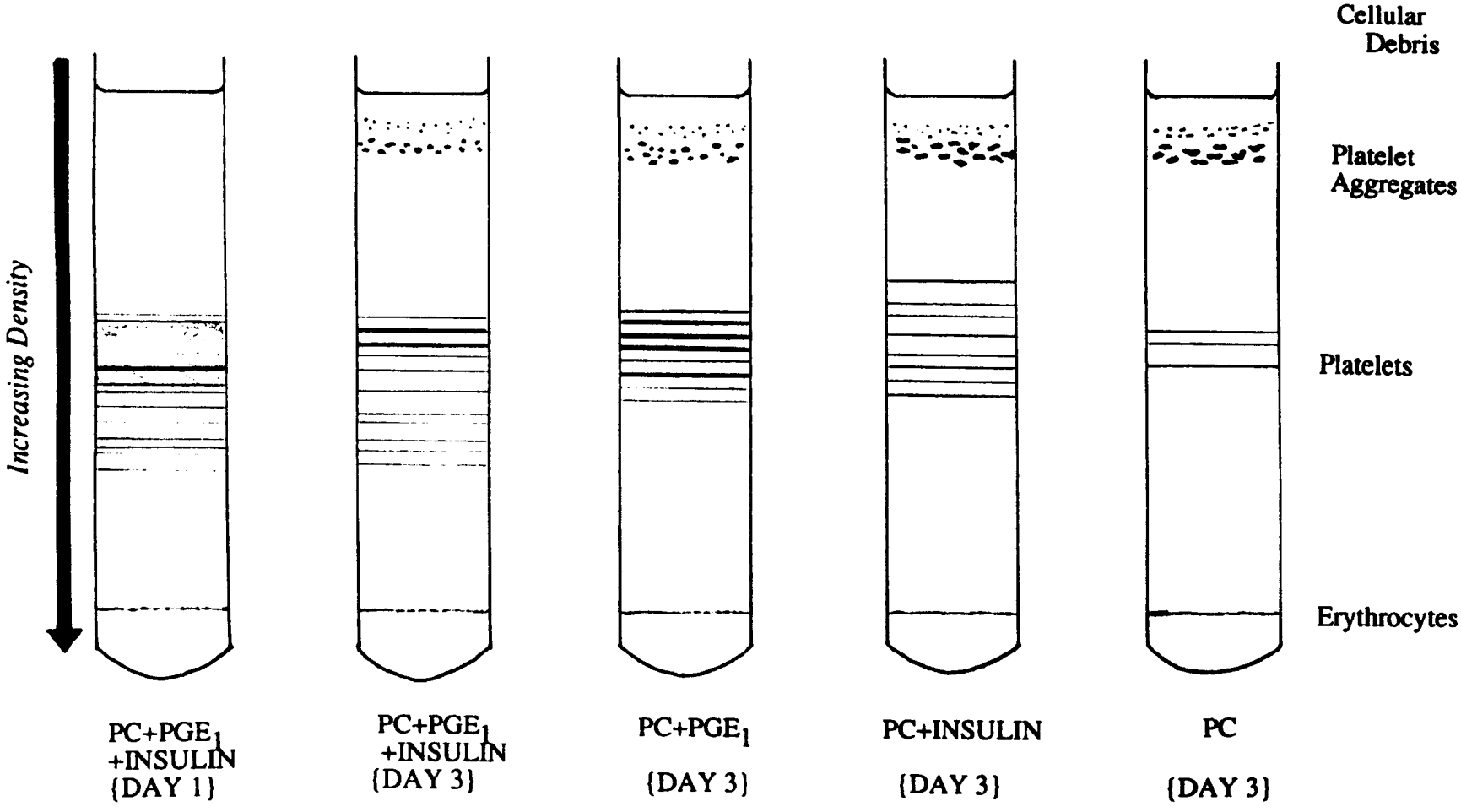


FIGURE 2.16 : Buoyant Density centrifugation of PCs. (Compare with Figure A2)

2.3.4. : The effect of Hirudin on PC preparation and stabilisation.

Hirudin, a 65 amino acid polypeptide isolated from the parotid gland of the leech (*Hirudo medicinalis*), is the most potent natural inhibitor of coagulation known[67]. A very stable 1:1 complex is rapidly formed with thrombin, therby abolishing its ability to cleave fibrinogen. Hirudin has also been shown to inhibit the thrombin-platelet interaction by interfering with the binding of thrombin to saturable, 'specific' receptors on the platelet surface, but not the nonsaturable, 'nonspecific' sites[65]. Hirudin also causes a rapid dissociation of thrombin bound to the saturable sites[66].

Considerable evidence has been gained in support of the hypothesis that thrombin generation plays a role in the 'storage lesion' of platelet concentrates. For these reasons the effect of hirudin on PC stability was investigated in the presence or absence of PGE_1.

Experiment 5

PRP was divided into four and treated with 'preservatives' to give the following platelet concentrate sampes;

(i) PC ; Control platelet concentrate,

(ii) PC+PGE_1 ; Platelet concentrate including 60nM PGE_1,

(iii) PC+H ; Platelet concentrate including 5 units/300ml Hirudin,

(iv) PC+PGE_1+H ; Platelet concentrate including 60nM PGE_1 and 5 units/300ml Hirudin.

Samples were taken at 2 hours, 1, 3, 6 and 9 days after collection and tested for platelet number, functionality and released β-TG.

Results

DAY	PC	PC+PGE_1	PC+H	PC+PGE_1+H
0	268	841	521	960
1	702	743	749	895
3	680	776	740	919
6	688	801	699	886

Table 2.21 : Platelet count (x $10^9/dm^3$) ; Experiment 2.3.4

DAY	PC	PC+PGE_1	PC+H	PC+PGE_1+H
0	0.030	0.205	0.085	0.220
3	0.055	0.138	0.054	0.152
6	0.055	0.112	0.080	0.114
9	0	0	0	0

Table 2.22.: Platelet aggregation in presence of 10μM ADP (ΔOD) ; Experiment 2.3.4.

DAY	PC	PC+PGE_1	PC+H	PC+PGE_1+H
0	15,800	420	11,500	150
1	18,000	5420	11,500	2000
6	22,000	6500	8750	11,500
9	40,000	4700	30,000	18,300

Table 2.23 ; β-TG released (ng/ml) ; Experiment 2.3.4.

These results are expressed graphically in figures 2.17-2.19.

FIGURE 2.17 : Platelet Count : Expt. 2.3.4.

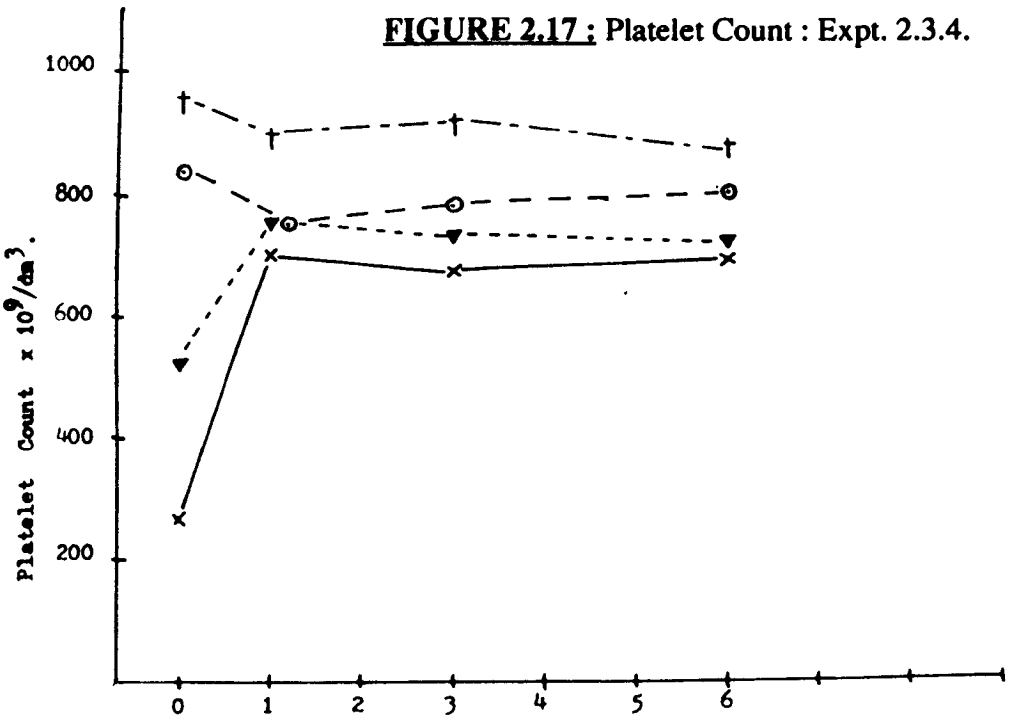
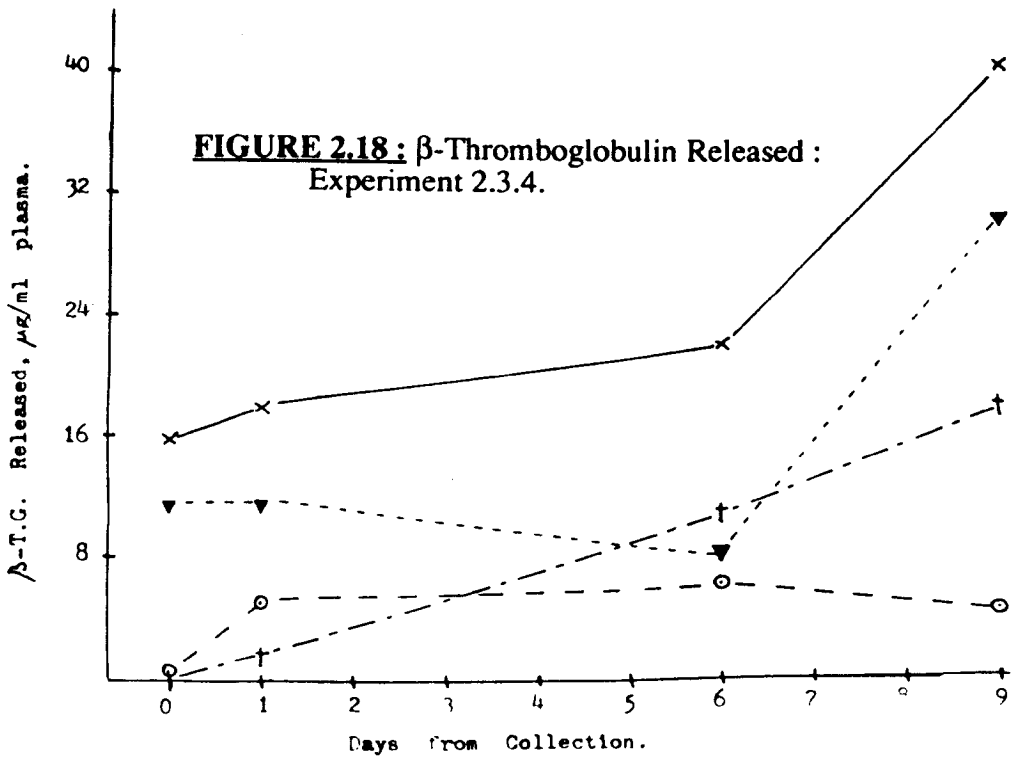


FIGURE 2.18 : β-Thromboglobulin Released : Experiment 2.3.4.



KEY: PC+PGE$_1$+H = †- - -†

PC+PGE$_1$ = ⊙— —⊙

PC+H = ▼- - - -▼

PC = ×——×

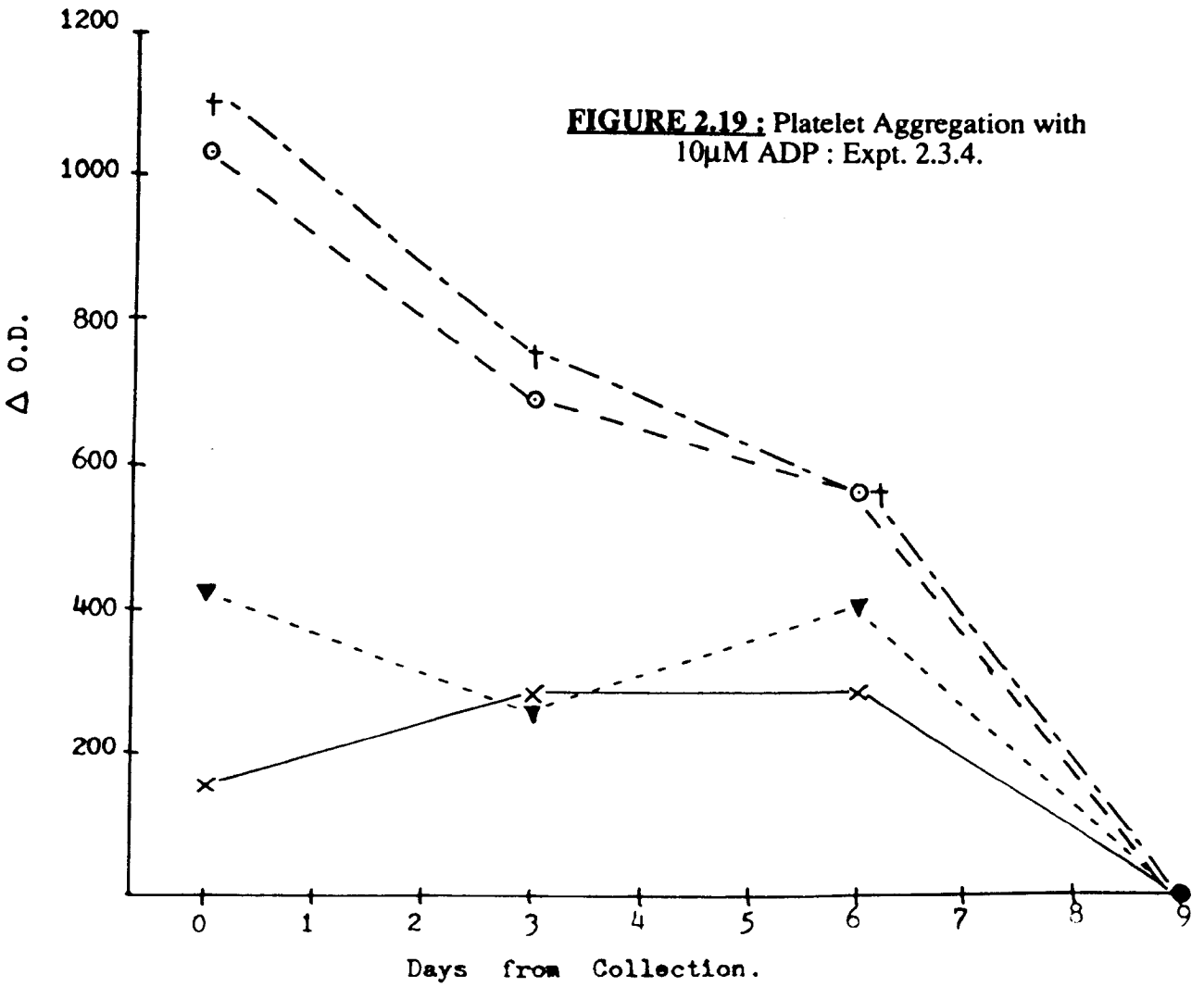


FIGURE 2.19 : Platelet Aggregation with 10μM ADP : Expt. 2.3.4.

KEY: PC+PGE$_1$+H = †— · —†
PC+PGE$_1$ = ⊙— —⊙
PC+H = ▼- - - -▼
PC = ×——×

The untreated sample showed poor initial resuspension, but recovered well by 24 hours. The presence of hirudin may have had some minor benefit towards resuspension, presumably acting by causing dissociation and inactivation of platelet-bound thrombin.

No improvement in aggregation was obtained through the presence of hirudin. The data for β-TG release shows, as expected, that PGE_1 protects against platelet activation during preparation. Hirudin alone is not able to do this, suggesting that thrombin does not play a major role in the preparative lesion. Evidence for the role of thrombin in the subsequent storage lesion is provided by the lack of β-TG release in the PC+H sample between days 0-6.

2.3.5. ; The effect of multiple additions of PGE_1 on platelet concentrate stabilisation.

PGE_1 appears to be doublely beneficial to PC, greatly enhancing platelet resuspension and also inhibiting the storage lesion, despite the apparent inactivation of PGE_1 after 48 hours or so. Other platelet 'preservatives' seem to be of benefit mainly in the resuspension of platelet pellets. Thus, an experiment was devised to find out if boosting PGE_1 levels after PC preparation would have any effect.

Experiment 7

The following platelet concentrate samples were prepared;

(i) PC ; Control platelet concentrate,

(ii) PC+PGE_1:1 ; Platelet concentrate including 60nM PGE_1,

(iii) PC+PGE_1:2 ; Platelet concentate including 60nM PGE_1 and additional 15nM PGE_1 every 24 hours,

(iv) PC+PGE_1:3 ; Platelet concentrate including 60nM PGE_1 and additional 30nM PGE_1 every 48 hours.

Samples were taken after 2 hours, 1, 3, 4, 5, 6, 7 and 8 days from collection and tested for platelet number and response to ADP/ Epinephrine .

Results

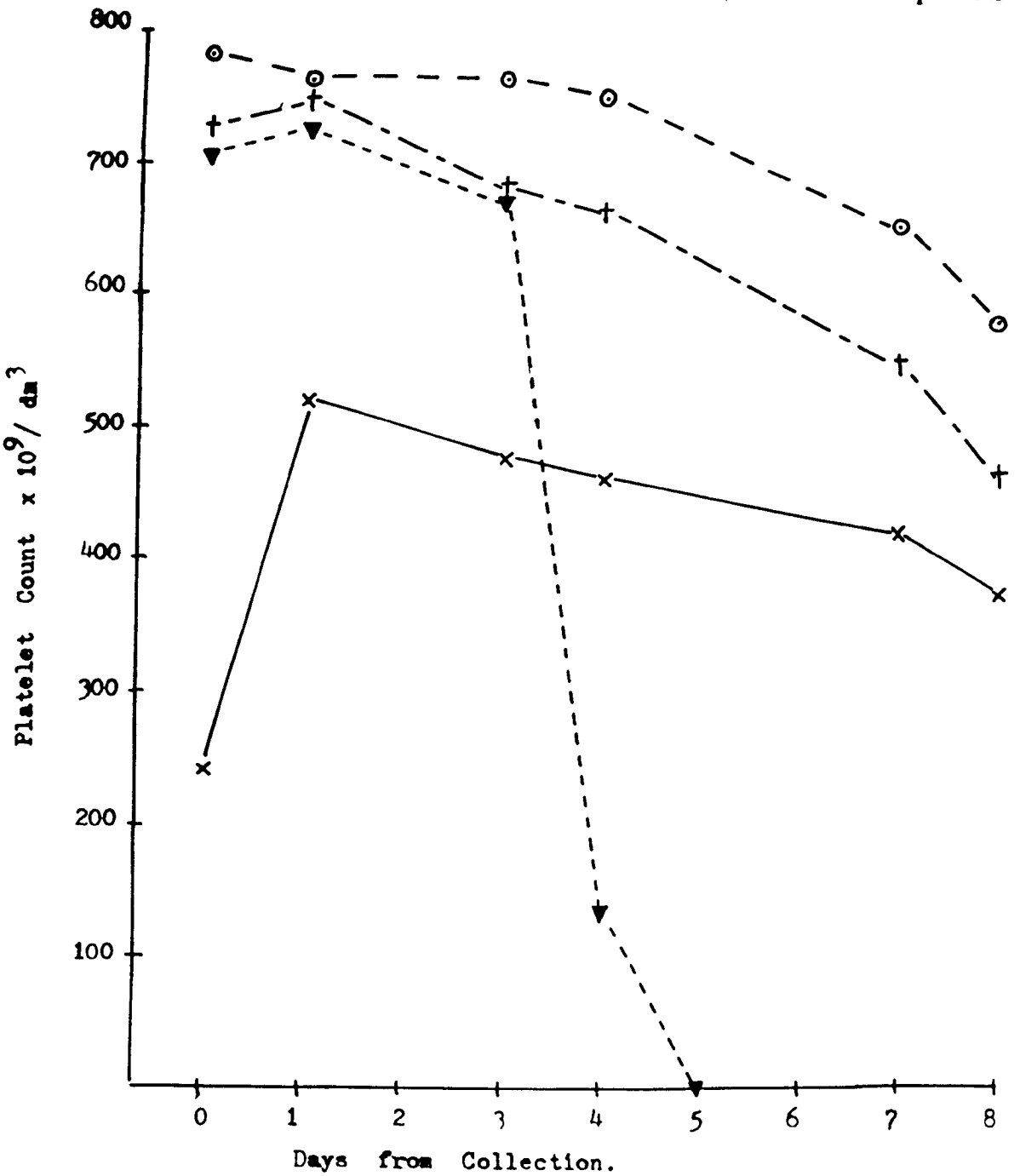
DAY	PC	PC+PGE_1:1	PC+PGE_1:2	PC+PGE_1:3
0	239	784	722	736
1	515	770	729	761
3	480	771	660	680
4	465	749	139	672
7	414	644	0	539
8	372	583	0	475

Table 2.24 ; Platelet count (x $10^9/dm^3$) ; Experiment 2.3.5.

DAY	PC	PC+PGE_1:1	PC+PGE_1:2	PC+PGE_1:3
0	0	0.200	0.189	0.178
1	0.037	0.120	0.089	0.114
3	0.035	0.072	0.069	0.060
4	0.030	0.060	0	0.045
5	0.029	0.082	0	0.052
6	0.025	0.077	0	0.069
7	0.016	0.073	0	0.060
8	0.010	0.055	0	0.048

Table 2.25 ; Platelet aggregation in presence of 5μM ADP and 2.5μM Epinephrine (ΔOD); Experiment 2.3.5.

FIGURE 2.20 : Platelet Count : Expt. 2.3.5



KEY: PC = ×——×
PC+PGE_1:1 = ⊙— —⊙
PC+PGE_1:2 = ▼- - - - -▼
PC+PGE_1:3 = †— - —†

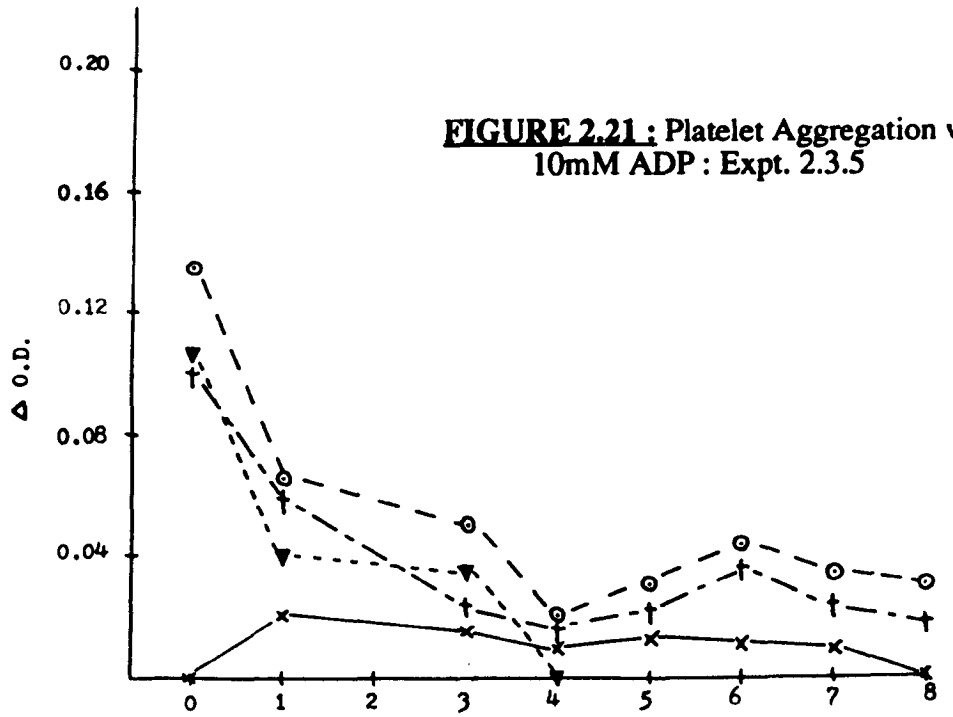


FIGURE 2.21 : Platelet Aggregation with 10mM ADP : Expt. 2.3.5

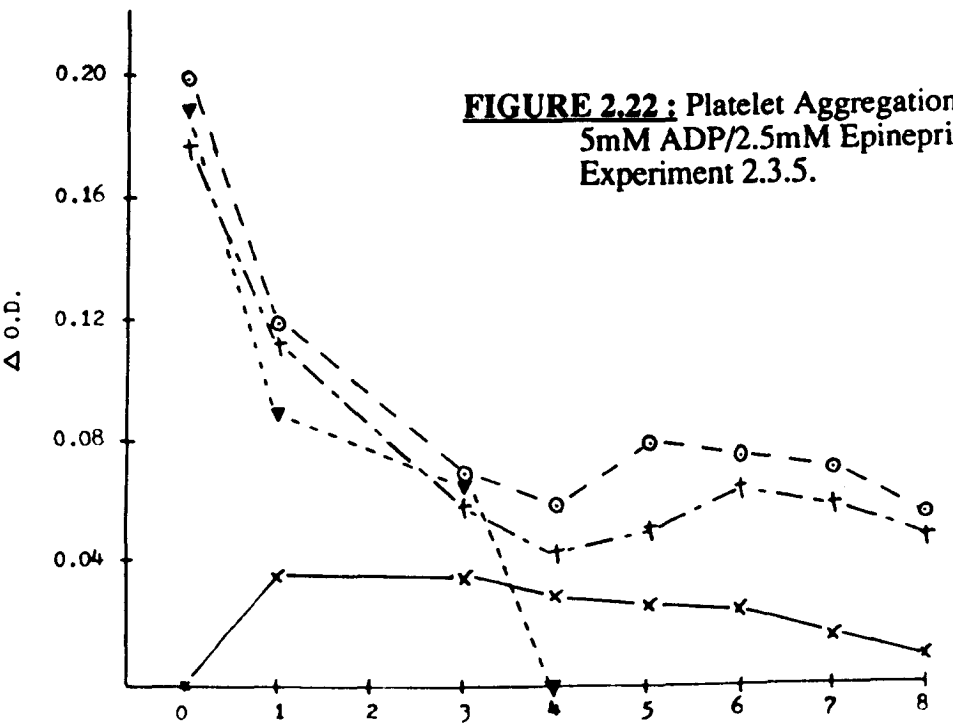


FIGURE 2.22 : Platelet Aggregation with 5mM ADP/2.5mM Epineprine: Experiment 2.3.5.

KEY: PC
$PC+PGE_1$:1
$PC+PGE_1$:2
$PC+PGE_1$:3

DAY	PC	PC+PGE_1:1	PC+PGE_1:2	PC+PGE_1:3
0	0	0.135	0.100	0.108
1	0.022	0.064	0.061	0.040
3	0.015	0.048	0.035	0.025
4	0.009	0.020	0	0.015
5	0.014	0.030	0	0.024
6	0.010	0.043	0	0.038
7	0.009	0.035	0	0.023
8	0	0.030	0	0.018

Table 2.26 : Platelet aggregation in presence of 10μM ADP (ΔOD)

These results are expressed graphically in figures 2.20-2.22.

Once again the untreated PC sample showed poor initial resuspension, but was able to recover to approximately 70% of platelet numbers in the PC+PGE_1 sample by 24 hours. Resuspension in all three PGE_1 preparations is very similar. The rapid decrease in viable platelet count in sample PC+PGE_1:2 was probably due to bacterial contamination of the plasma caused by the repeated PGE_1 infusions and sample taking.

Until this event, all three PGE_1-treated samples showed almost identical aggregation profiles, indicating that increasing the PGE_1 level after the initial preparative steps has no beneficial effect.

2.3.6. : A comparison of CPDA-1 and CPD anticoagulants.

Menitove *et al*[68-70] used CPD anticoagulant in the preparation of their PCs. The tests described here have all employed CPDA-1 anticoagulant which differs only in the presence of 277mg/dm^3 adenine hydrochloride. Despite this seemingly small change in conditions, the PCs prepared with the latter anticoagulant have shown vastly improved functionality

compared to that observed by Menitove's group. Therefore it was decided to compare platelets concentrated in the presence of CPD with CPDA-1 concentrated platelets.

Experiment 8.

The following platelet concentrate samples were prepared;

(i) PC ; Control platelet concentrate (CPD),

(ii) PC+PGE_1 ; Platelet concentrate including 60nM PGE_1 (CPD)

(iii) PC+A ; Platelet concentrate including 277mg/dm^3 Adenine hydrochloride (CPDA-1),

(iv) PC+PGE_1+A ; Platelet concentrate including 60nM PGE_1 and 277mg/dm^3 Adenine hydrochloride (CPDA-1).

Samples were taken after 2 hours, 1, 2, 3, 4, 5 and 8 days and tested for pH, platelet number and functionality.

Results

The pH of all four samples was 7.60-7.65 at t=2 hours, showing that addition of adenine-HCl had no detrimental effect.

DAY	PC	PC+PGE_1	PC+A	PC+PGE_1+A
0	310	1152	1098	985*
1	658	1095	1112	950
3	738	1053	1030	895
5	702	990	975	910
8	582	927	940	866

Table 2.27 ; Platelet count (x 10^9/dm^3) ; Experiment 2.3.6.

* Sample PC+PGE_1+A contained only 85% of the PRP of the other concentrates, hence the intial platelet count is lower.

DAY	PC	PC+PGE_1	PC+A	PC+PGE_1+A
0	0.008	0.250	0.240	0.180
1	0.035	0.195	0.185	0.160
2	0.065	0.235	0.180	0.175
3	0.050	0.195	0.155	0.140
4	0.044	0.170	0.127	0.124
5	0.035	0.160	0.095	0.120
8	0.005	0.160	0.007	0.018

Table 2.28 : Platelet aggregation in presence of 5μM ADP and 2.5μM Epinephrine (ΔOD): Experiment 2.3.6.

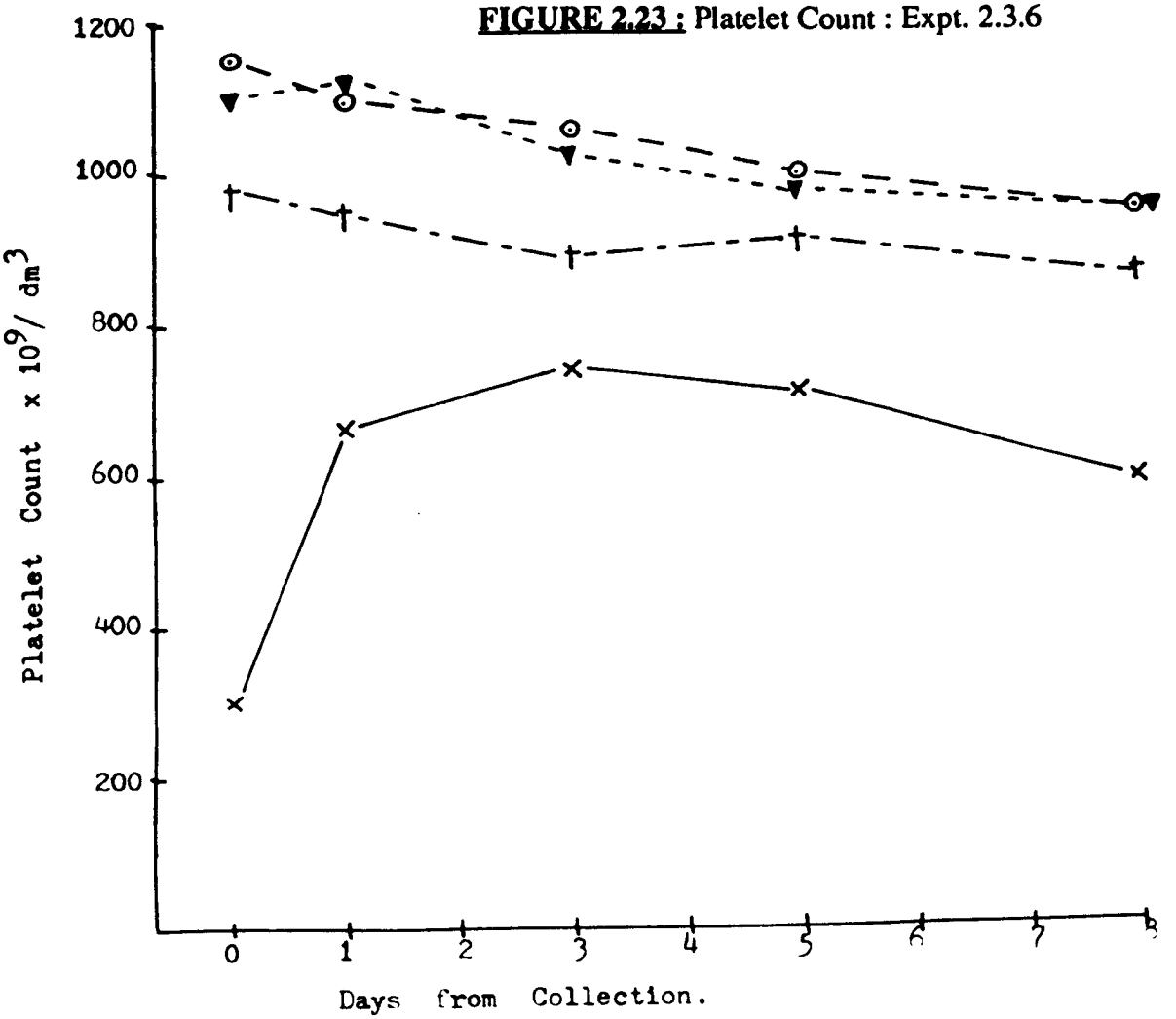
DAY	PC	PC+PGE_1	PC+A	PC+PGE_1+A
0	0	0.125	0.195	0.095
1	0.012	0.090	0.135	0.075
2	0.020	0.115	0.120	0.095
3	0.018	0.090	0.080	0.065
4	0.022	0.082	0.065	0.070
5	0.016	0.078	0.050	0.065
8	0	0.075	0.004	0.015

Table 2.29 : Platelet aggregation in presence of 10μM ADP (ΔOD).

These results are expressed graphically in figures 2.23-2.25.

Surprisingly, the PC+A sample gave perfect resuspension, in contrast to the untreated PC control, which showed relatively poor recovery. The PC+A sample exhibited the steady decrease in functionality as previously seen with good quality PCs [experiments 2.3.1(a) and 2.3.1(c)].

FIGURE 2.23 : Platelet Count : Expt. 2.3.6



KEY: PC = X——X
PC+PGE_1 = ⊙— —⊙
PC+A = ▼- - - -▼
PC+PGE_1+A = †— · —†

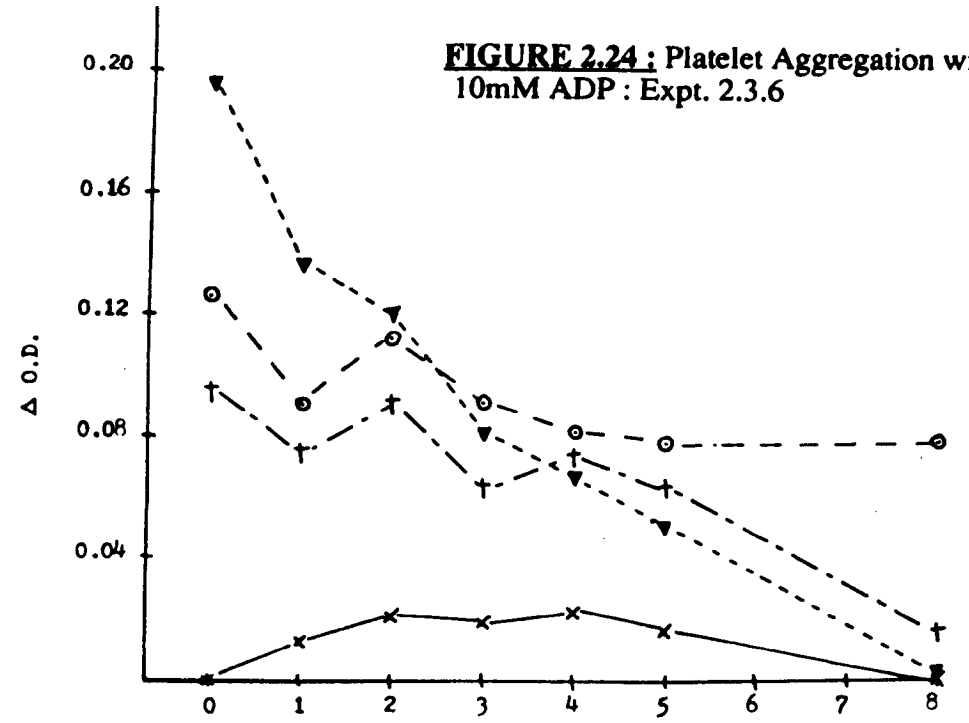


FIGURE 2.24 : Platelet Aggregation with 10mM ADP : Expt. 2.3.6

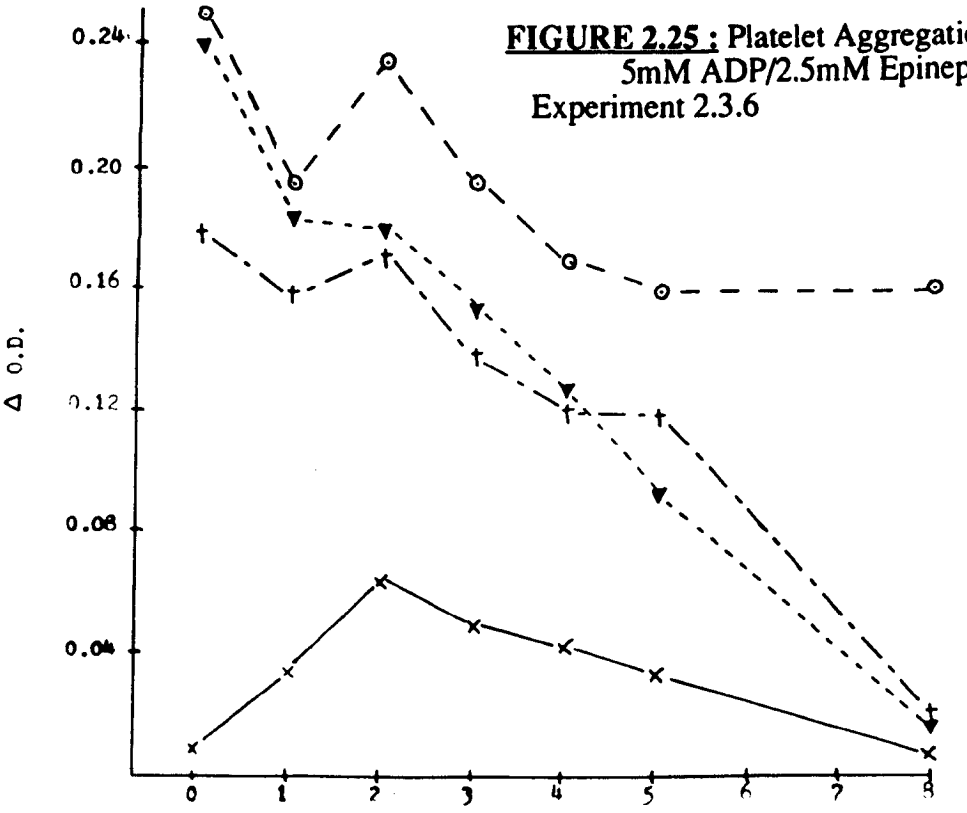


FIGURE 2.25 : Platelet Aggregation with 5mM ADP/2.5mM Epinephrine: Experiment 2.3.6

Days from collection.

KEY: PC
PC+PGE_1
PC+A
PC+PGE_1+A

Once more, evidence for initial inhibition of aggregation was seen in the PC+PGE_1 and PC+PGE_1+A samples.

The apparent protective efect of adenine during PC preparation may be due to competition between adenine and ADP for the platelet's ADP receptor. Plasma ADP could originate from the platelets themselves, or from erythrocytes during the preparation of PRP.

2.4 General Conclusions.

In the absence of platelet protecting agents, three types of platelet resuspension were observed;

(a) Good resuspension: Observed in experiments 1, 3 and 8 (CPDA), in which full resuspension was achieved within 2 hours.

(b) Fair resuspension: Observed in experiments 2, 4, 6, 7 and 8 (CPD), in which complete or satisfactory resuspension was achieved by 24 hours.

(c) Poor resuspension: Observed only in experiment 5, where satisactory resuspension did not occur.

The possible reasons for this variability in resuspension is discussed later. However, all 17 samples prepared in the presence PGE_1 showed maximal resuspension within 2 hours, including the samples in which a second preserving agent (verapamil, nifedipine, insulin or hirudin) was present.

Interestingly, some paired reagents gave superior resuspension to PGE_1 alone, suggesting that PGE_1 is not able to fully dissociate platelet aggregates on its own. This could indicate the presence of a population of platelets particularly resistant to disaggregation, possibly bound to contaminant erythrocytes in the PC.

Unlike PCs, in which the pH remains fairly constant, autologous PPP, used for dilutions prior to aggregometry, shows a steady rise in pH. The reason for this is unclear, but certainly the presence of platelets prevents this effect. This buffering capacity of platelets may be due to the presence of the platelet Na^+/H^+ antiport[71]. On activation, platelets

exchange cytosolic H^+ for plasma Na^+, raising internal pH and aiding cytosolic Ca^{2+} mobilisation. Thus, the absence of a significant rise in pH of stirred PCs may be due to an extrusion of H^+ from the platelets themselves. Conversely, a small rise in plasma pH may induce the activation of the Na^+/H^+ antiport, forcing protons out of the platelet. In this way, the compensation for rises in plasma pH may provide a mechanism or the storage lesion of PCs.

The inconsistency in quality of PCs could be due to antigenic interactions between pooled platelets from different donors. Although all donors were ABO-matched, all blood cells possess many other antigenic determinants.

Another possibility is variations in the time between venupuncture and PC preparation. Owing to logistical dificulties in transportation of blood from collection centres to the Blood Bank for processing, it was not possible to ensure a constant duration of time pre-concentration. Very fresh whole blood samples will contain endogenous prostacyclin, which may give protection to platelets during PRP and PC preparation. Prostacyclin is rapidly degraded to inactive 6-keto-$PGF_{1\alpha}$[21] in plasma, and so PCs prepared slightly later may be more difficult to resuspend.

Similarly, the inhibition of aggregation by 60nM PGE_1 disappears within 48 hours, or so, of PC preparation, as shown by the "recovery" of aggregation potential after 2-3 days in PGE_1-treated PCs. Whether this is due to degradation of PGE_1, or down-regulation of platelet PGE_1 receptors is unclear. Evidence for the latter hypothesis is given by the observation that increasing the concentration of PGE_1 after the initial resuspension (experiment 7) had no effect on either inhibition of aggregation or prolongation of shelf-life.

There appear to be two quite distinct problems in the concentration of platelets for transfusion, the "preparative lesion" and the "storage lesion". The preparative lesion occurs during the pelleting of platelets from PRP to give the platelet concentrate itself, and is almost completely abolished by the presence of PGE_1. This lesion is characterised by massive β-TG release, indicative of platelet activation and the release reaction, and may be due to small amounts of plasma ADP and other agonists, platelet-bound thrombin, or platelet-surface antigenic interactions between platelets from different donors.

The storage lesion, which is characterised by a small but steady decrease in platelet number and a loss of platelet activation potential, seems to be due to a combination of fluctuations in plasma pH, thrombin generation and depletion of glycogen stores.

A third factor which must be taken into account is the "experimental lesion", which arises due to the repeated sampling of PCs and removal of up to 5ml per day for testing. Not only does this increase the risk off bacterial contamination, but it steadily decreases the volume of plasma, relative to the volume of the container, increasing the likelihood of potentially damaging interactions between platelets and the container.

Several authors have shown that platelets transfused into patients recover functionality to some degree[59,69,70]. The reason or this is uncertain, but it seems likely that platelets replenish spent metabolites and granule contents from the recipient's plasma. For example, the presence of insulin may trigger the platelets to synthesise more glycogen. Menitove *et al*[68-70] reported that PGE_1 reduced post-transfusion recovery of platelets in an experimental model, possibly due to a transient lowering of the platelet's threshold for activation following transfusion. Owing to these results, this group argued against the use of PGE_1 as a platelet preservative[70]. However this group only report PCs showing good resuspension and claim a complete loss of responsiveness to 10μM ADP within 4 days, even in the presence of PGE_1, and no significant difference in response to ADP/epinephrine between PGE_1-treated and untreated samples[68]. These results contradict the results reported here.

The general quality of PCs prepared by existing techniques is not really satisfactory. Many preparations never reach full resuspension of platelets, and even good preparations only have a shelf-life of 5 days maximum. With resuspension difficulties, this leaves a useful life-time of as little as 2-3 days when they are suitable for transfusion.

Addition of a suitable platelet antagonist to the anticoagulant would result in full, immeadiate resuspension. Hence, PCs could be used within hours of preparation, and have an extended shelf-life, with improved aggregation response for as long as 7 days in the case of 60nM PGE_1. This period may be extended still further in the absence of the "experimental lesion". Thus, the useful life-time of a PC is more than doubled, and the waste of the few PCs

that never reach satisfactory resuspension is eliminated. Evidently, the savings that this would entail must be weighed against the cost of adding preservatives to PCs.

Using prices obtained from the "Sigma" catalogue, the reagents used in the study would cost as little as;

PGE_1, 60nM = 13p/PC,

Verapamil, 200μM = 16.5p/PC,

Insulin, 100iu = 31p/PC,

Hirudin, 5 units = 35p/PC.

All the preservatives used are regularly prescribed drugs and show no deleterious effects *in vivo* at the levels used.

Thus, further studies on the post-transfusion recovery of platelets treated with PGE_1 (or PGE_1 plus a second platelet preservative, eg. Insulin), similar to those undertaken by Menitove *et al*[68-70] should be undertaken to prove that PGE_1-treated PCs are at least as good as untreated PCs in clinical trials. If this proves to be the case, then the routine preparation of PCs in the presence of PGE_1 could be of major financial benefit to the blood-transfusion service.

THE THIOBARBITURIC ACID ASSAY FOR MALONDIALDEHYDE IN BLOOD SERUM

3.1 Background

Lipid peroxidation plays an important role in the biosynthesis of TxA_2 in platelets (figure 3.1). Several lipid peroxides and hydroperoxides are formed as intermediates in this pathway. Overproduction of these may play a part in the onset of diabetic microangiopathy since secondary lipid peroxidation can cause damage to cell membranes and their associated enzymes and structural proteins[73].

A by-product of prostanoid biosynthesis is malondialdehyde (MDA)[75]. Hence, there is a permanent basal level of MDA in the blood, owing to the continuous low-level synthesis of prostacyclin and TxA_2. However, during a thrombotic episode involving *in vivo* platelet activation, the levels of TxA_2, and hence MDA, would be expected to rise transiently but dramatically.

Several authors have reported increased TxA_2 formation by diabetic platelets or an increased circulating level of TxA_2[45,46]. However, other groups report a decrease in TxA_2 by diabetic platelets[45]. Even so, the tendency towards thrombosis is increased because anti-aggregatory PGI_2 synthesis is supressed, in diabetes, to a greater degree than TxA_2 synthesis[45].

Radioimmunoassay kits for the detection of TxB_2 (the more stable degradation product of TxA_2) are available. However, the cost of this technique was prohibitive for a survey of this size.

Yagi[72-74] has described a simple, sensitive assay, capable of detecting nanomolar levels of MDA in serum. This technique was used to compare serum MDA levels of diabetics with and without microangiopathy, and non-diabetic controls.

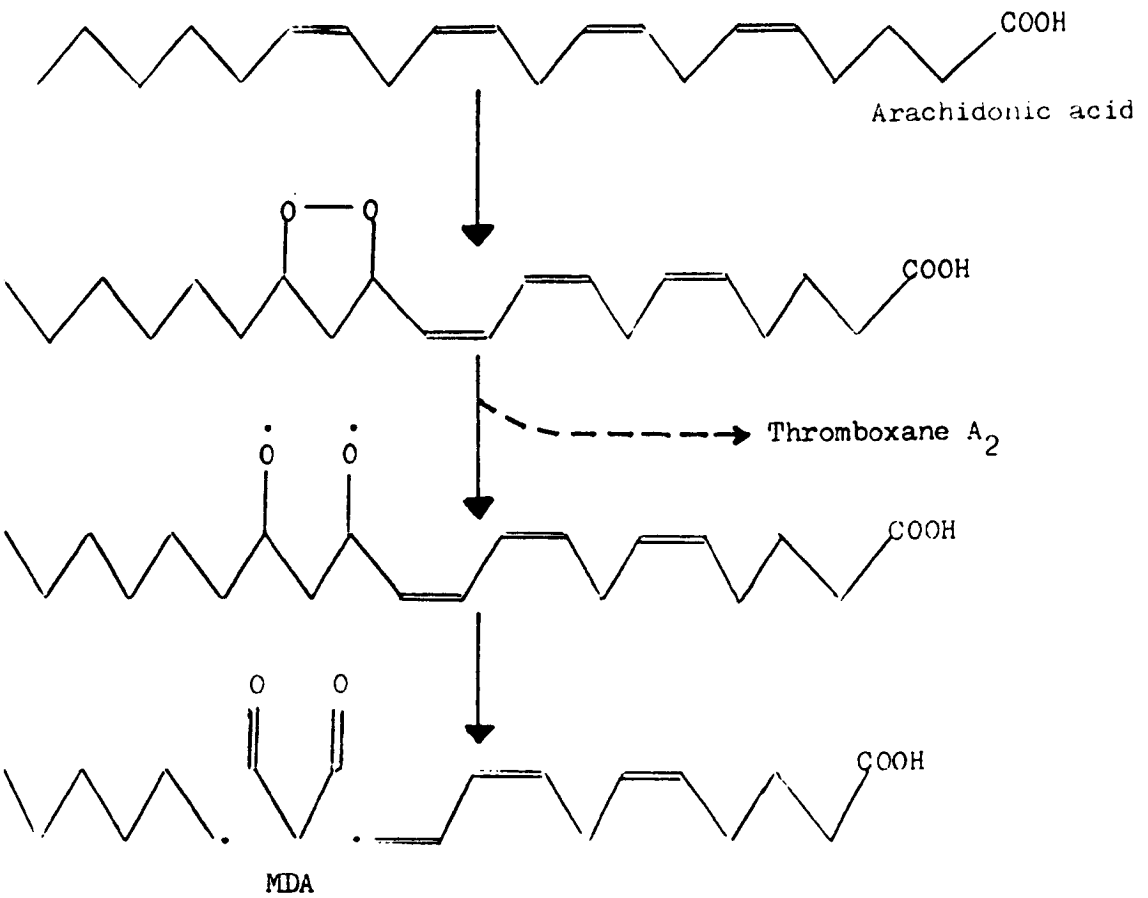


FIGURE 3.1 : Peroxidation of Arachidonic acid and proposed mechanism of MDA formation

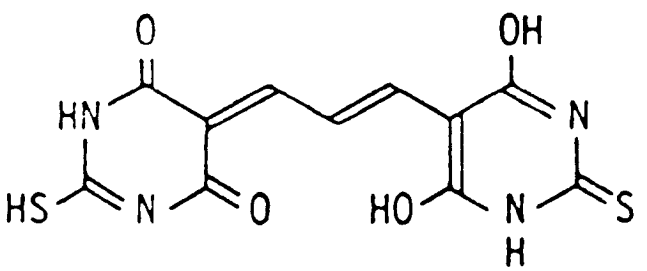


FIGURE 3.2 : Structure of the product of the reaction of MDA with TBA.

3.2.1.: The Assay

MDA and certain lipid peroxides react with thiobarbituric acid (TBA) to give a red pigment[75] (figure 3.2). When excited by light of wavelength 515nm, this adduct emits light at 553nm. Hence a simple fluorimetric assay for MDA is possible.

Since other serum components are known to react with TBA[74,76], serum lipids and proteins are isolated by phosphotungstic acid/sulphuric acid precipitation prior to exposure to TBA. In this way, water-soluble, TBA-reacting substances are eliminated from the assay.

Optimum coloured adduct formation is obtained by incubating samples with TBA at pH 3.5, 95^{o}C for one hour. The coloured pigment is extracted with n-butanol, and its fluorescence intensity compared to that of a tetramethoxypropane (TMP) standard, which is quantatitively converted to MDA during the assay[74].

3.2.2.: Reagents

N/12 Sulphuric acid.

10% Phosphotungstic acid solution (w/v).

TBA reagent; Freshly prepared mixture of equal volumes of glacial acetic acid and 0.67% TBA solution (w/v).

n-Butanol.

Stock TMP solution (0.5 nmol/ml).

3.2.3.: Method

(a) 1.5ml of venous blood was collected into an Eppendorf tube. Serum was obtained, after clotting, by spinning for 10 minutes in a MSE microcentaur bench centrifuge.

(b) Duplicate 20µl serum samples were taken and put into separate 12.5ml polypropylene centrifuge tubes. 4ml of N/12 sulphuric acid was added to each tube and the mixture was gently mixed by inversion.

(c) To this mixture was added 0.5ml of 10% phosphotungstic acid. After mixing, the tubes were centrifuged at 3000rpm for 10 minutes at room temperature in a Sorvall GSA rotor.

(d) The supernatant was discarded and the precipitate resuspended in 2ml of N/12 sulphuric acid and 0.3ml of 10% phosphotungstic acid. After mixing, a second 10 minute, 3000rpm spin was performed.

(e) The second sediment was taken up in 4ml of distilled water and 1.0ml of TBA reagent was added. The tube contents were mixed and heated at 95^{o}C for 60 minutes in a water/steam bath.

(f) After cooling on ice, 5.0ml of n-butanol was added, the mixture was shaken vigorously, and then centrifuged at 3000rpm for 15 minutes in the Sorvall GSA rotor.

(g) The alcohol layer (upper) was removed and taken for fluorimetric analysis on the Perkin-Elmer LS5 fluorescence spectrophotometer, at 515nm excitation and 553nm emission.

The blank used was n-butanol, and a MDA standard was obtained by reacting 0.5nmoles of TMP through steps (e)-(g).

Results and Calculations[74]

F = fluorescence intensity of standard,

f = fluorescence intensity of sample,

Serum [MDA] = 0.5 x f/F x 1.0/0.02 = f/F x 25 nmoles/ml serum.

Eighteen assays, carried out simultaneously on a single 1.5ml blood sample, showed that the assay was consistent to within 5% reproducability (s.d.).

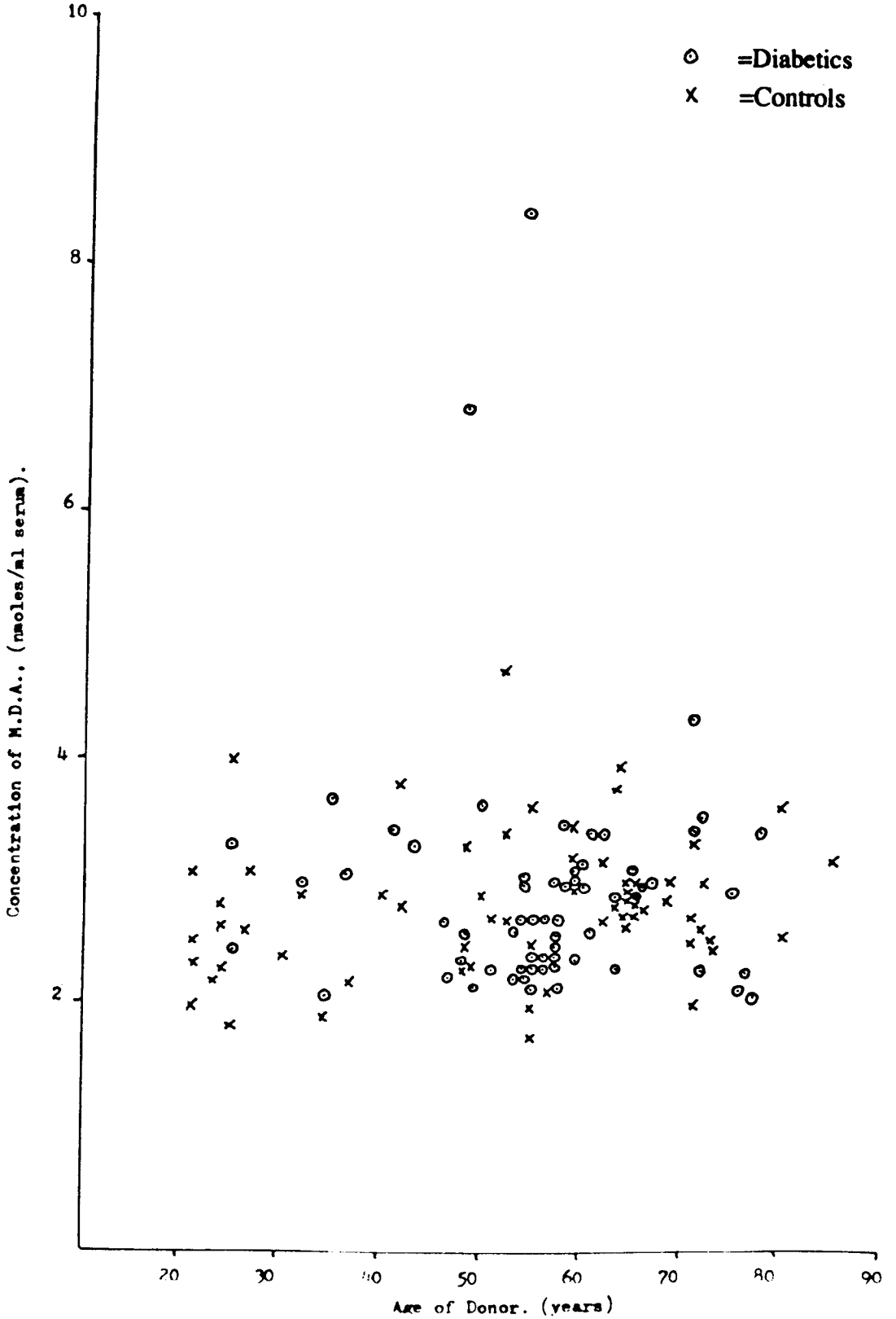


FIGURE 3.3 : Correlation of serum MDA with age of donor.

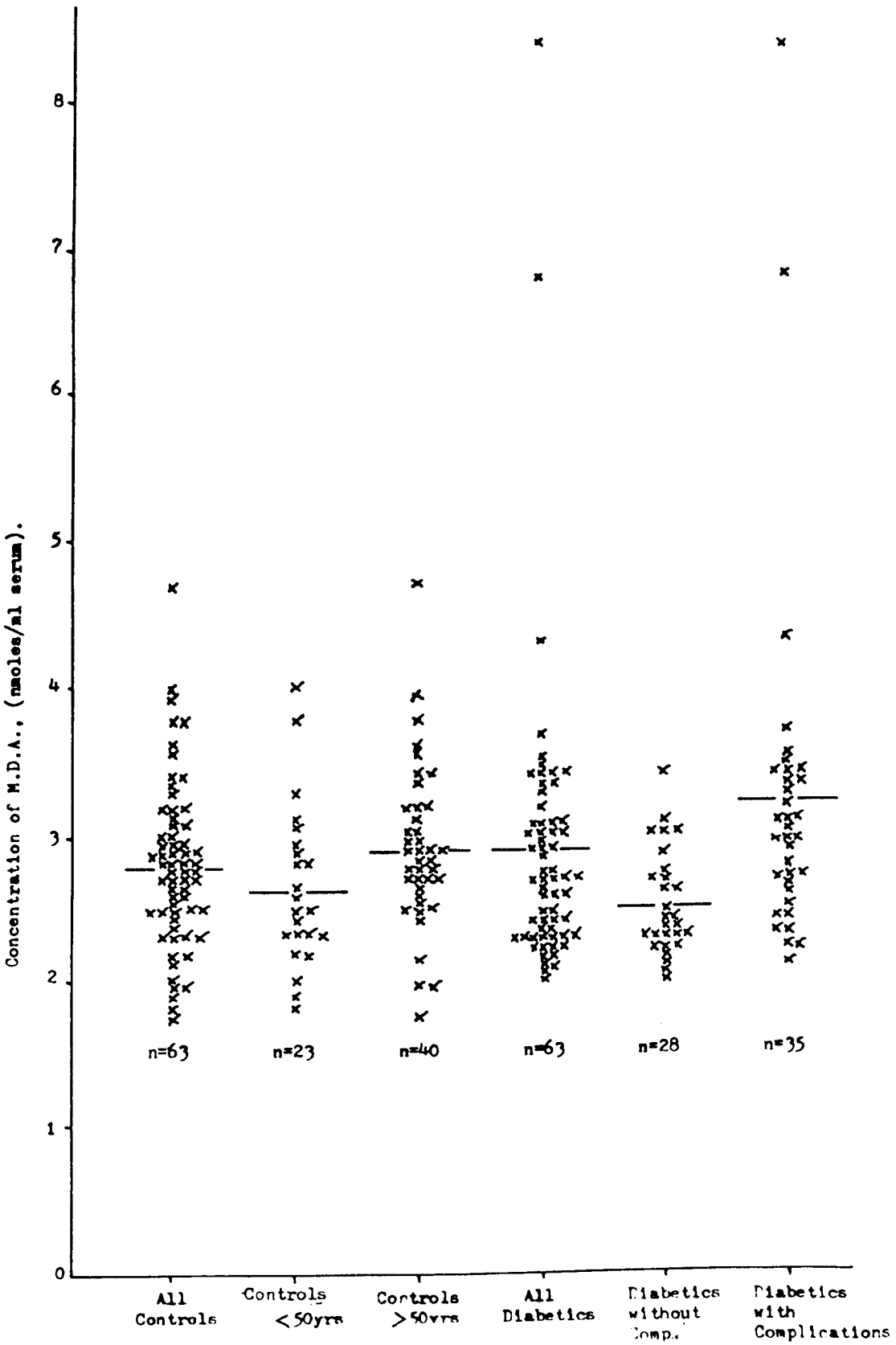


FIGURE 3.4 : Serum MDA levels in diabetics and controls

GROUP	AGE	SERUM [MDA] nmoles/ml	NUMBER OF SAMPLES ASSAYED
All non-diabetic controls	52.25+/-18.01	2.81+/-0.56	63
Controls < 50 years	31.78+/-10.08	2.64+/-0.55	23
Controls > 50 years	64.02+/-8.32	2.91+/-0.55	40
All diabetics	55.68+/-12.28	2.89+/-0.99	63
Diabetics, no complications	55.28+/-11.35	2.51+/-0.36	28
Diabetics with complications	56.00+/-13.29	3.19+/-1.21	35

Table 3.1 : Serum MDA levels of diabetics and controls.(means +/- s.d.)

3.4 : Discussion

Serum was used in the assay, in preference to plasma, in order to measure the capacity of the donor's platelets to produce TxA_2 (and hence MDA) on activation induced by the clotting of whole blood. *Ex vivo* plasma MDA levels would give an indication of *in vivo* TxA_2 production, but could also be due to MDA produced as a by-product of prostacyclin production by vascular endothelial cells.

Non-diabetic donors were selected to give an appropriate age-match with the diabetics. However, figure 3.3 shows that there is no significant correlation between serum MDA concentration and donor age (controls, correlation coefficient = 0.202, diabetics, correlation coefficient = 0.030).

The diabetics in the survey show a similar range of serum MDA concentrations to the controls, with the exception of two patients with severe microangiopathy (see figure 3.4). If these two patients are discounted, the mean MDA concentrations in serum from diabetics

with complications falls to 2.90+/-0.51 nmoles/ml, the same as age-matched controls. Only these two patients show a significant increase in MDA compared to controls ($p > 0.05$).

Thus it appears that, in general, there is no difference in the capacity of diabetics and non-diabetic controls to produce MDA, and hence TxA_2, on clotting.

The two abnormal results require some explanation. Both patients are IDDMs, in their 50s, with extensive retinopathy and mild proteinuria. However, similar patients, and several diabetics with more severe complications did not show the elevated serum MDA levels.

There are four possible mechanisms for the production of elevated MDA levels (see figure 3.5).

(1) Overactive Phospholipase A_2: Resulting in the production of elevated levels of free arachidonate and hence more TxA_2 and MDA.

(2) Overactive cyclooxygenase: Producing more PGG_2 from the available arachidonate.

(3) An increase in non-enzymatic peroxidation, due to elevated levels of oxygen radical species within the platelet.

(4) Defective TxA_2 synthetase, producing less TxA_2, and hence allowing more PGH_2 to be non-enzymatically degraded to MDA.

Mechanisms (1) and (2) would result in elevated TxA_2 as well as MDA, whereas mechanisms (3) and (4) would lead to decreased TxA_2 production, but increased MDA synthesis.

The absence of an elevated serum MDA level in controls and the majority of diabetics tested could be due to the phenomenon being a transient malfunction in platelet metabolism, possibly induced by periods of poor diabetic control. A longtitudinal survey of poorly controlled diabetics, involving multiple blood samplings over a period of months, would give some indication as to the viability of this hypothesis.

Chapter 4

THE EFFECT OF VERAPAMIL ON PLATELET AGGREGATION IN DIABETICS AND NON-DIABETIC CONTROLS.

4.1 ; Background

An increase in the cytoplasmic free Ca^{2+} concentration is an essential component of platelet activation. Resting, unstimulated platelets have a free Ca^{2+} level of less than 0.1μM[17]. Stimulation of platelets by an agonist such as ADP or collagen causes a rapid increase in cytoplasmic calcium due to influx from plasma and release of stored calcium from sites within the platelets[17]. This increase in calcium is directly or indirectly responsible for platelet shape change, the release reaction and aggregation (see section 1.4 for full explanation).

The calcium-channel-blockers (CCBs) are a heterogeneous group of drugs that share the common characteristic of inhibiting the influx of calcium ions across the plasma membrane of excitable cells[78-83]. Thus, CCBs are regularly prescribed for the treatment of angina and hypertension due to their ability to make the heart work more economically and cause vasodilation.

Verapamil, marketed as Isoptin[78], was the prototype which paved the way for all calcium antagonists subsequently introduced, such as nifedipine (Adalat), dilitazem and the dihydropyridines. Many groups have reported that CCBs are able to inhibit platelet aggregation *in vivo* as well as *in vitro* and prevent thrombosis in experimental models[78-83]. Abnormal Ca^{2+}-handling has been proposed as a mechanism for abnormal platelet aggregation in arterial thrombosis[85] and diabetes mellitus patients[52].

In order to test the viability of using CCBs as anti-thrombotic, and hence possibly anti-microangiopathic drugs in diabetes patients, a series of experiments were performed to establish the relative sensitivities of platelets from diabetics and healthy controls to one of the most commonly prescribed CCBs, verapamil.

4.2 ; Materials and Methods

4.2.1 ; Reagents

Adenosine diphosphate reagent (Sigma);

Special platelet aggregation reagent, reconstituted with water to give a buffered 0.2mM solution of ADP.

Verapamil hydrochloride (Sigma);

18mg/ml in saline (36.6mM).

Calcium ionophore, A23187: calcium salt (Sigma);

0.1mg/ml in saline (0.2mM).

4.2.2. : Methods

PRP was prepared from citrated whole blood as described in appendix 1.

Initial experiments involved investigating the effect of an increasing dose of A23187 or verapamil on platelet aggregation in the Platelet Aggregometer at the Coventry and Warwickshire Hospital. This was achieved by adding increasing doses of the stock reagents to stirred 1ml PRP samples at 37°C in the presence of 2μM ADP. (see figure 4.1).

In order to establish whether there was any difference in the response to verapamil in diabetics compared to non-diabetic cotrols, the following experiments were performed.

The degree of aggregation induced by 2μM and 10μM ADP was established, 10μM being sufficient to cause full aggregation in most donors. This data was expressed as "2/10 ADP" aggregation ratio where;

$$2/10\ ADP = \frac{\Delta OD \text{ in presence of } 2\mu M\ ADP}{\Delta OD \text{ in presence of } 10\mu M\ ADP} \times 100\%$$

The ΔOD in the presence of various doses of verapamil was established, and the data processed to give two indications of the inhibitory potential of verapamil; AD_{50}, the dose of verapamil required to give a 50% reduction in 2/10 ADP ratio, and AD_{100}, the abolition dose, at which verapamil completely prevented platelet aggregation in the presence of 2μM ADP (see figure 4.2).

4.3 : Results

2/10 ADP ratio, AD_{50} and AD_{100} are tabulated together with aggregation type (see appendix 1), age and clinical details in tables 4.1 and 4.2.

The reproducibility of the technique was tested by assessing the platelet responses of two young non-diabetic controls at 7 points over a 12 week period (table 4.1 and figure 4.3).

The mean responses to ADP and verapamil of the diabetic platelets were calculated and compared with an age-matched control population (ie. without the younger, multiply-assayed donors). These results are summarised in table 4.3.

DONOR	AGE	PLATELET NUMBER ($x10^9/dm^3$)	AGGREGATION TYPE WITH 2µM ADP	2/10 ADP RATIO(%)	AD_{50} g/ml Verap.	AD_{100}
AJS	22	335	Reversible	39	n/a	205
AJS	22	316	Reversible	52	15	275
AJS	22	340	Reversible	38	n/a	255
AJS	22	379	Reversible	44	n/a	280
AJS	22	321	Reversible	42	n/a	195
AJS	22	276	Reversible	45	n/a	220
AJS	22	299	Reversible	37	n/a	175
MEAN	**22**	**323.7 +/- 32.7**	**Reversible**	**42.4 +/- 5.2**	**n/a**	**229.3 +/- 41.1**
GMW	24	499	Reversible	53	70	430
GMW	24	503	Reversible	57	135	440
GMW	24	489	Reversible	57	90	400
GMW	24	448	Reversible	55	35	350
GMW	25	443	Reversible	55	50	335
GMW	25	397	Reversible	53	15	290
GMW	25	385	Reversible	60	95	350
MEAN	**24.5**	**452.0 +/- 47.9**	**Reversible**	**55.7 +/- 2.5**	**70.0 +/- 40.6**	**370.7 +/- 54.5**
RJB	22	435	Reversible	42	n/a	360
RJB	22	420	Biphasic	79	205	470
RJB	22	476	Biphasic	68	130	410
MEAN	**22**	**443.7 +/- 29.0**	**Rev./Biph.**	**63.0 +/- 19.0**	**111.7 +/- 103.7**	**413.3 +/- 55.0**
BEPS	48	392	Biphasic	73	120	375
JF	60	315	Reversible	84	240	465
DF	56	323	Irreversible	85	240	570
DF	56	519	Irreversible	81	242	410
KA	31	341	Reversible	29	n/a	320
CC	68	236	Irreversible	85	310	830
VC	63	469	Biphasic	86	245	640
ALT	26	399	Biphasic	68	70	265

Table 4.1: The effects of Verapamil on Platelet Aggregation in Non-diabetic Controls.

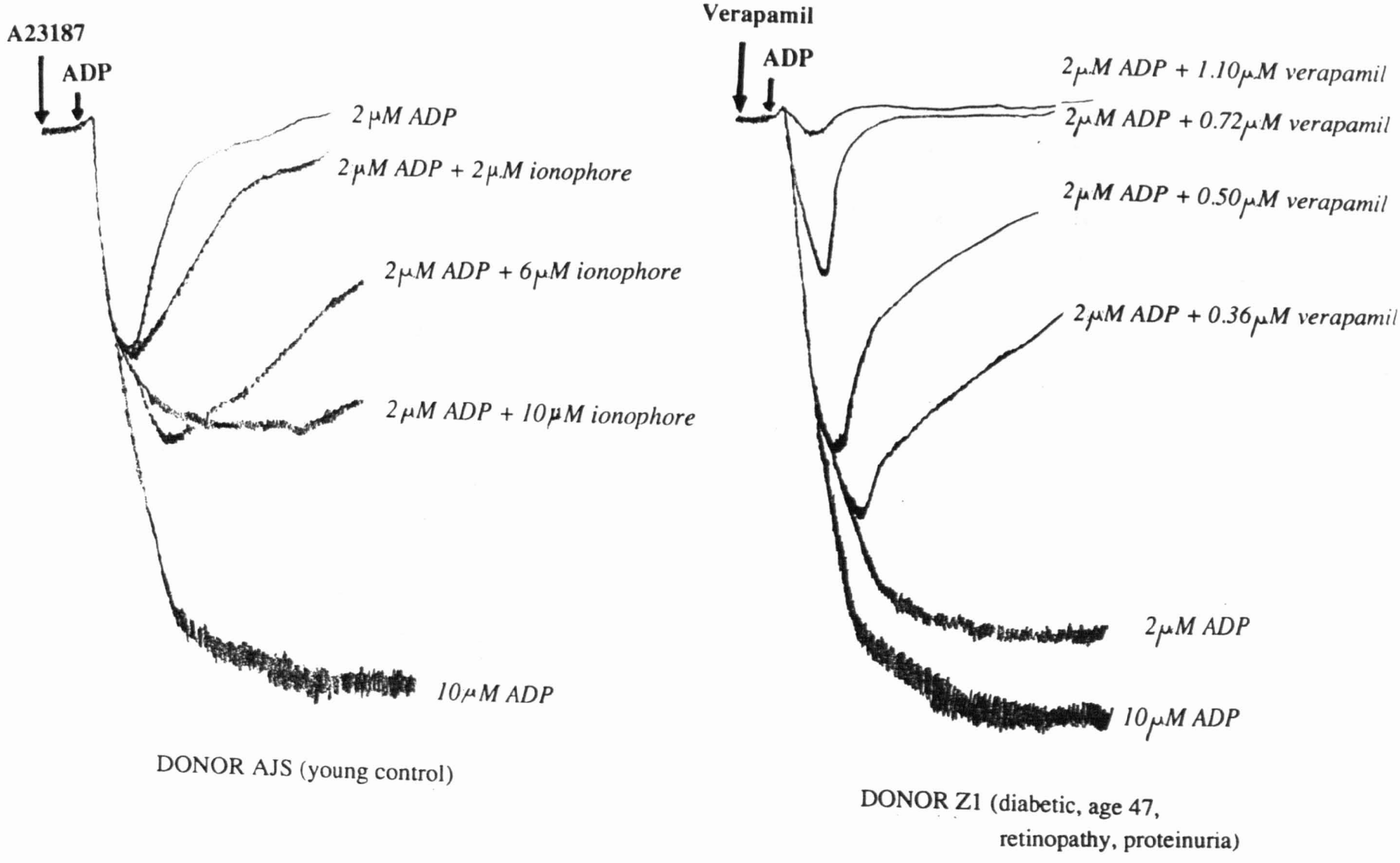
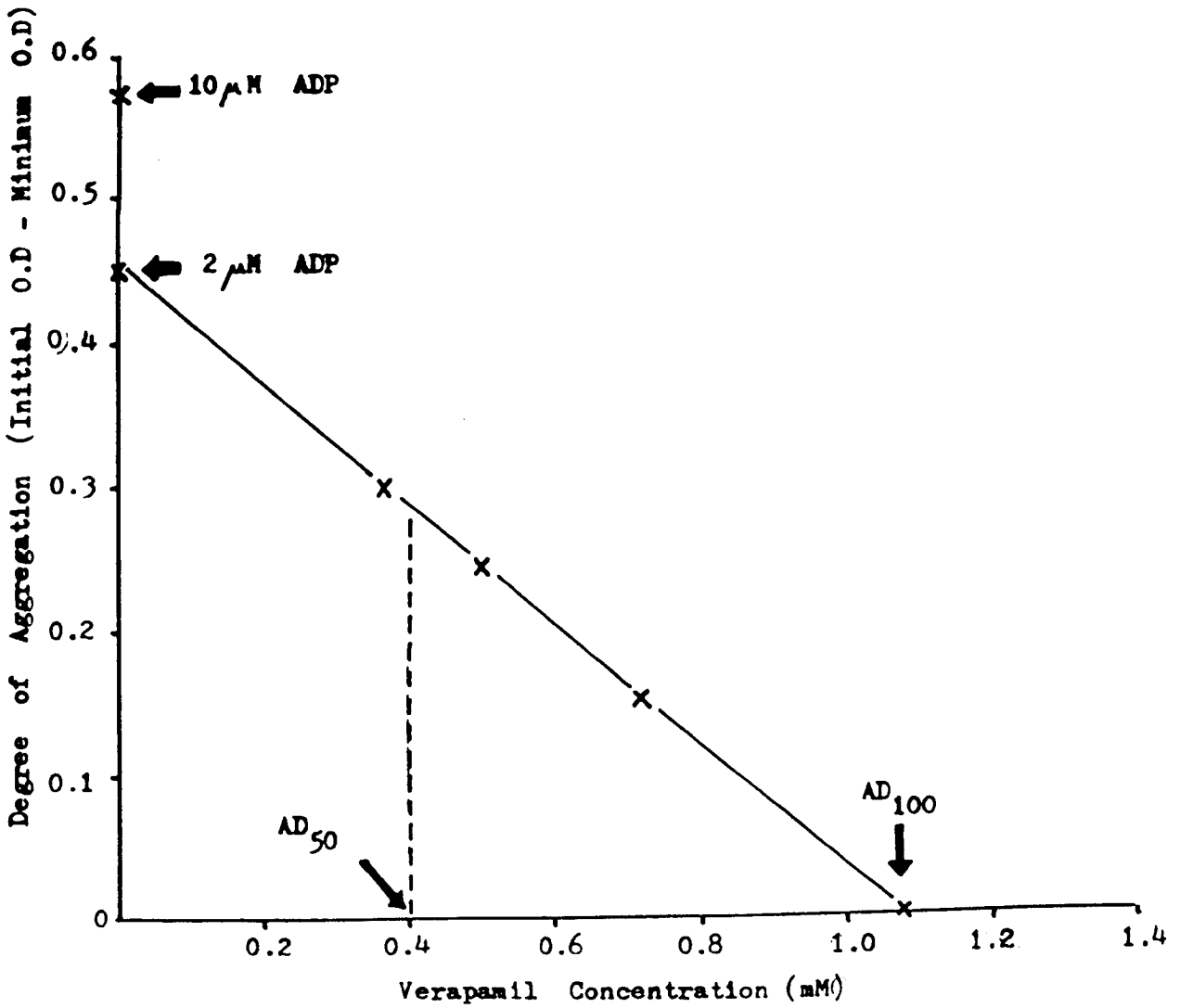


FIGURE 4.1 : The effects of A23187 and Verapamil on ADP-induced platelet aggregometry.



. **FIGURE 4.2 :** Verapamil Titration,
results and calculations

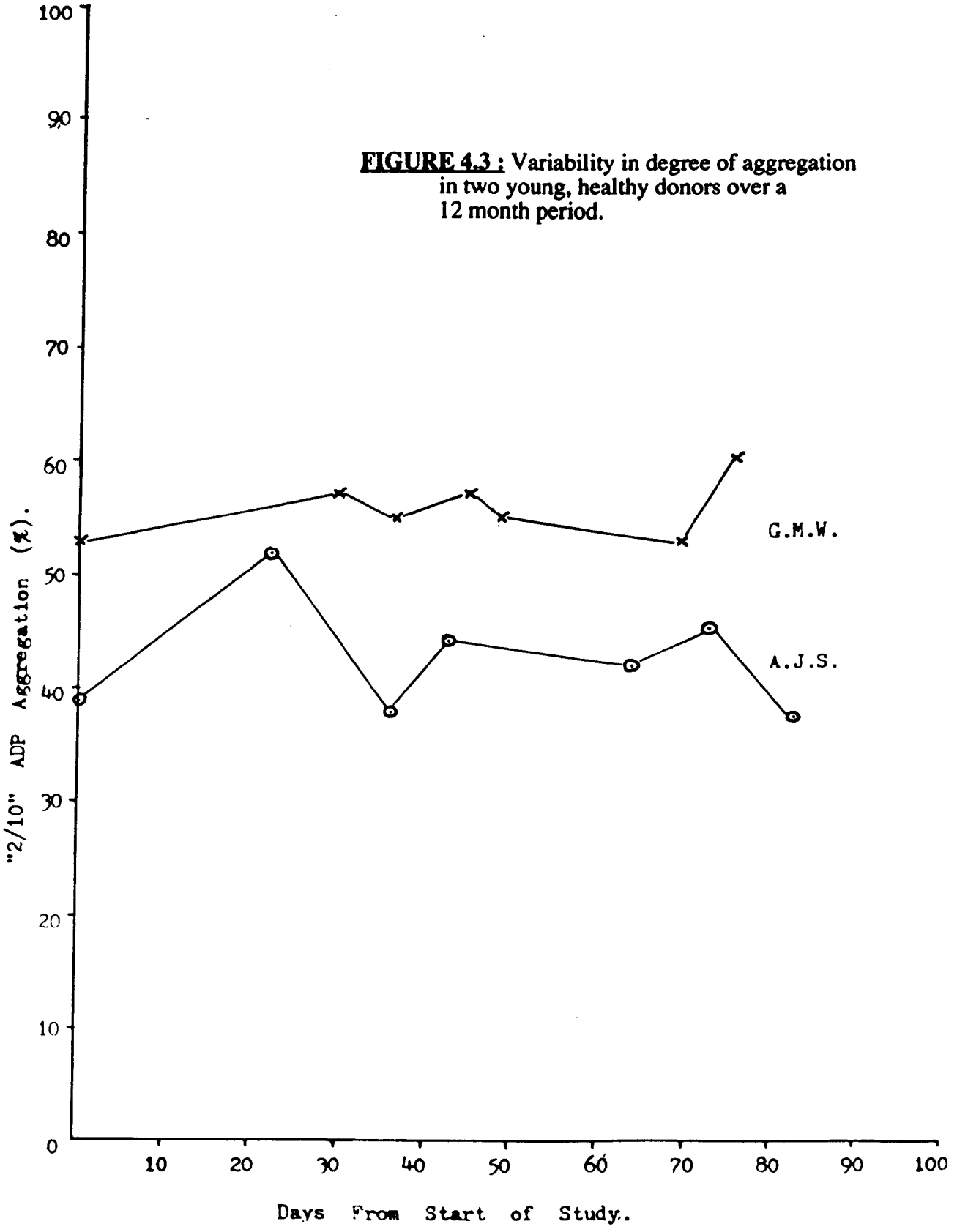


FIGURE 4.3 : Variability in degree of aggregation in two young, healthy donors over a 12 month period.

DONOR	AGE	TYPE	PLATELET NUMBER ($x10^9/l$)	AGGREG. TYPE	2/10 ADP RATIO(%)	AD_{50} g/ml Verap.	AD_{100}
A41	61	I+++	395	Irrev.	96	290	610
A41	61	I+++	369	Irrev.	90	275	585
A42	46	N+++	427	Irrev.	82	210	500
A43	71	I++	260	Biph.	60	125	470
A44	23	I-	337	Rev.	62	120	420
A45	50	N-	281	Rev.	90	285	515
A46	59	N-	362	Irrev.	90	180	400
A47	59	I+	306	Irrev.	83	355	650
A49	48	N+	305	Rev.	61	190	375
A50	67	I++	421	Irrev	94	225	480
A51	60	N-	609	Rev.	45	n/a	425
A52	50	N++	502	Irrev.	78	190	380
A53	30	I++	652	Irrev.	81	130	345
A55*	73	I++	289	Irrev.	88	185	395
A56	70	N++	326	Rev.	59	105	330
A57	59	N++	378	Rev.	75	335	635
A58	56	I+++	329	Rev.	70	175	480
A59*	59	I+++	568	Rev.	45	n/a	390
A60	28	I-	288	Rev.	39	n/a	355
A61*	55	I+++	205	Irrev.	96	345	525
A62	48	N+	241	Irrev.	100	260	480

Table 4.2 ; The Effects of Verapamil on Platelet Aggregation in Diabetic Patients.

KEY: * indicates patients taking the anti-hypertensive CCB, Adalat (nifedipine)

"TYPE" refers to type of diabetes,

N=NIDDM,

I=IDDM,

and to degree of microangiopathy,

- = no microangiopathy,

+ = background retinopathy,

++ = severe retinopathy,

+++ = retinopathy and proteinuria.

	NUMBER OF SAMPLES	AGE	PLATELET NUMBER (x 10^9/l)	2/10 ADP RATIO (%)	AD_{50} g/ml Verapamil	AD_{100}
CONTROLS	8	51.0 +/- 15.1	374.2 +/- 90.4	73.9 +/-+/- 19.3	183.4 +/- 107.0	484.4 186.7
DIABETICS	21	53.9 +/- 13.6	373.8 +/- 120.3	75.4 +/-+/- 18.5	189.5 +/- 108.0	464.0 96.1

Table 4.3 : Summary of results.

4.4.; Discussion

Figure 4.1 illustrates the opposing effects of adding increasing doses of A23187 and verapamil to ADP-activated PRP samples. The ionophore is able to prevent the reversal of aggregation seen at low dose ADP in healthy controls, but does not cause the secondary wave of aggregation at the concentrations investigated. Verapamil, by contrast, turns irreversible aggregation into the spontaneously reversible aggregation characteristic of "healthy" platelets.

The similarity between diabetics and age-matched controls in both their response to ADP and their inhibition of aggregation by verapamil, suggests that the calcium-dependent mechanisms of platelet activation are not perturbed in diabetes. This contrasts with the work of Bergh *et al*[52], who reported that IDDM without clinical complications showed abnormalities in Ca^{2+}-handling, involving an increased Ca^{2+} influx and a protracted initial efflux. However, this survey involved the relatively long-term (15 minutes-2 hours) investigation of $^{45}Ca^{2+}$ influx into unstimulated platelets, and the corresponding efflux. In the study reported here, all aggregation events are complete within 5 minutes of the addition of an agonist (ADP), as is the uptake and release of Ca^{2+} by the platelet.

Shanbaky *et al*[85] demonstrated that elevated cytoplasmic Ca^{2+} in resting platelets was a major risk factor for arterial thrombosis, lowering the threshold for activation by physiological agonists. This was also found to be the case in diabetics. The results reported here indicate that diabetics, even with microvascular complications, do not show an enhanced platelet aggregation or a decreased sensitivity to inhibition by verapamil, suggesting similar resting Ca^{2+} levels in platelets from diabetics and age-matched controls.

Thus, it appears that if enhanced platelet aggregation *in vivo* occurs in diabetics, it is not, in general, due to an elevated basal Ca^{2+} level. However, CCBs may still have a role to play in the prevention or treatment of diabetic microangiopathy. It is well documented that verapamil is able to reduce platelet aggregation *in vivo*, possibly acting synergisticly with PGI_2 and other endogenous anti-aggregators[81]. Verapamil appears to inhibit platelet activation by at least two mechanisms. In addition to its familiar effect of blocking Ca^{2+}-channels, it is able to block the α_2-adrenergic receptor of platelets, thus directly inhibiting activation by epinephrine[82,83]. It has also been proposed that verapamil is an antagonist of platelet serotonin receptors[84].

Even if the primary cause of platelet activation *in vivo* in diabetes is not an elevated basal Ca^{2+} level, treatment with CCBs would be beneficial in two ways. The tendency for platelets to aggregate in response to an *in vivo* stimulus would be diminished, reducing the chances of circulating microaggregates, and in addition, the CCBs vasodilatory properties would reduce the risk of any microaggregates causing a blockage.

Possible evidence of the beneficial effect of CCB therapy in diabetes is provided by the results from patients already taking the drugs. Of the three patients in this survey taking Adalat, one (A59) shows a surprisingly low degree of platelet aggregation in response to ADP, considering the extent of microvascular complications. This may represent an improvement in haemostasis since the development of the complications. In addition, patient R13 (see section 6.3) shows an almost complete lack of response to 2μM ADP, which again may be due to Adalat therapy. However, not all diabetics on CCBs show this improvement in platelet status, and all the patients noted are already suffering from some degree of microangiopathy.

The only way to discover whether CCBs could have a role in the prevention of microangiopathy is to perform a long-term survey on a number of diabetics treated with or without CCBs starting prior to the development of microvascular complications. Such a survey was beyond the scope of this project.

Chapter 5

DOES DIABETIC PLASMA CONTAIN A PLATELET AGGREGATION-ENHANCING FACTOR?

5.1 : Background.

Blood plasma contains numerous factors which potentially can induce the activation of platelets *in vivo*. In addition to the recognised clotting factors and platelet agonists involved in haemostasis[45,90,91], "abnormal" agonists such as bacterial endotoxins[92], autoantibodies[93,94], soluble immune complexes[95] and lipoproteins[26,97] may modify platelet function.

All of the above may be postulated to play a role in the development of circulating platelet microaggregates in diabetic patients. Diabetic patients are particularly prone to recurrent viral and bacterial infection. Gram-negative bacteria produce an endotoxin, lipopolysaccharide in nature[6], that has been shown to activate platelets in whole blood *in vitro*[92]. This effect requires the presence of other blood cells in addition to platelets (see section 7.3.5.) and may explain the thrombocytopenia seen after certain types of infection.

Autoantibodies may arise in diabetics against infused insulin[95], non-enzymatically glycosylated plasma proteins[94] and cellular receptors[94]. Platelets are known to possess receptors for the Fc portion of immunoglobulin G (IgG)[95] and the induction of platelet aggregation by soluble immune complexes is well known[95,96]. Numerous workers have shown significant increases in the level of soluble immune complexes in the plasma of diabetic patients[45,95].

Diabetics have been reported to have higher than normal levels of pro-atherogenic low density lipoprotein (LDL), and low levels of anti-atherogenic high density lipoprotein (HDL)[45,98,99]. Platelets possess specific binding sites for lipoproteins[97,100]. Lipoprotein binding changes membrane cholestrol content and platelet responses[95,96]. LDL increases thrombin-induced platelet aggregation, whereas HDL has the opposite effect. However, the direct importance of LDL on diabetic platelet activation is questionable. Non-enzymatic glycosylation of LDL is increased in diabetics, inhibiting its association with cellular receptors[94]. The resulting high levels of blood cholestrol become deposited in various

tissues, particularly the vascular lumen, making high plasma LDL an important factor in the development of atherosclerosis or macrovascular disease[98,99]. Recently, much interest has been focused on lipoprotein(a) [Lp(a)], a varient of LDL that promotes coagulation by competing with plasminogen[101]. Lp(a) is increased in the plasma of diabetics where it may play a part in thrombogenesis[101].

The most clear evidence for a factor in diabetic plasma that could influence platelet function, but was not found in non-diabetics, has come from the work of Levin *et al*[102]. They reported that the degree of irreversible aggregation of a normal donor's platelets after ADP stimulus was increased when additional diabetic plasma was present. This factor has been partially purified, has a molecular weight of 21kD, and may represent an activated coagulation component or a prostaglandin metabolism modulator, but its exact nature is unclear[102].

The following experiments investigate the effects of mixing diabetic plasma with platelets from non-diabetics and vice-versa.

5.2. ; Materials and Methods

5.2.1. ; Reagents

3.8% Tri-sodium citrate solution;

Adenosine diphosphate reagent (Sigma);

Special platelet aggregation reagent, reconstituted with water to give a buffered 0.2mM solution of ADP.

Hirudin (Sigma),

5 units/ml in saline.

Protein-A sepharose CL-4B (Sigma);

2mg protein-A per ml gel: One ml gel binds approximately 20mg of human IgG.

Heparin-agarose (type II) (Sigma);
Approximately 803 g/ml of packed gel.

5.2.2. ; General Method

Citrate anticoagulated venous blood was obtained with informed consent from diabetic patients and non-diabetic controls. PRP and PPP were prepared as described in appendix 1.

"Mixing" experiments were performed by adding 0.35ml of non-diabetic PRP to 0.65ml of diabetic PPP (or vice-versa), in a siliconised aggregometry cuvette. Mixed samples were incubated at 37°C for varying times prior to the onset of aggregation induced by 2μM ADP and stirring at 1000rpm.

t=0 samples correspond to the aggregation obtained with 0.35ml PRP diluted with 0.65ml of autologous PPP.(see also figure 5.1).

5.2.3. ; Adaptations to general method to determine the nature of the aggregation-enhancing factor in diabetic plasma.

(i) In order to prevent platelet activation by thrombin, hirudin was added to certain experiments at a final concentration of 50 milliunits/ml plasma.

(ii) IgG and soluble immune complexes were removed from plasma by passing 1ml of PPP through a 2ml Protein-A sepharose column prior to mixing with PRP in some experiments.

(iii) 1ml of PPP was passed through a Heparin-agarose column to remove antithrombin III (ATIII) from the plasma prior to mixing with PRP in certain experiments.

5.3. ; Results

(a) Column Efficiency; The protein concentrations of PPP passed through each column, and of full PPP were assayed using the Lowry protein assay[147] and comparison with a bovine serum albumin standard curve.

Duplicate samples at each of two different concentrations of plasma were assayed to give quadruplicate results.

The protein concentrations were found to be;

(1) Full plasma = 105.8 mg protein/ml PPP.

(2) Plasma after Heparin-agarose = 75.8 mg protein/ml PPP.

(3) Plasma after Protein-A sepharose = 55.2 mg protein/ml PPP.

(b) Mixing Experiments; The results of mixing PRP and PPP from diabetics and controls are expressed graphically in figures 5.2a-5.2n. They are derived by measuring the change in optical density (ΔOD) at 600nm of diluted PRP samples after the addition of 2μM ADP (see figure 5.1).

The increase in aggregation typically seen when control platelets are mixed with diabetic plasma is defined below, so as to normalise results;

$$\text{Increase} = \frac{\Delta OD_{max} - \Delta OD_{t=0}}{\Delta OD_{t=0}} \times 100\%$$

The decrease in aggregation typically seen when diabetic platelets are incubated with control plasma is defined as;

$$\text{Decrease} = \frac{\Delta OD_{t=0} - \Delta OD_{min.}}{\Delta OD_{t=0}} \times 100\%$$

Where $\Delta OD_{max.}$ = The maximum change in optical density in heterologous mixed samples on the addition of 2μM ADP.

$\Delta OD_{min.}$ = The minimum change in optical density in heterologous mixed samples on the addition of 2μM ADP.

$\Delta OD_{t=0}$ = The change in optical density in autologous diluted samples on the addition of 2μM ADP.

If, conversely, diabetic platelets decrease in sensitivity to ADP on mixing with control plasma, or control platelets increase in sensitivity on mixing with diabetic plasma, the results derived as above are expressed as a negative number.

(c) Control mixing experiments; The results of mixing PRP and PPP from two different non-diabetic controls to check if the effects seen in experiments 5a-5n are diabetes specific are seen in figures 5o-5q.

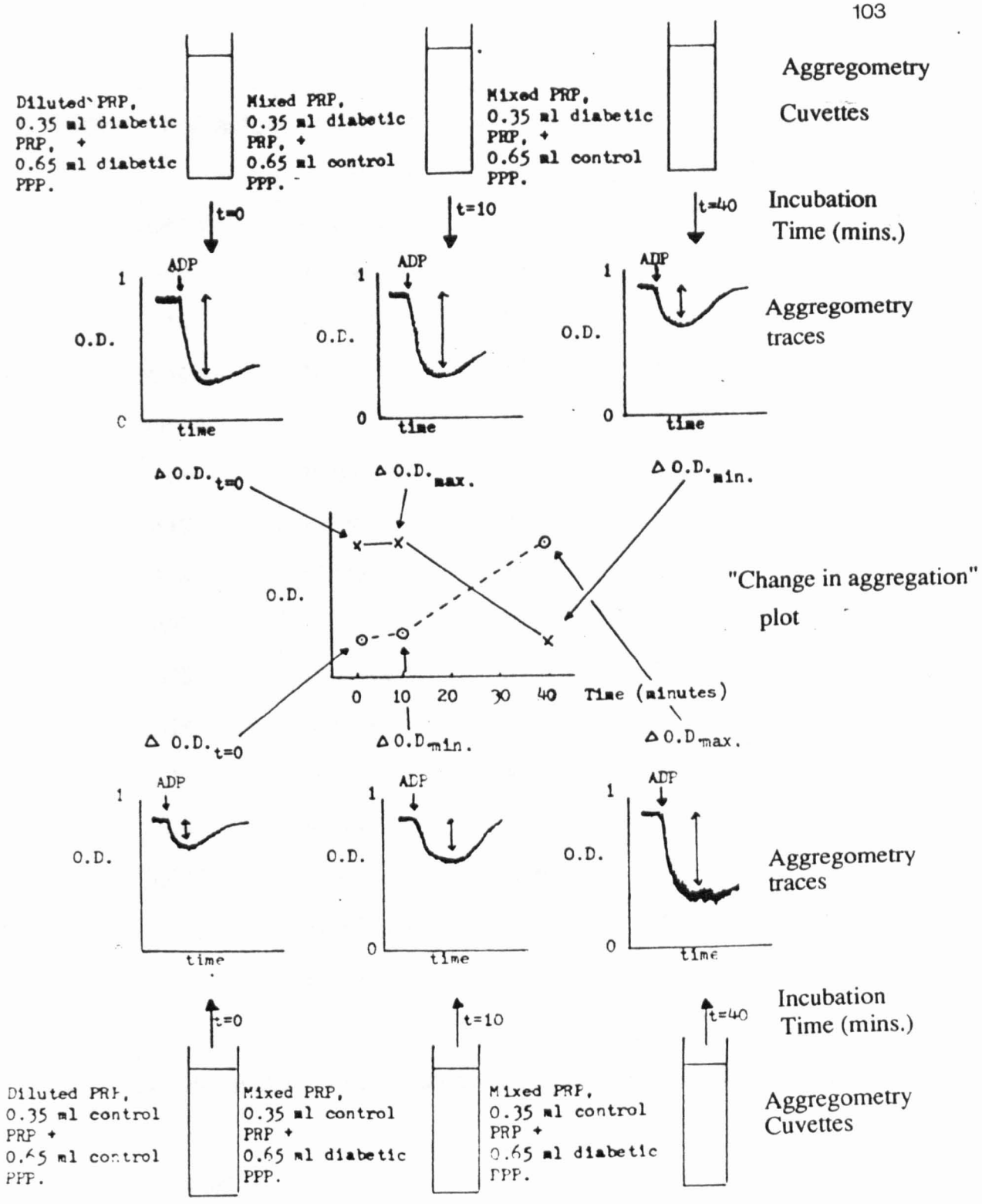


FIGURE 5.1: Assay Protocol for "Mixing" experiments.

EXPERIMENT	CONTROL	AGGREGATION INCREASE	DIABETIC	AGGREGATION DECREASE
(a)	GMW	-(11.9)%	A52	50.9%
(b)	AJS	32.1%	A53	11.4%
(c)	AJS	123.5%	A56	6.0%
(d)	GMW	38.5%	A54	40.7%
(e)	GMW	0	X1	26.7%
(f)	AJS	19.0%	X2	32.0%
(g)	AJS	79.2%	X4	-(5.3)%
(h)	AJS	67.5%	X11	9.1%
(i)	AT	0	X12	25.7%
(j)	AJS	-(41.7)%	X13	53.8%
(k)	AJS	-(29.2)%	X14	21.4%
(l)	AJS	20.5%	X15	10.2%
(m)	AJS	93.3%	X17	14.9%
(n)	GMW	110.9%	X16	10.7%
Mean +/- s.d.		**35.8% +/- 52.0%**		**22.0% +/- 17.4%**

Table 5.1 : Summary of mixing experiments (terms defined on previous page).

5.4. ; Discussion

In all cases except experiment 5n, the diabetic donor showed a higher initial aggregation prior to mixing than the young, healthy controls involved. This is to be expected, since the degree of aggregation in response to ADP is known to increase in diabetics, and also to increase with age. Although most of the diabetics were significantly older than the controls, the initial aggregation response was similar, and in addition, the degree of microangiopathy did not have a consistent effect on initial aggregation.

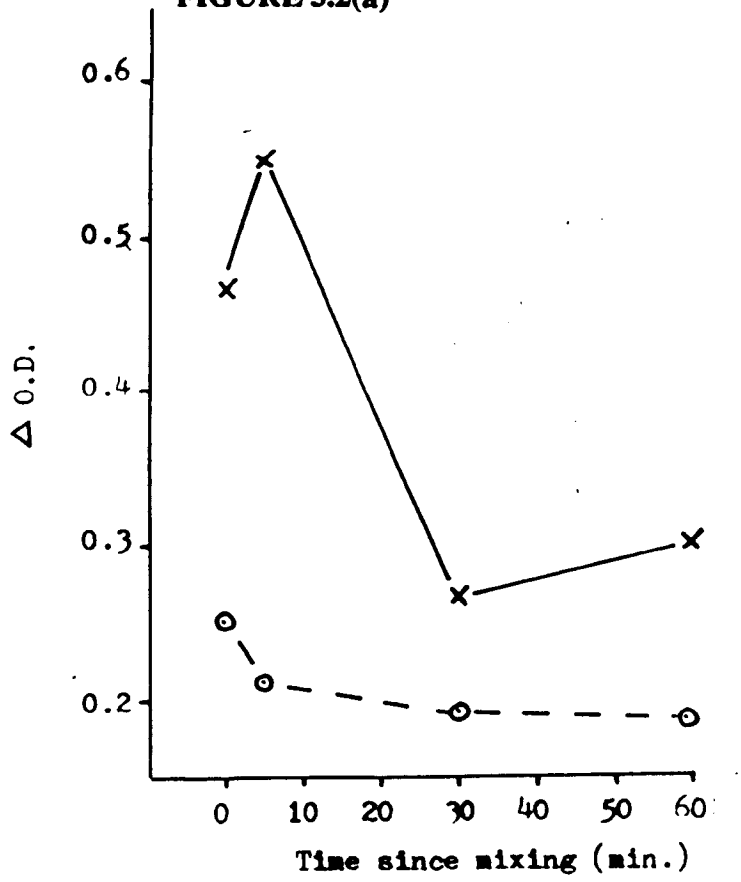
Non-diabetic, healthy control plasma reduced the aggregation of diabetic platelets in all but one case, 5g. In contrast, diabetic plasma caused an increase in non-diabetic, healthy control platelet aggregation in 9 cases, a decrease in 3 cases, and had no effect in two others.

This suggests that diabetic plasma contains a "factor", absent in control donors, that can potentiate platelet aggregation. Alternatively, diabetics may lack a particular aggregation inhibitor that is present in control donors. However care should be taken in describing this factor as diabetes-specific since figures 5o-5q clearly show that mixing PRP and PPP from two healthy controls gives similar results, with the plasma from the control showing the greater degree of initial aggregation increasing the aggregation of the platelets from the control with less initial aggregation, and vice versa.

Depletion of IgG decreases the effect of diabetic plasma on normal platelets (5c, 5d) but increases the effect of control plasma on diabetic platelets (5c). These results suggest that both diabetic and non-diabetic plasma contain IgG or immune complexes that can potentiate aggregation, since the presence of IgG-depleted plasma causes a reduced t=40 aggregation in both mixtures.

Depletion of ATIII (5e-5g) or addition of hirudin (5h-5n) generally had no effect on t=40 aggregation, suggesting that the aggregation enhancing factor is not thrombin. However, in one experiment (5h), a major reduction in diabetic plasma -enhanced aggregation is obtained in the presence of hirudin, possibly indicating the presence of thrombin in this paticular diabetic patient's plasma.

FIGURE 5.2(a)



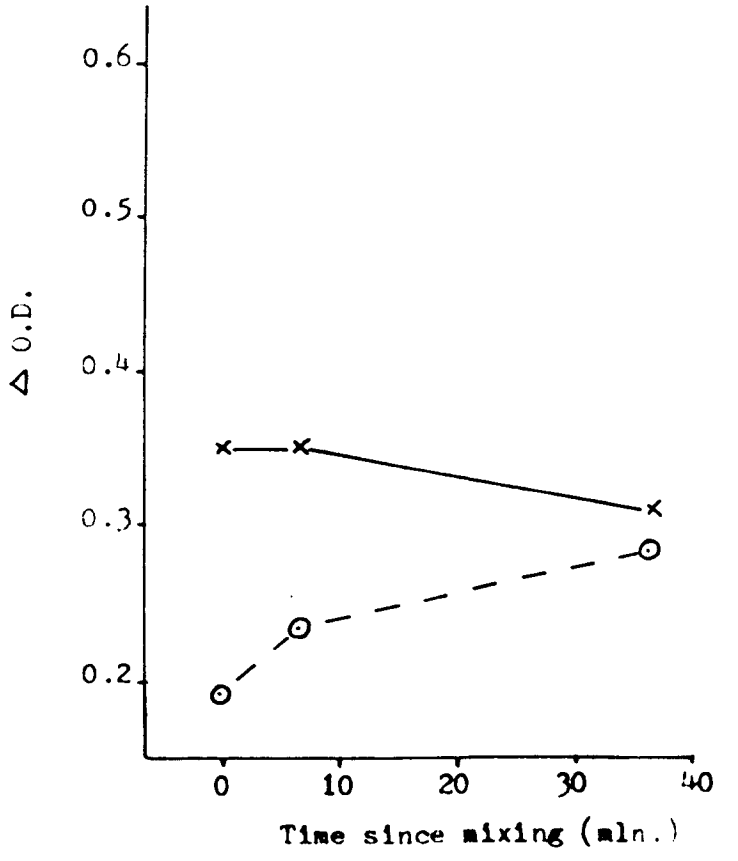
⊙ = Control, GMW, age 24.

X = Diabetic, A52, age 51, NIDDM, retinopathy

Increase in control aggregation = -(11.9)%

Decrease in diabetic aggregation = 50.9%

FIGURE 5.2(b)



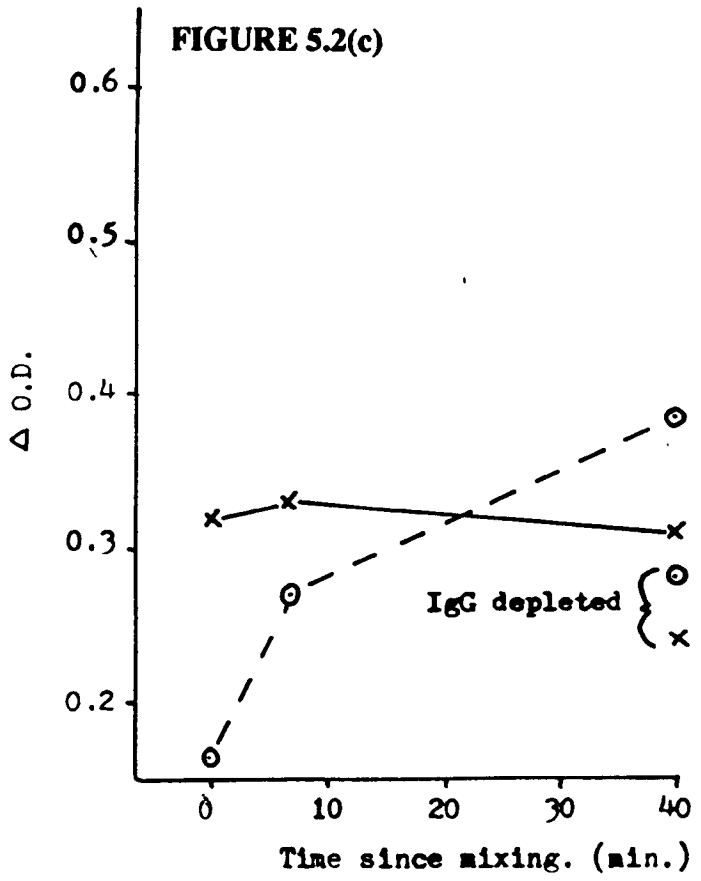
⊙ = Control, AJS, age 22.

X = Diabetic, A53, age 30, IDDM, proliferative retinopathy

Increase in control aggregation = 32.1%

Decrease in diabetic aggregation = 11.4%

FIGURE 5.2(c)



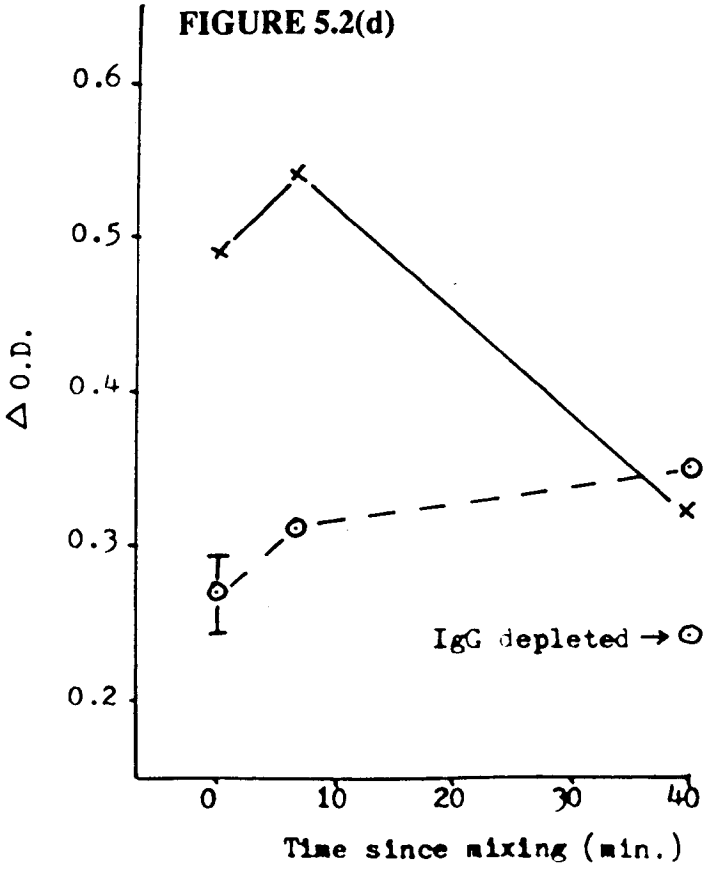
⊙ = Control, AJS, age 22.

× = Diabetic, A56, age 70, NIDDM, proteinuria

Increase in control aggregation
= 123.5% (whole plasma)
= 64.7% (IgG depleted plasma)

Decrease in diabetic aggregation
= 6.0% (whole plasma)
=27.3% (IgG depleted plasma)

FIGURE 5.2(d)



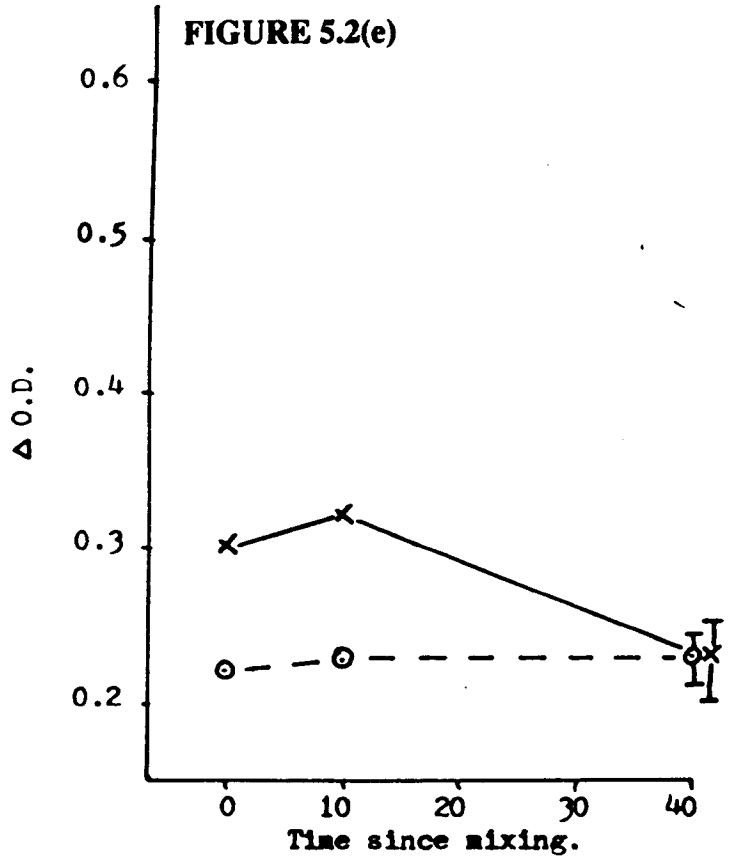
⊙ = Control, GMW, age 24.

× = Diabetic, A54, age 61, NIDDM, no complications

Increase in control aggregation
= 38.5% (whole plasma)
= -(11.1)% (IgG depleted plasma)

Decrease in diabetic aggregation = 40.7%

FIGURE 5.2(e)



⊙ = Control, GMW, age 24.

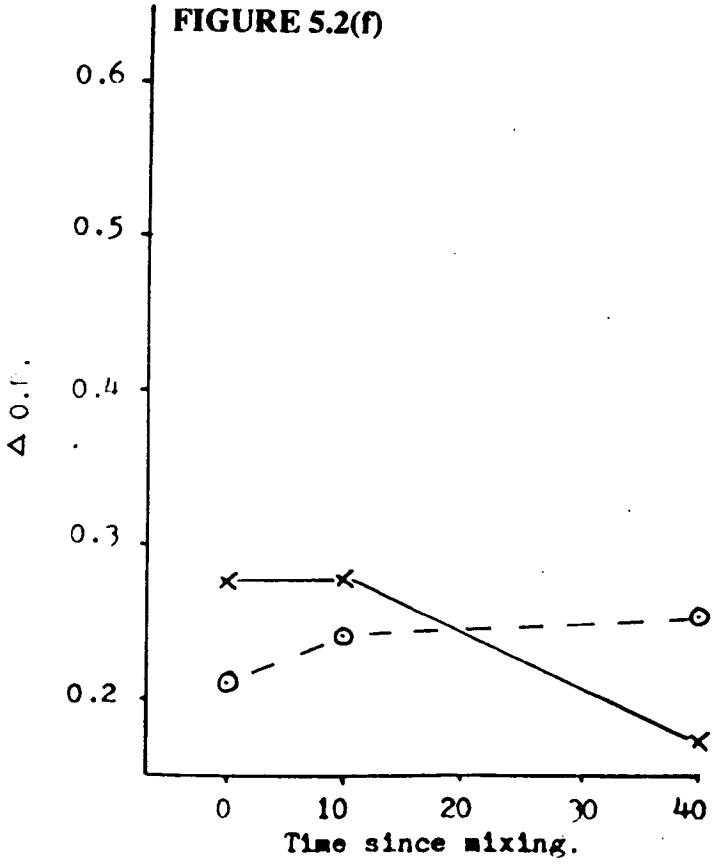
× = Diabetic, X1, age 56, IDDM, retinopathy, proteinuria, leg amputated

Increase in control aggregation = 0

Decrease in diabetic aggregation = 26.7%

Antithrombin III depletion has no effect on t=40 aggregation in either sample.

FIGURE 5.2(f)



⊙ = Control, AJS, age 22.

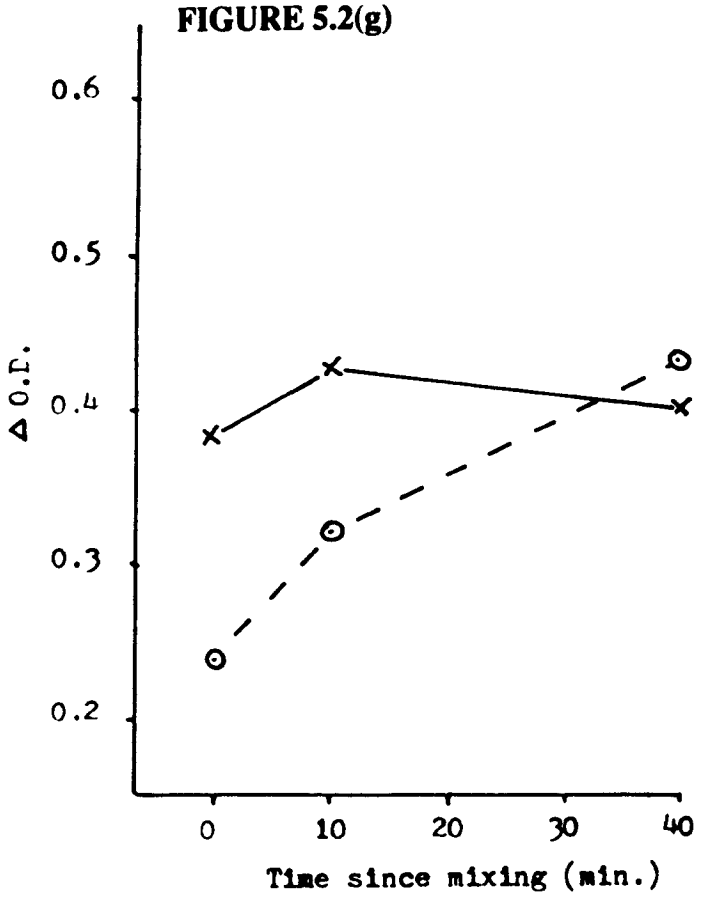
× = Diabetic, X2, age 60, IDDM, retinopathy, proteinuria, hypertension.

Increase in control aggregation = 19.0%

Decrease in diabetic aggregation = 32.0%

Antithrombin III depletion has no effect on t=40 aggregation in either sample.

FIGURE 5.2(g)



⊙= Control, AJS, age 22.

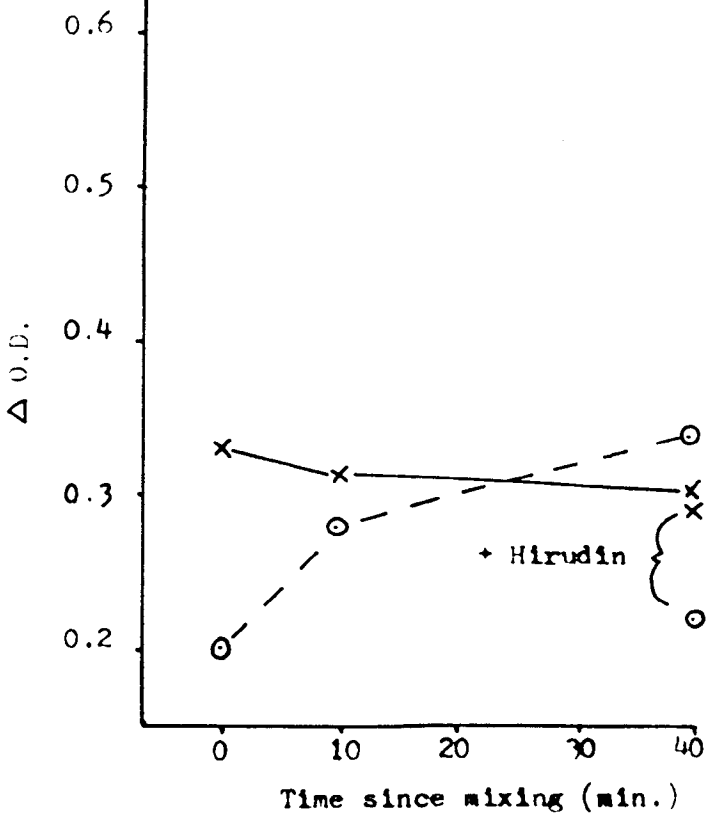
✕ = Diabetic, X4, age 56, IDDM, retinopathy, proteinuria, hypertension

Increase in control aggregation = 79.2%

Decrease in diabetic aggregation = -(5.3)%

Antithrombin III depletion has no effect on t=40 aggregation in either sample.

FIGURE 5.2(h)



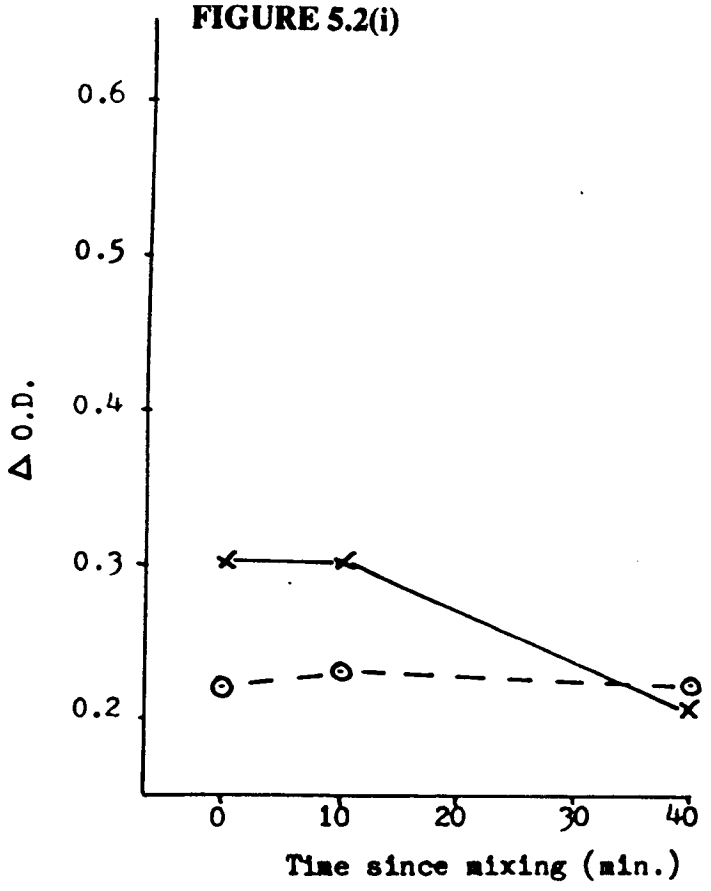
⊙ = Control, AJS, age 22.

✕ = Diabetic, X11, age 58, NIDDM, retinopathy

Increase in control aggregation = 67.5% (-Hirudin)
= 10.0% (+Hirudin)

Decrease in diabetic aggregation = 9.1% (-Hirudin)
= 9.1% (+Hirudin)

FIGURE 5.2(i)



⊙ = Control, AT, age 24.

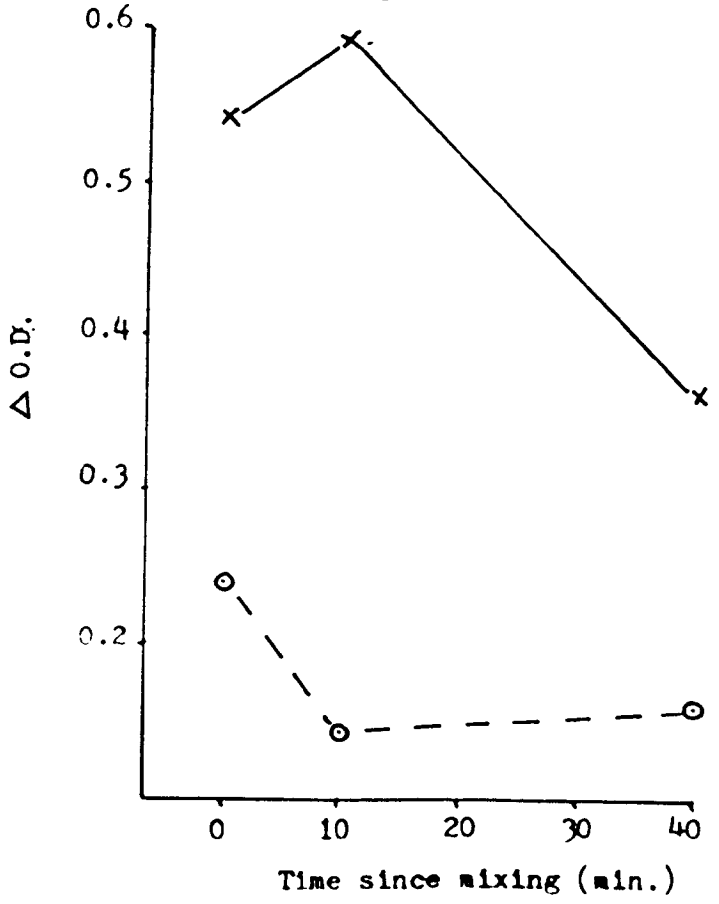
X = Diabetic, X12, age 54, NIDDM, retinopathy

Increase in control aggregation = 0

Decrease in diabetic aggregation = 25.7%

Addition of hirudin has no effect on t=40 aggregation in either sample

FIGURE 5.2(j)



⊙ = Control, AJS, age 22.

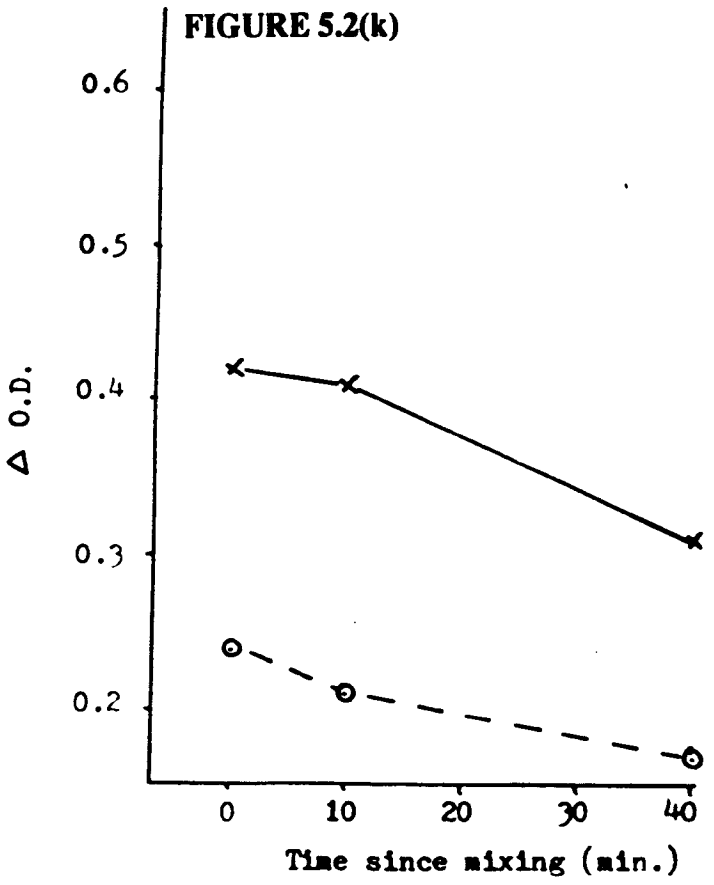
X = Diabetic, X13, age 49, NIDDM, retinopathy

Increase in control aggregation = -(41.7)%

Decrease in diabetic aggregation = 53.8%

Addition of hirudin has no effect on t=40 aggregation in either sample

FIGURE 5.2(k)



⊙ = Control, AJS, age 22.

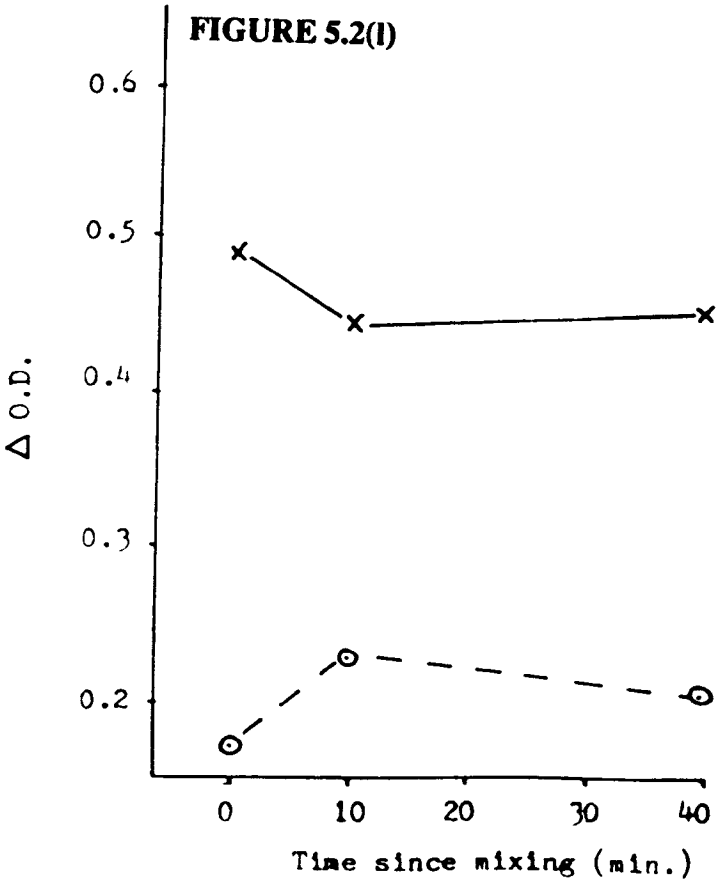
× = Diabetic, X14, age 37, IDDM, retinopathy, hypertension.

Increase in control aggregation = -(29.2)%

Decrease in diabetic aggregation = 21.4%

Addition of hirudin has no effect on t=40 aggregation in either sample

FIGURE 5.2(l)



⊙ = Control, AJS, age 22.

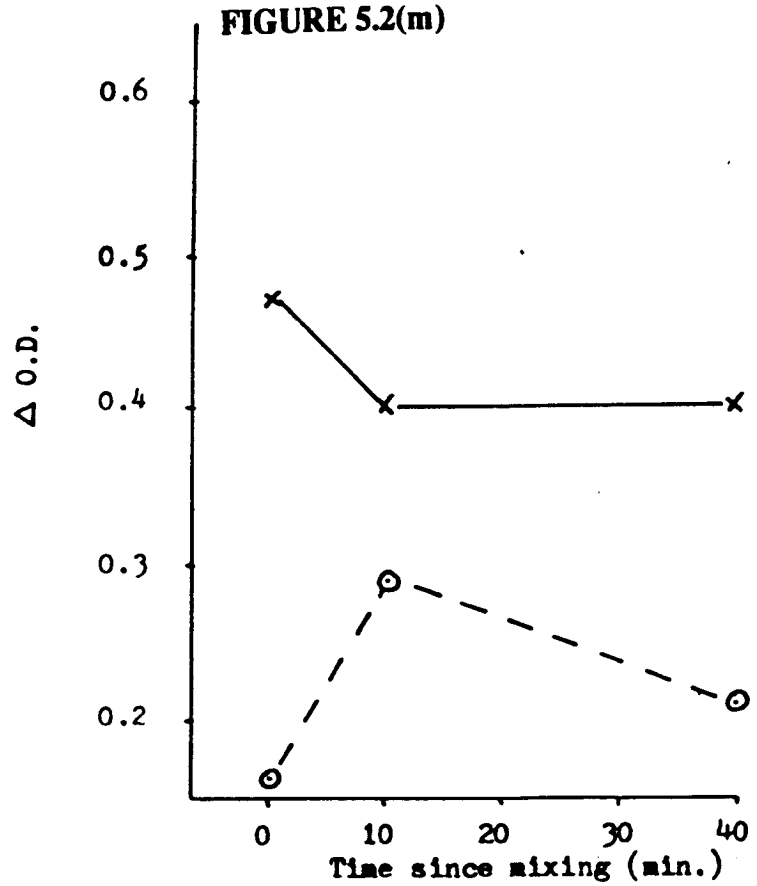
× = Diabetic, X15, age 63, NIDDM, retinopathy, proteinuria, recent stroke.

Increase in control aggregation = 20.5%

Decrease in diabetic aggregation = 10.2%

Addition of hirudin has no effect on t=40 aggregation in either sample

FIGURE 5.2(m)



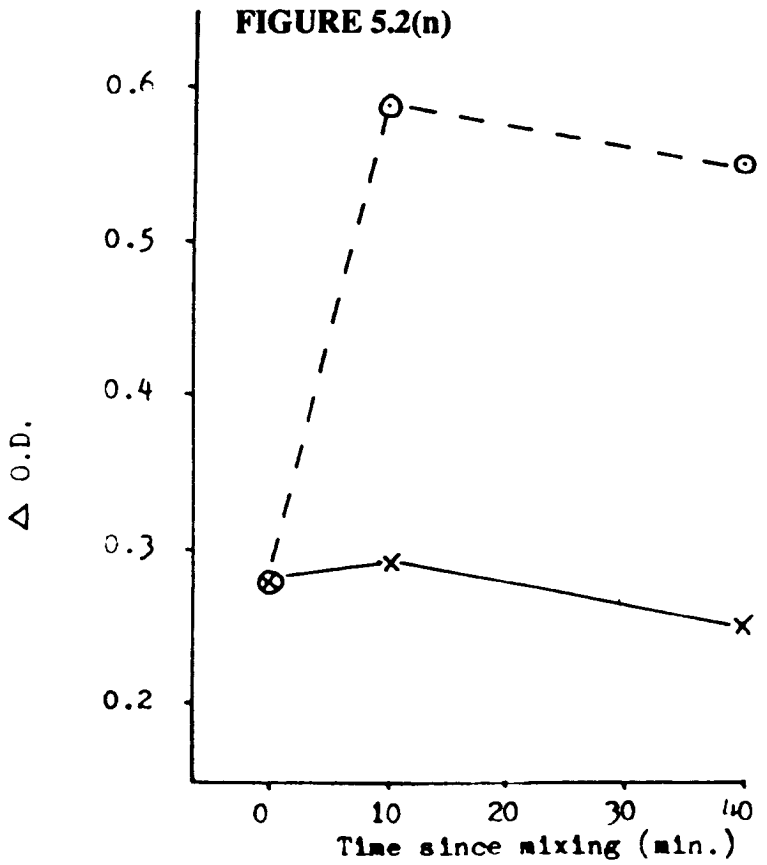
⊙ = Control, AJS, age 22.

× = Diabetic, X17, age 61, IDDM, retinopathy

Increase in control aggregation = 93.3%

Decrease in diabetic aggregation = 14.9%

FIGURE 5.2(n)



⊙ = Control, GMW, age 24.

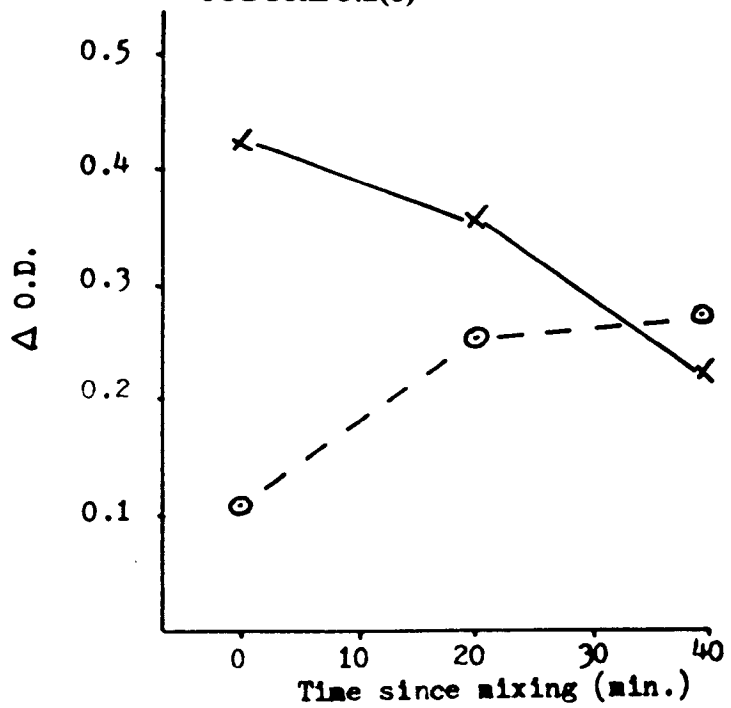
× = Diabetic, X16, age 58, NIDDM, retinopathy

Increase in control aggregation = 110.9%

Decrease in diabetic aggregation = 10.7%

Addition of hirudin has no effect on t=40 aggregation in either sample

FIGURE 5.2(o)



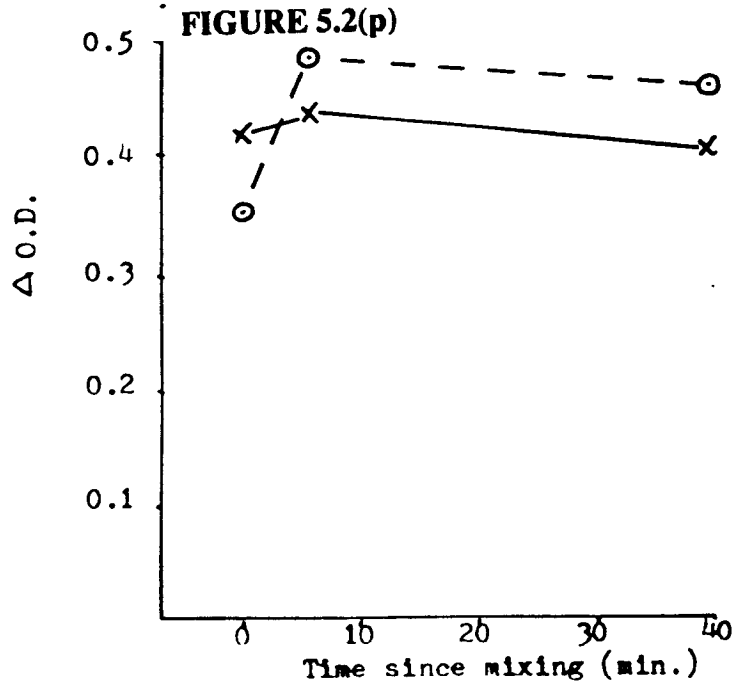
⊙ = Control 1, AJS, age 22.

✕ = Control 2, DF, age 56,

AJS, aggregation increase = 14.5%

DF, aggregation decrease = 47.6%

FIGURE 5.2(p)



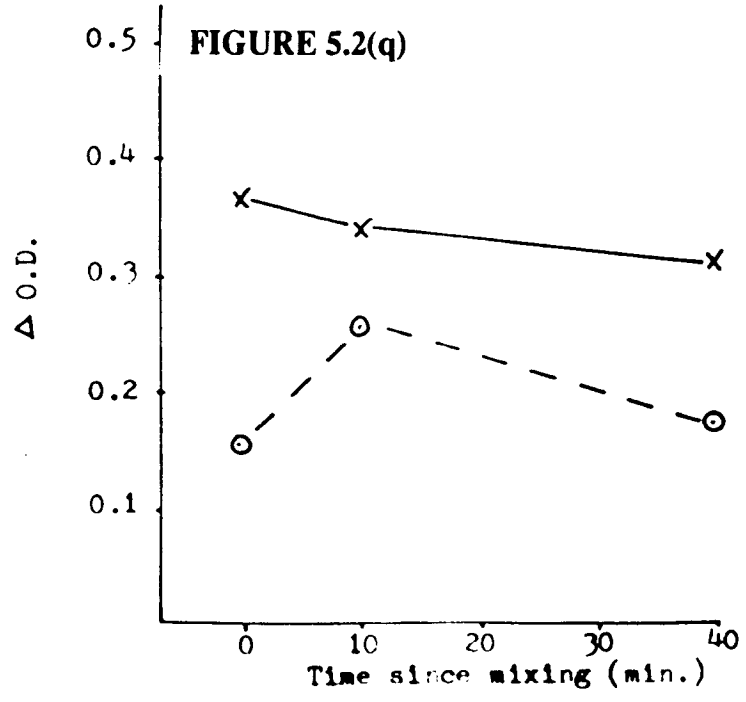
⊙ = Control 1, RJB, age 22.

✕ = Control 2, DF, age 56.

RJB, aggregation increase = 37.1%

DF, aggregation decrease = 4.8%

FIGURE 5.2(q)



⊙ = Control 1, AJS, age 22.

✕ = Control 2, RJB, age 22.

AJS, aggregation increase = 73.3%

RJB, aggregation decrease = 16.2%

Chapter 6

THE EFFECTS OF INSULIN ON BLOOD PLATELETS

6.1 ; Background

Numerous types of human blood cells have been shown to possess specific insulin receptors, including monocytes,lymphocytes and erythrocytes. Hajek *et al*[103] demonstrated that platelets also possess specific insulin receptors with a high affinity dissociation constant of Kd= $3x10^9$ M^{-1}, and 570 +/- 100 sites per platelet. These figures can also be expressed as approximately 25 sites per μm^2 platelet surface area, a figure very similar to that seen in other blood cells.

Udvardy *et al*[104] have shown that platelets from NIDDM patients possess far fewer insulin receptors, only 30 +/- 11 sites per cell, compared to 420 +/- 116 for the control donors in their study. The affinity of the platelet insulin receptor was also significantly reduced in NIDDM.

Many receptors exert their actions through specific guanine nucleotide regulatory proteins (G-proteins). These G-proteins , upon interaction with an appropriate occupied receptor, bind GTP in order to attain an activated state capable of modulating the activity of a paticular effector system[105,106].

There is a large family of G-proteins associated with cells, some of which have a clearly defined function. Platelets are known to express the stimulatory G-protein, G_s, whose activity can be stimulated by prostacyclin and PGE_1, the inhibitory G-protein, G_i, stimulated by epinephrine and thrombin, and a G-protein, termed G_p, which can stimulate inositol phospholipid metabolism triggered by agonists such as vasopressin and thrombin[105].

Houslay *et al*[105,106] have suggested that insulin might affect certain tissues by interacting with G-proteins, since insulin is able to inhibit adenylate cyclase and activate a high affinity plasma membrane cAMP-phosphodiesterase in hepatocytes. They termed this paticular G-protein G_{ins}[105]. If insulin affects the equivalent platelet enzymes in the same way, it would cause a lowering of platelet cAMP levels and would, at an appropriate concentration, be expected to potentiate platelet activation.

However, several authors have reported that insulin is able to decrease platelet aggregation both *in vivo* and *in vitro*[107-109], in response to ADP and other agonists, leading to the theory that hyperinsulinaemia reduces platelet aggregation *in vivo* when euglycaemia is maintained. A proposal arising from these studies was that increased aggregation and decreased disaggregation in diabetics may be due to defective enzyme activities in diabetic platelets that could be corrected by insulin[108].

Contrary to this idea, insulin concentrations are commonly above normal in NIDDM patients who are obese as well as in IDDM patients using insulin injections. It has long been hypothesised that high levels of circulating insulin are related to the development of atherosclerosis through its effect on the endothelial cells of the blood vessels, either by causing damage to the blood vessels, or by acting on other factors involved in the development of atherosclerosis such as lipoproteins and smooth muscle cell proliferation[110,111]. The evidence in favour of the view that insulin is atherogenic comes mainly from epidemiological studies in diabetics and non-diabetics, but results have been inconsistent[111].

It was decided to investigate the effects of insulin on platelet aggregation *in vitro* with PRP from both diabetics and controls. With a view to the suggestion that insulin might act on platelets through a G-protein linked to adenylate cyclase, it was decided to include PGE_1 in the system. PGE_1 is known to exert its inhibitory effects on platelets via an activation of adenylate cyclase and an increase in cAMP[4,112]. This system is more akin to the physiological situation in which potential platelet agonists are balanced by antagonists such as prostacyclin. Previous studies have concentrated only on the effects of insulin alone on agonist-induced platelet aggregation in healthy controls.

6.2. ; Materials and Methods

6.2.1. ; Reagents

Adenosine Diphosphate Reagent (Sigma);

Special platelet aggregation reagent, reconstituted with water to give a buffered 0.2mM solution of ADP.

Prostaglandin E_1 (Sigma);

Stored as a 0.5mM stock solution in 50% ethanol at -20°C. Diluted to 6μM PGE_1 with saline prior to use.

Insulin (Sigma);

From bovine pancreas, approximately 24 i.u./mg. Suspended in saline prior to use to give stock concentrations of 100μM and 1μM.

6.2.2. ; Method

PRP and PPP were prepared as described in appendix 1.

1.0ml PRP samples were placed in siliconised aggregometry cuvettes and pre-incubated at 37°C for 10 minutes.

"Baseline" aggregation was measured by adding 10μl of the stock ADP solution to a PRP sample, stirring at 1000rpm, 37°C, and recording the resultant change in optical density at 600nm, ΔOD_1 (see figure 6.1).

The inhibitory action of PGE_1 was measured by pre-incubating a second 1.0ml PRP sample with 30 or 60nM PGE_1 for 3 minutes prior to the addition of 2μM ADP (ΔOD_2).

The effect of insulin on the system was measured by adding either 10^{-6} or 10^{-8} molar insulin to a PRP sample and stirring for 3 minutes prior to the addition of PGE_1 and subsequently ADP (ΔOD_3).

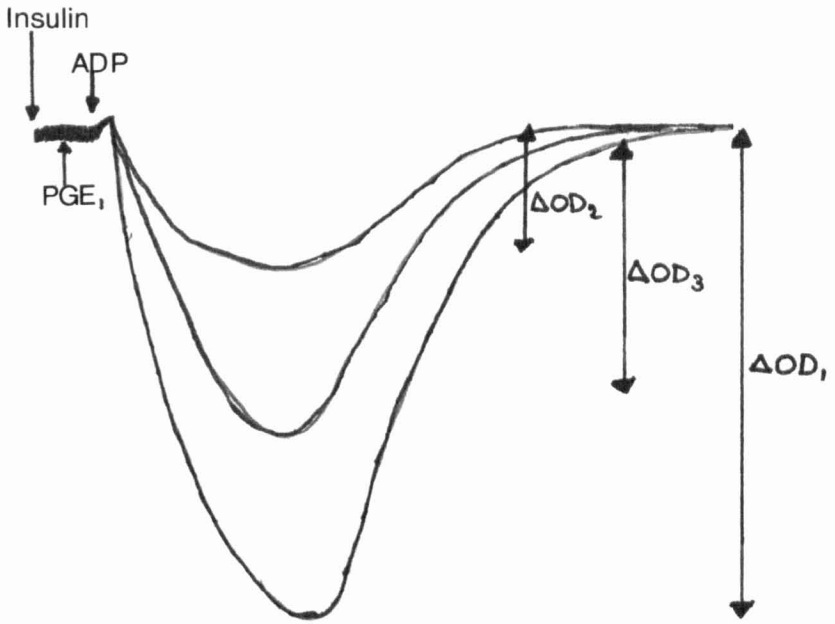


Figure 6.1. ; The effects of ADP, PGE_1 and insulin on platelet aggregation.

The degrees of aggregation in the presence of the various reagents were recorded and, in addition, the percentage inhibition of aggregation by PGE_1 was calculated according to the formula;

$$PGE_1 \text{ inhibition effect} = \frac{(\Delta OD_1 - \Delta OD_2)}{\Delta OD_1} \times 100\%$$

Also, the effect of insulin on PGE_1 induced inhibition of aggregation was evaluated to give a "degree of recovery" according to the formula;

$$\text{Insulin/}PGE_1 \text{ antagonism factor} = \frac{\Delta OD_3 - \Delta OD_2}{\Delta OD_1 - \Delta OD_2} \times 100\%$$

By this formula, an insulin-induced increase in aggregation will give a positive number, whereas a decrease will be negative.

6.3. : Results

Results are expressed in tables 6.1-6.5 and in figure 6.2.

6.4. : Discussion

Unfortunately it was not possible to obtain an age-matched control population for this survey. However, the phenomena observed do not appear to be significantly age-dependent. The diabetics, both NIDDM and IDDM, show significantly reduced responses to PGE_1 at the concentrations studied. These observations are in agreement with results in the literature, showing decreased sensitivity to the physiological analogue of PGE_1, prostacyclin, in diabetics[45]. Prostacyclin and PGE_1 are known to share a common receptor[112], but their lack of effect on diabetic platelets does not appear to be mediated via altered PGI_2/PGE_1 receptors. This suggests a fault in either the coupling of the receptor to adenylate cyclase, a fault in the adenylate cyclase itself, or an increased platelet phosphodiesterase activity, all of which would lead to a lower cAMP level and hence a greater tendency towards aggregation in diabetics.

A second condition in which decreased platelet prostacylin sensitivity has been reported is Behcet's Syndrome[113], a vasculitic disorder in which thrombosis of both arteries and veins occurs.

Only the IDDM patients show a noticable increase in response to ADP (ΔOD_1) ($p>0.1$), but this is only just significant. Previous studies have reported increased platelet response to ADP in diabetes, but an equal proportion of researchers claim little or no difference compared to age-matched controls[45]. The inconsistency of these reports may be due to variations in the quality of diabetic control of the patients and to different drug therapy, particularly where drugs known to modify platelet function are concerned. In addition to common anti-aggregatory, but non-diabetes specific drugs such as aspirin[21] and calcium-antagonists[78-83], certain anti-diabetic drugs such as metformin are known to modify platelet function[114].

Donor	Age	Degree of Micro-angiopathy	ΔOD_1	30nM PGE_1			60nM PGE_1		
				% PGE_1 Inhibition	% Recovery (10nM Ins.)	% Recovery (1μM Ins.)	% PGE_1 Inhibition	% Recovery (10nM Ins.)	% Recovery (1μM Ins.)
AJS	23	-	.275	-	-	-	83.6	-	10.9
AJS	23	-	.230	69.6	-6.20	0	91.3	-4.80	0
GP	23	-	.365	-	-	-	37.0	-7.40	-
RJB	23	-	.375	25.3	-21.0	-21.0	74.7	-1.80	1.80
FW	49	-	.530	66.0	-	0	85.8	-	-
BEPS	50	-	.260	46.1	16.7	20.8	73.0	-2.60	7.90
MH	44	-	.200	67.5	3.7	-3.7	75.0	10.0	20.0
GMW	26	-	.390	35.9	-	50.0	82.0	-	14.0
AJS	23	-	.280	51.8	-	17.2	78.6	-	-6.80
DF	57	-	.280*	67.8	-5.3	-15.8	91.1	-	0
FW	49	-	.210*	64.3	-	-11.1	100.0	-	4.80

Table 6.1 ; The effects of ADP, PGE_1 and Insulin on platelet aggregation in healthy controls.

* = 1μM ADP

Donor	Age	Degree of Micro-angiopathy	ΔOD_1	30nM PGE_1			60nM PGE_1		
				% PGE_1 Inhibition	% Recovery (10nM Ins.)	% Recovery (1μM Ins.)	% PGE_1 Inhibition	% Recovery (10nM Ins.)	% Recovery (1μM Ins.)
R4	62	++	.330	46.9	-	32.2	78.8	-	9.6
R6	18	++	.550	1.8	-100.0	100.0	21.8	41.7	79.2
R8	39	+	.275	14.5	-62.5	-50.0	49.1	-18.5	-44.4
R13	45	++	.290*	32.7	-	5.3	70.7	-	0
R14	71	++	.550	7.3	-25.0	0	54.5	50.0	50.0
R16	29	++	.405	43.2	45.7	-	50.6	4.8	19.5
R17	37	++	.530	45.3	-8.3	-8.3	58.5	0	3.2
R36	62	++	.445	41.6	59.4	48.6	58.4	-	50.0
R37	28	+++	.160	43.8	-	35.7	-	-	-
R38	49	++	.575	47.8	-	7.3	79.1	-	-2.2
R40	55	+	.675	9.6	-	76.9	85.2	-	-1.0

Table 6.2 ; The effects of ADP, PGE_1 and Insulin on platelet aggregation in IDDM patients.

* = 5μM ADP

Donor	Age	Degree of Micro-angiopathy	ΔOD_1	30nM PGE_1			60nM PGE_1		
				% PGE_1 Inhibition	% Recovery (10nM Ins.)	% Recovery (1μM Ins.)	% PGE_1 Inhibition	% Recovery (10nM Ins.)	% Recovery (1μM Ins.)
R5	52	++	.290	10.3	-	100.0	31.0	-	66.7
R7	70	++	.490	6.1	0	133.0	32.6	59.4	21.9
R9	62	-	.150	40.0	0	-16.7	93.3	3.6	7.1
R10	62	-	.400	42.5	-5.9	26.5	75.0	-3.3	8.3
R11	70	+	.420	25.0	-	-	64.3	5.5	11.1
R12	70	+	.375	29.3	113.6	63.6	49.3	-16.2	0
R15	51	++	.330	33.3	4.5	127.3	43.9	6.9	24.1
R39	65	++	.575	0	-	0	69.6	-	75.0
R41	45	+	.380	19.7	33.3	106.7	57.9	4.5	20.4
R43	62	++	.270	-	-	-	50.0	22.2	29.6

Table 6.3 ; The effects of ADP, PGE_1 and Insulin on platelet aggregation in NIDDM patients.

VARIABLE	CONTROLS	IDDM	NIDDM
Age	35.45 +/- 14.07	45.00 +/- 16.56 N.S.	60.90 +/- 8.81 p>0.001
ΔOD_1	0.323 +/- 0.102	0.449 +/- 0.158 p>0.1	0.368 +/- 0.118 N.S.
% Inhibition by 30nM PGE_1	54.92 +/- 16.11	30.41 +/- 18.19 p>0.005	22.91 +/- 15.02 p>0.001
% Inhibition by 60nM PGE_1	79.28 +/- 16.27	60.67 +/- 18.75 p>0.025	56.69 +/- 19.45 p>0.01
"Recovery": 30nM PGE_1 (10nM Insulin)	-2.42 +/- 13.88	-15.11 +/- 61.39 N.S.	24.25 +/- 45.91 N.S.
"Recovery": 30nM PGE_1 (1μM Insulin)	4.04 +/- 22.10	24.78 +/- 43.59 p>0.01	67.55 +/- 58.31 p>0.1
"Recovery": 60nM PGE_1 (10nM Insulin)	-1.32 +/- 6.69	15.60 +/- 29.10 N.S.	10.30 +/- 22.52 N.S.
"Recovery": 60nM PGE_1 (1μM Insulin)	5.84 +/- 8.23	16.40 +/- 35.03 N.S.	26.42 +/- 25.14 p>0.05

Table 6.4. Quantitative Summary of Results.

	POSITIVE INSULIN EFFECT	POSSIBLE EFFECT	NO EFFECT
CONTROLS	3	2	7
IDDM	5	3	3
NIDDM	9	3	1

Table 6.5. Qualitative Summary of Results.

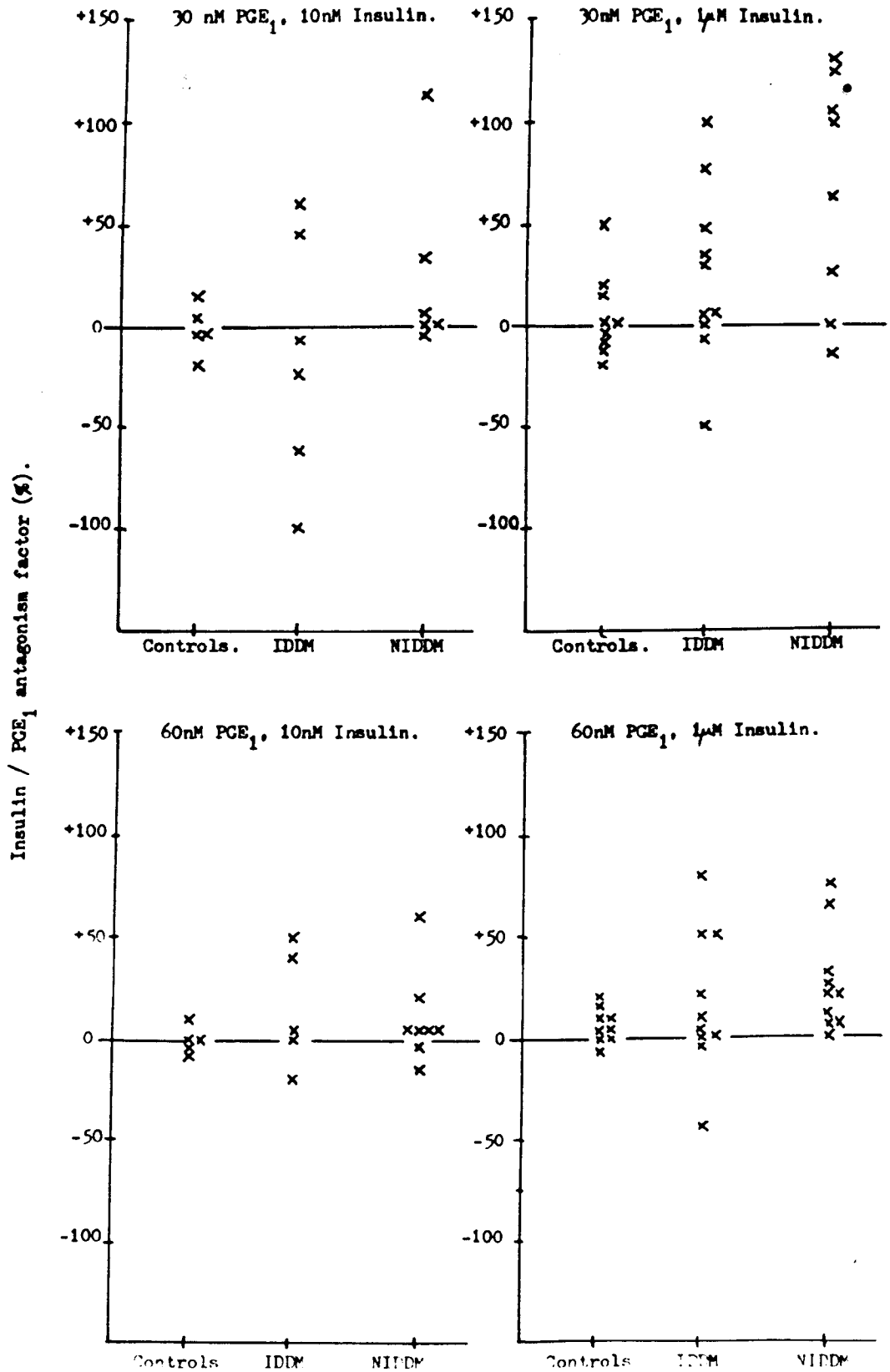


FIGURE 6.2: Summary of Results

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Chapter 7

SPONTANEOUS PLATELET AGGREGATION IN WHOLE BLOOD

7.1 ; Background

When investigating spontaneous or abnormal platelet aggregation, particularly *in vivo*, it is important not to forget the role played by the other cellular constituents of the blood. Preparation of PRP both increases the concentration of platelets relative to whole blood, and removes almost all the erythrocytes and white cells.

In addition to the documented platelet abnormalities seen in diabetes mellitus[38,39,45], there have been numerous reports of atypical red[121] and white corpuscle[116-120] properties and behaviour, some of which may have important haemostatic consequences in diabetes.

On stimulation by an appropriate agent, human neutrophils undergo the "respiratory burst". This consists of a massive increase in oxygen consumption by the cells, resulting in the production of a range of partially reduced "active" oxygen species, implicated in bacterial killing; singlet oxygen ($^1\Delta O_2$), peroxide radical ($HO\cdot_2$), hydroxyl radical ($OH\cdot$) and superoxide anion ($O_2^{-}\cdot$)[122]. In addition to their bactericidal activity, these substances are potentially responsible for tissue damage, such as induction of vascular permeability, destruction of endothelial cells, enzyme inactivation and platelet activation[122].

The effect of $OH\cdot$ on platelets has been well studied by Violi *et al*[123]. Platelets produce free radicals as by-products of the cyclo-oxygenase pathway and $OH\cdot$ scavengers significantly inhibit platelet aggregation, suggesting a self-activating role for free radicals produced by platelets. Hence, bacterial infection and neutrophil activation may play a part in platelet aggregation *in vivo*.

During the respiratory burst, neutrophils emit light, a phenomenon known as chemiluminescence. By measuring this light emission using a scintillation counter, Littarru's group[118,119] were able to show that neutrophils from diabetic patients gave a higher "resting" or unstimulated chemiluminescence and a lower stimulated response than non-diabetic controls. This behaviour was more evident in diabetics with recurrent infections. The persistant basal chemiluminescence of diabetic neutrophils suggests that they are

permanantly "poised" or partially activated *in vivo*, a condition possibly analagous with the "hypersensitive" platelets seen in the condition, resulting in a small but potentially important increase in circulating free radicals and the ensuing likelihood of tissue damage.

Other reported neutrophil abnormalities in diabetes include defective chemotaxis, adherence[117] and phagocytosis[120]. However , Valerius *et al*[116] have reported that there is no difference in chemotaxis of neutrophils between diabetics and controls. Similar inconsistences have been reported for other factors of the neutrophils bactericidal mechanism.

In addition to blockages caused by platelet aggregates, both red and white cells may occlude small blood vessels. Normally, the fluidity of erythrocyte and leukocyte membranes allows them sufficient deformability to pass through capillaries of smaller diameter than the cells themselves. A reduction in erythrocyte deformability has long been recognised as a side-effect of diabetes mellitus[45,121] and Vermes *et al*[120] have recently shown that leukocytes from diabetics show a decreased deformability similar to that seen in diabetic red cells. This phenomenon correlated well with diabetic microvascular disease.

Erythrocyte deformability appears to be directly linked to insulin concentrations since the abnormal deformability in IDDM can be rapidly reversed by good diabetic control achieved with an artificial pancreas[120]. Even when hyperglycaemia was maintained, insulin infusion was able to increase red cell deformability, indicating that low insulin, rather than high glucose is to blame for the abnormality[120]. However, it is unlikely that insulin will have much effect on the decreased erythrocyte deformability seen in NIDDM because these patients possess significantly fewer red cell insulin receptors than healthy controls[104]. In addition, diabetics have been shown to possess a defective erythrocyte membrane Na^+/K^+-ATPase with a significantly increased activity[124]. This activity is returned to normal by insulin therapy.

The potential role of erythrocytes in haemostasis and spontaneous platelet aggregation (SPA) has long been recognised. As long ago as 1961, Gaarder *et al*[11] identified ADP from erythrocytes as an important trigger for platelet activation. Much more recently, Goldsmith *et al*[125,126] have shown that SPA can occur in whole blood in the absence of red cell lysis or

platelet granule release. The activating factor was found to be ADP, suggesting a sublytic release from the erythrocytes themselves.

A number of observations at autopsy have suggested that platelet aggregates form *in vivo* and may have a patholgical role in sudden death in man[4]. In addition, small platelet aggregates may block smaller blood vessels such as retinal capilliaries, a factor believed to play a major role in the development of diabetic microangiopathy. Wu and Hoak[127] were the first to describe a method for quantifying platelet aggregates *ex vivo*. There have however, been frequent criticisms of the Wu and Hoak method ranging from artifactual formation of red cell debris, counted mistakenly by elctronic counters as platelets, to loss of certain platelet subpopulations by centrifugation during preparation of samples. Modified versions of the Wu and Hoak technique have described "circulating" platelet aggregates in a variety of clinical conditions including myocardial infarction, stroke, deep vein thrombosis, sickle cell anaemia and diabetes mellitus, but doubts still exist as to whether or not the aggregates actually exist *in vivo*, or are fomed during venupuncture[131].

If the latter is the case, then the presence of aggregates is more likely to represent the existence of "hypersensitive" or pre-activated platelets, which aggregate spontaneously and rapidly *ex vivo*.

Erythrocytes may have a second role to play in haemostasis. In addition to platelet aggregates, platelets have frequently been seen in aggregates with erythrocytes (see figure 7.1). The attatchment of platelets to erythrocytes could lead to drastic changes in the deformability of the red cell, causing occlusion of small blood vessels.

In vitro SPA occurs to a much greater extent in whole blood than in PRP[128-130]. Saniabadi *et al*[130] have shown that increasing the number of red cells present in relation to PRP increases the degree of SPA seen. In addition, this SPA can be partially inhibited by the presence of the ADP-destroying enzyme, apyrase[129] strongly implicating a red cell/platelet interaction and ADP release as important steps leading to SPA.

The following experiments seek to characterise SPA in whole blood in healthy controls and three classes of patients at risk of thrombotic episodes; diabetics, psoriasis sufferers[132], and people at risk of thrombosis taking the anticoagulant drug Warfarin[133].

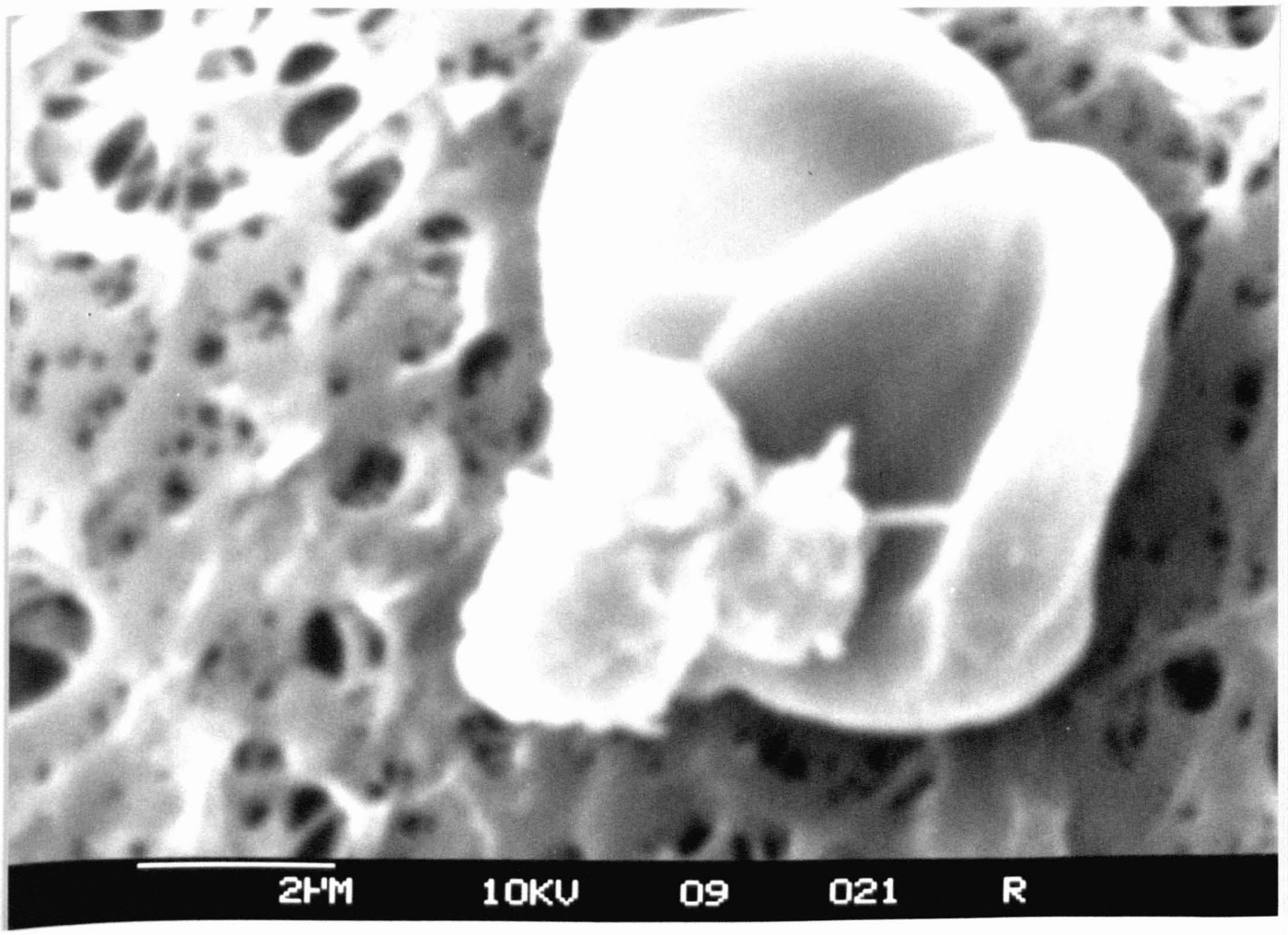


FIGURE 7.1: Electron micrograph of a platelet microaggregate in association with two erythrocytes.

7.2.1. : General Method

Whole blood samples were obtained from informed donors by venepuncture. Unless otherwise stated, all samples were collected into 3.8% citrate anticoagulant (final concentration 0.38%).

1.0 to 1.5ml aliquots of blood were removed, placed in siliconised, capped aggregometry cuvettes (LIP ltd.) and agitated by rolling or gentle stirring at 22 or 37^{o}C.

At regular intervals, the platelet numbers were measured by passing 100μl of each sample through the Technicon H*1 or Technicon H*1 Jr. whole blood haematological analysers at the Coventry and Warwickshire hospital.

The maximum degree of SPA is measured as the lowest number of "free" platelets seen during the experiment, and expressed as a percentage of the initial or highest number of free platelets seen during a paticular experiment.

7.2.2. : The Technicon H*1 Whole Blood Analyser

The H*1 counts red cells, white cells and platelets by sophisticated laser light-scattering techniques. The H*1 Jr., most frequently used during the following experiments lacks the H*1's ability to differentiate between the different types of white cells[133,134].

The performance characteristics of the H*1, from Technicon's own technical specifications, are given in table 7.1.

In addition to counts of the diferent types of blood cells, the H*1 provides histograms of red cell volume, haemoglobin concentration, and platelet volume.

The platelet counting method gives linearity from $10x10^{9}$/l to $700x10^{9}$/l.

PARAMETER (units)	LEVEL (mean)	WITHIN-RUN STANDARD DEVIATION	WITHIN-RUN STANDARD DEVIATION, 95%C.I.*
Red Cells ($x10^{12}/l$)	4.31	0.041	0.05
Platelets ($x10^{9}/l$)	97	3.7	4.5
Platelets ($x10^{9}/l$)	338	7.0	8.6
White cells ($x10^{9}/l$)	7.59	0.14	0.17

*, 95% Confidence Interval; The s.d. has a 95% probability of being smaller than this value.

Table 7.1. ; Technicon H*1 specifications

7.2.3. ; Reagents

3.8% trisodium citrate solution: Used in the ratio of 1ml to 9ml blood to give 0.38% citrated whole blood.

Sterile vacutainers (B.D. ltd.) 4.5ml volume:

(i) Citrate

(ii) Lithium Heparin

(iii) EDTA

Prostaglandin E_1 solution: 15μM stock, diluted to 150nM in blood, prepared fresh each day.

Adenosine diphosphate solution (Sigma): Special platelet aggregation reagent, reconstituted with water to give a buffered 0.2mM ADP solution.

Apyrase, grade V from potato (Sigma): 200 units/ml stock suspension.

Pyruvate kinase, type III (Sigma): 1000 units/ml stock suspension.

Phospho(enol)pyruvate, mono(cyclohexylammonium) salt (Sigma): 10mM stock solution, prepared fresh each day.

Insulin, from bovine pancreas (Sigma): 150 i.u./ml stock suspension, prepared fresh each day.

Lipopolysaccharide, from *Escherichia coli*, serotype 0111:B4 (Sigma): 0.5mg/ml stock solution.

Red blood cells, human : 4% RBC suspended in phosphate-buffered saline (Sigma);

(i) Group "O", glutraldehyde treated,

(ii) Group "O", trypsin and gluteraldehyde treated,

(iii) Group "O", neuraminidase and gluteraldehyde treated.

D-glucose (Fisons): 3.5M stock solution.

D-galactose (Sigma): 1.0M stock solution.

N-acetyl-glucosamine: 1.0M stock solution.

7.2.4. ; Reconstitution of "treated" red blood cell samples.

12.5ml of the 4% RBC suspension was centrifuged at 200g for 5 minutes to give approximately 0.4ml of "packed" red cells. The supernatant was removed and the pellet resuspended in 0.6ml of PRP from the appropriate donor.

7.3.: Results

7.3.1. ; Experiment 7.1. ; The temperature dependence of SPA in whole blood.

Citrated blood samples were obtained from three donors, AJS, RJB and BEPS, and aliquots of each were mixed at 22 and 37°C to establish the differences in SPA at each temperature.

Results

TIME (minutes)	PLATELET COUNTS ($x10^9/l$)					
	AJS		RJB		BEPS	
	22°	37°	22°	37°	22°	37°
15	165	151	211	218	215	216
30	155	152	202	216	227	214
45	148	116	205	199	208	196
60	150	110	191	193	199	181
90	147	96	198	91	189	174
135	-	-	187	155	187	150
180	142	95	186	143	-	-
195	-	-	-	-	192	109

Table 7.2. ; The temperature dependence of SPA.

These results are expressed graphically in figure 7.2.

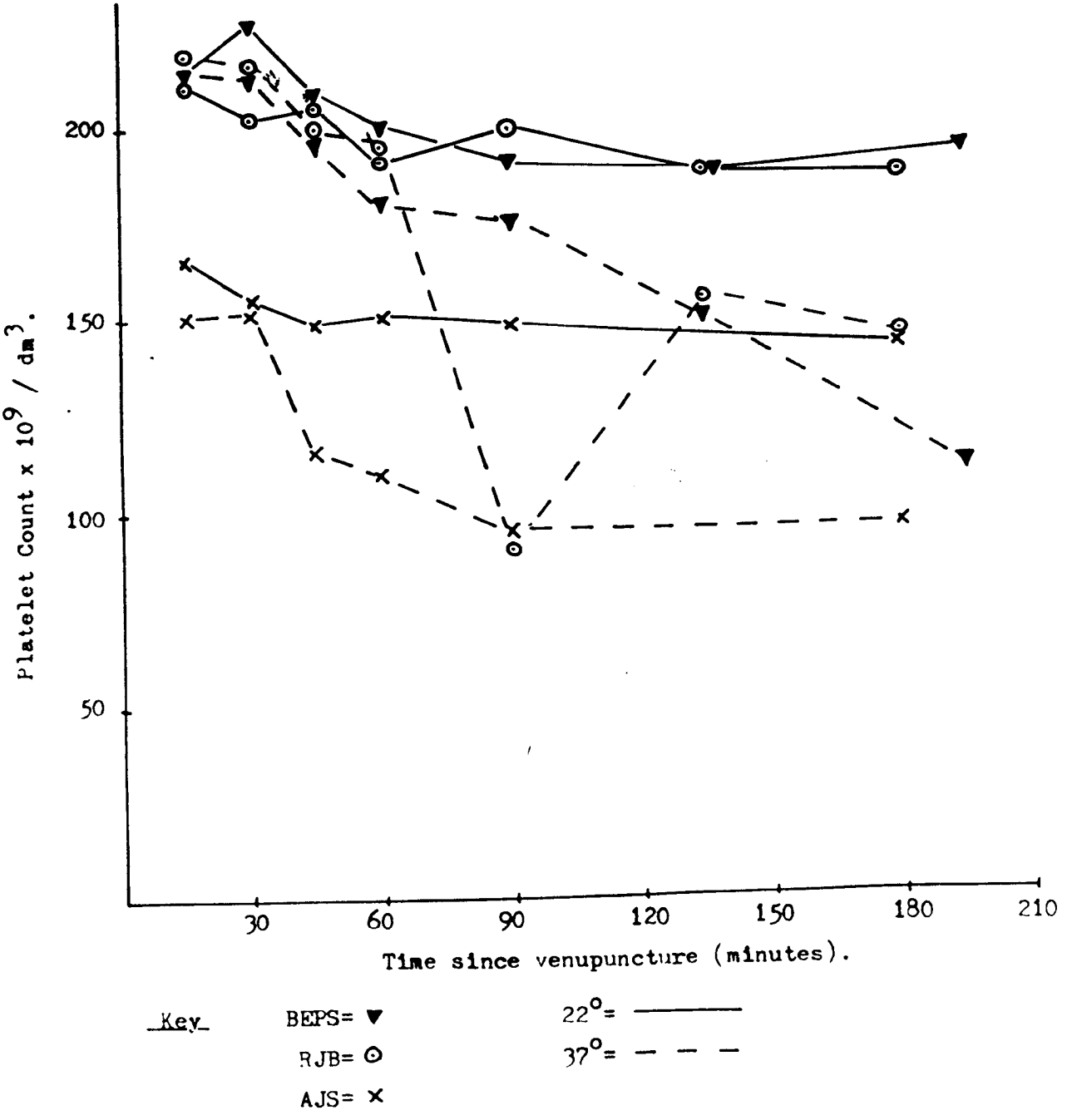
The degree of SPA in each sample was as follows;

AJS; $SPA_{max(22)}$= 13.9% $SPA_{max(37)}$= 37.7%

RJB; $SPA_{max(22)}$= 11.8% $SPA_{max(37)}$= 58.3%

BEPS; $SPA_{max(22)}$= 17.6% $SPA_{max(37)}$= 49.5%

FIGURE 7.2: A comparison of whole blood SPA at 22^{o} and $37^{o}C$ in 3 healthy controls.



Disscusion

Clearly, whole blood SPA occurs to a greater degree at physiological temperature rather than room temperature. In addition to the possiblity of enhanced sub-lytic release of ADP from red cells, the platelet's own metabolism will be most effective at 37°C. Hence, stimulated platelets will form and release secondary agonists more rapidly.

7.3.2. ; Experiment: Whole blood SPA in the presence of different anticoagulants.

7.3.2.1. ; A comparison of Citrate and Heparin anticoagulants.

Two blood samples were collected from the same donor, BEPS. One was collected into a standard citrate containing tube, the other into a heparinised tube. Aliquots of each were stirred at 37°C for 3 hours and H*1 counts were taken regularly.

Results

ANTICOAGULANT	TIME SINCE VENUPUNCTURE (minutes)				
	30	50	70	110	175
Citrate	177	153	84	64	70
Heparin	152	103	53	55	64

All platelet numbers $x10^9/l$.

Table 7.3. ; SPA in the presence of citrate and heparin anticoagulants.

Citrate: SPA_{max}= 63.8%

Heparin: SPA_{max}= 65.1%

Discussion

Hence, there appears to be little difference in the degree of SPA observed using these two anticoagulants. Possibly the heparin-treated sample shows a faster SPA than citrated blood.

7.3.2.2.; A comparison of citrate and EDTA anticoagulants.

(a) Blood samples were collected from donors AJS and BEPS (twice) into standard vials containing anticoagulants EDTA, citrate, or citrate plus added 150nM PGE_1. Each sample was split two ways and either rolled at 22°C or stirred at 37°C. Platelet counts were compared before and after 30 minutes of mixing.

Results

	Donor and date.					
SAMPLE	BEPS, 31.10.88		AJS, 31.10.88		BEPS, 1.11.88	
	20min.	50min.	20min.	50min.	20min.	50min.
EDTA stirred	223	226	145	176	221	248
EDTA rolled	218	200	148	150	215	198
Cit. stirred	58	149	150	140	193	160
Cit. rolled	47	132	147	142	196	165
Cit. stirred +PGE_1	198	287	153	148	234	228
Cit. rolled +PGE_1	153	247	150	142	220	221

All Platelet counts $x10^9/l$

Table 7.4. : The effect of EDTA and PGE_1 on SPA. Platelet numbers $x10^9/l$.

(b) Blood samples from donor AJS were collected into standard EDTA or citrate + PGE_1 (150nM) vials and aliquots were stirred at 37°C to investigate the difference between EDTA and PGE_1-dependent spontaneous platelet disaggregation.

Results

TIME(minutes)	CITRATE + PGE_1	EDTA
15	154	163
30	165	156
45	163	154
105	169	171
170	162	196

All Platelet counts $x10^9/l$

Table 7.5. ; Time dependance of PGE_1 and EDTA-induced spontaneous platelet disaggregation: Platelet numbers $x10^9/l$.

See also figure 7.3.

7.3.2.3. ; Discussion

The three types of anticoagulant investigated work by different methods. Heparin, a negatively-charged polysaccharide, enhances the inhibitory action of antithrombin III, which inactivates thrombin by forming an irreversible complex with it[3].In contrast, both citrate and EDTA work by binding calcium ions. Calcium (or coagulation factor IV) is involved at several sites in both the intrinsic and extrinsic pathways of coagulation. In addition, several stages of platelet activation and aggregation are Ca^{2+}-dependent (see section 1.4.).

It is clear from the results shown here that citrate and EDTA have very different effects on SPA. Whereas citrate, like heparin, allows SPA to occur, apparently unhindered, EDTA severely inhibits SPA or, under the right conditions, causes a spontaneous disaggregation of platelet aggregates.

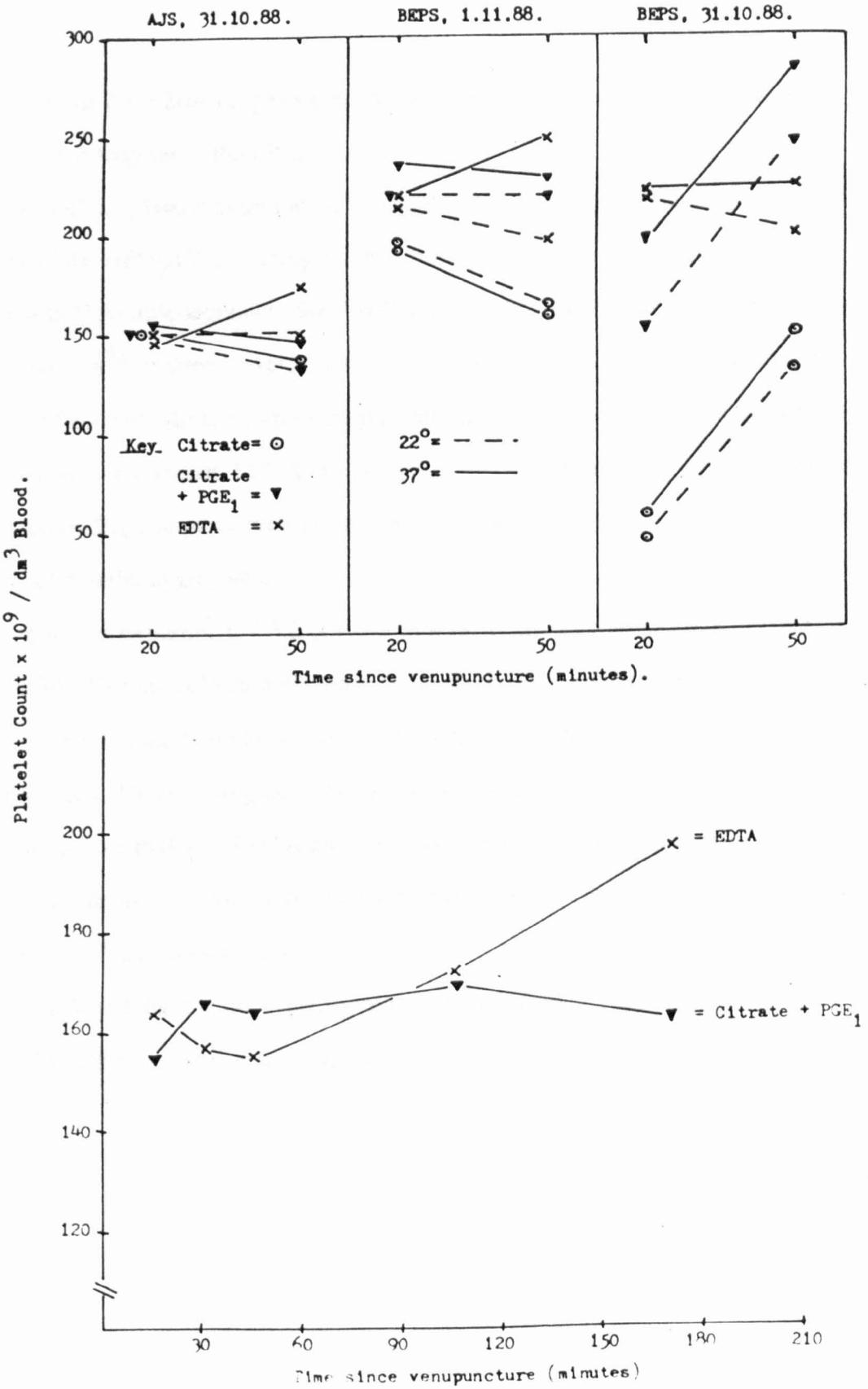


FIGURE 7.3: The effects of PGE_1 and EDTA on SPA.

Experiment 7.3.2.2(b) suggests that the capacity of EDTA to dissociate agregates is even greater, in the long term, than PGE_1.

EDTA inhibits platelet aggregation by tightly chelating all the plasma Ca^{2+} essential for formation of the $GPII_b/GPIII_a$ complex, the fibrinogen receptor. Hence, platelet aggregation in EDTA-anticoagulated blood will not pass the "shape-change" phase. Although citrate also binds Ca^{2+}, it does so very weakly in comparison to EDTA. Hence, Ca^{2+} is easily removed from the citrate complex by platelets and can be used for formation of the fibrinogen receptor. In contrast, EDTA is a powerful enough chelator of Ca^{2+} to remove it from the $GPII_b/GPIII_a$ complex, even when fibrinogen is bound. Hence, EDTA is able to dissociate small platelet aggregates.

The results of experiment 7.3.2.2(a) are somewhat anomalous. Despite the two samples from BEPS being collected on consecutive days, great differences in platelet behaviour are seen. On the first day, a tremendous degree of SPA had occured even before mixing commenced. These aggregates were obviously very transient since gentle mixing, even in the absence of PGE_1 or EDTA caused a massive disaggregation. On the second day, blood taken from the donor's other arm behaved perfectly normally, very much as the sample from donor AJS had the previous day.

It was deduced that the poor quality of the earlier platelets from donor BEPS was due to defective phlebotomy, possibly a collapsed vein.

7.3.3. ; Further studies on whole blood SPA.

7.3.3.1 ; A longtitudinal study to determine individual variations in SPA.

Citrated whole blood was obtained from donors AJS and BEPS on numerous occasions over an eight month period. The degree of SPA was ascertained after mixing at either 22 or 37°C.

Results

Donor AJS ; age 23/24.

DATE	EXPERIMENTAL TEMPERATURE	INITIAL PLATELET COUNT ($x10^9/l$)	%SPA	TIME FOR MAXIMUM SPA (minutes)
29.09.88	37°	142	15.5	165
07.10.88	37°	112	22.3	45
14.10.88	37°	157	37.6	90
07.11.88	37°	151	37.1	180
22.11.88	37°	168	28.6	120
11.01.89	37°	171	32.2	100
05.05.89	37°	138	31.1	120
31.10.88	22°	147	3.4	90
07.11.88	22°	165	13.9	180
28.04.89	22°	153	9.1	50
21.06.89	22°	158	8.9	90

Table 7.6. ; SPA. individual variation in donor AJS.

Donor BEPS ; age 50/51

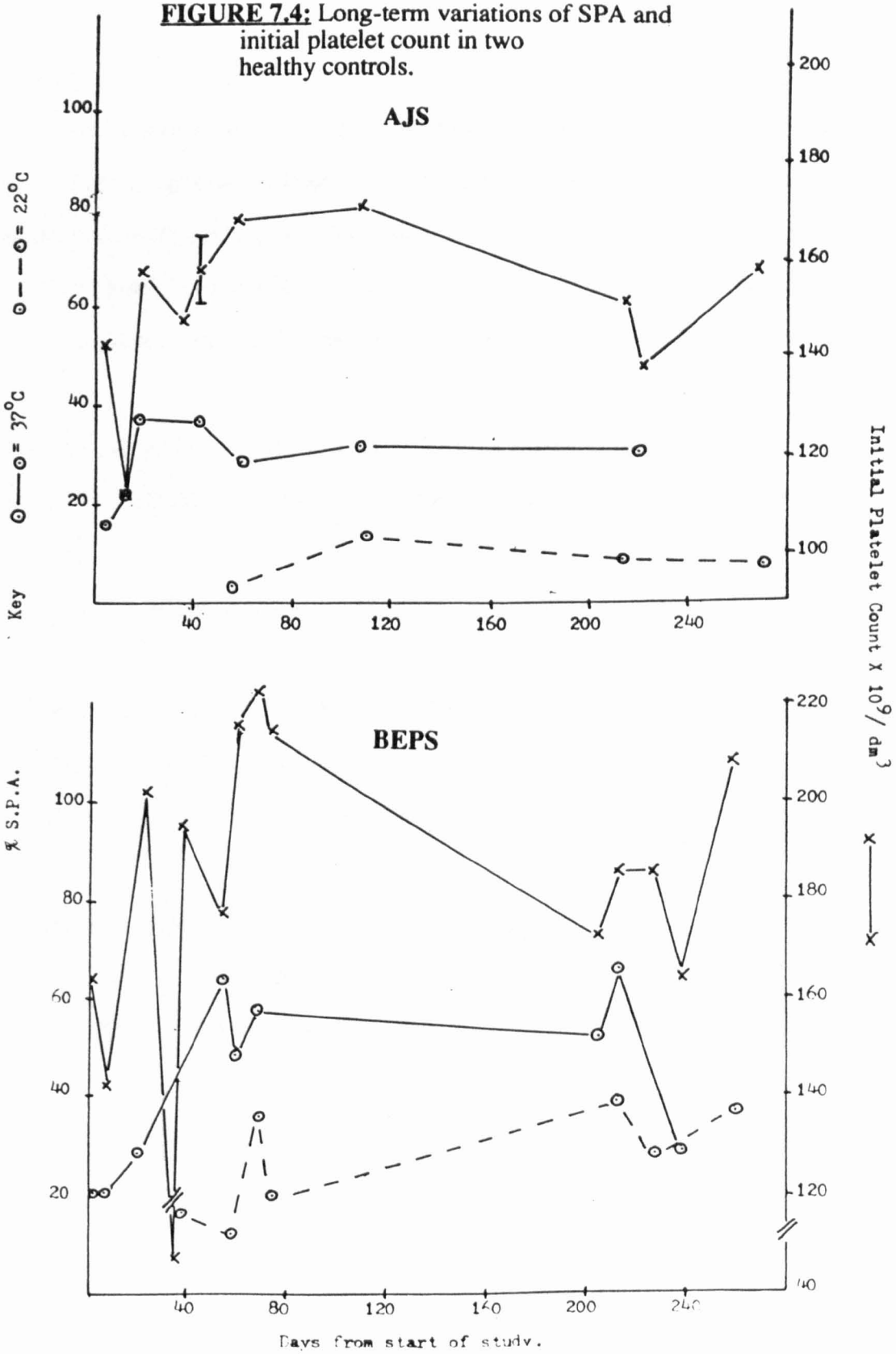
DATE	EXPERIMENTAL TEMPERATURE	INITIAL PLATELET COUNT ($x10^9/l$)	%SPA	TIME FOR MAXIMUM SPA (minutes)
26.09.88	37°	164	20.1	180
03.10.88	37°	142	20.4	180
18.10.88	37°	202	27.7	160
18.11.88	37°	177	63.8	110
24.11.88	37°	216	48.6	195
01.12.88	37°	224	57.7	200
17.04.89	37°	173	52.6	100
24.04.89	37°	112	66.5	120
22.05.89	37°	164	28.0	120
31.10.88	22°	47	-*	0
01.11.88	22°	196	15.8	50
24.11.88	22°	215	11.8	135
01.12.88	22°	222	35.5	120
06.12.88	22°	209	21.5	180
24.04.89	22°	186	39.2	120
09.05.89	22°	186	28.0	120
09.06.89	22°	208	37.5	110

* see section 7.3.2.3.

Table 7.7. : SPA, individual variation in donor BEPS.

These results are expressed graphically in figure 7.4.

FIGURE 7.4: Long-term variations of SPA and initial platelet count in two healthy controls.



Discussion

Figure 7.4 shows the variations in platelet number and SPA of the two control donors. Clearly there are large fluctuations in both SPA and platelet number. Platelet concentrations are kept relatively constant *in vivo* by the hormone thrombopoietin, which stimulates the formation of new platelets from megakaryocytes when needed[3,4]. Many of the fluctuations are undoubtedly due to the presence of some platelets as microaggregates (see section 7.3.4.1.).

SPA at 37°C is consistently higher than at 22°C as expected, and is paticularly consistent in donor AJS. Donor BEPS shows rather more variability in both platelet number and degree of SPA.

The mean values for platelet number and SPA are as follows;

AJS Platelet number = 151.1 +/- 16.6 x10^9/l (n=11)

SPA, 37°C = 8.8 +/- 4.3 % (n=4)

SPA, 22°C = 29.2 +/- 8.0 % (n=7)

BEPS Platelet number = 192.3 +/- 24.5 x10^9/l (n=16)

SPA, 37°C = 27.0 +/- 10.9 % (n=7)

SPA, 22°C = 42.8 +/- 18.8 % (n=9)

7.3.3.2. : SPA in diabetic patients.

Method as for section 7.3.3.1.

Results

PATIENT	AGE	TYPE	INITIAL PLATELET COUNT ($x10^9/l$)	EXPERIMENTAL TEMPERATURE	%SPA	TIME FOR MAX. SPA (minutes)
H3	71	N+	162	37°	39.5	165
H4	46	I+++	221	37°	3.2	160
H5	46	I+	308	37°	38.0	175
H6	61	I++	146	37°	34.9	105
H7	78	N+	163	37°	46.0	170
H8	71	N+	224	37°	68.3	75
H11*	56	I+++	379	37°	30.6	180
H12	40	I+	186	37°	41.4	60
A14	35	I+++	546	37°	14.3	80
R1	64	N-	192	37°	41.0	130
H5	46	I+	262	22°	8.0	175
H6	61	I++	198	22°	10.6	105
H8	71	N+	201	22°	42.7	75
H11*	56	I+++	371	22°	10.2	180
R1	64	N-	207	22°	28.0	45
J1	80	I++	198	22°	23.7	120
J2	63	I+	251	22°	32.7	115
J3*	57	I+	329	22°	37.1	120
J4	72	I+	180	22°	36.7	120
J6	46	I++	168	22°	23.8	90
J7*	70	N++	291	22°	21.6	80
J8	30	I++	162	22°	23.4	90

Table 7.8. : Whole blood SPA in diabetic patients.

Key: N = NIDDM, I = IDDM, - = no complications,

+ = background retinopathy, ++ = severe retinopathy,

+++ = retinopathy plus proteinuria.

* = on adalat.

7.3.3.3. : SPA in non-diabetic patients at risk of thrombosis (taking warfarin).

Method as for section 7.3.3.1.

Results

PATIENT	AGE	CONDITION	INITIAL PLATELET COUNT ($x10^9/l$)	EXPERIMENTAL TEMPERATURE	%SPA	TIME FOR MAX. SPA (mins.)
HQ	60	F.E.	306	37°	34.0	165
VT	74	DVT, F.E.	218	37°	34.4	45
LO	32	P.E.	365	22°	37.8	90
JS	72	P.E.	190	22°	75.3	90
AMF	24	P.E.	220	22°	38.2	180
JM	59	P.E.	309	22°	28.5	130
HQ	60	F.E.	306	22°	16.0	165
JC	56	DVT	341	22°	53.1	90

Table 7.9. ; Whole blood SPA in patients taking Warfarin.

Key : F.E.= Femoral embolism

P.E.= Pulmonary embolism

DVT= Deep vein thrombosis

7.3.3.4. ; SPA in psoriasis patients.

Method as section 7.3.3.1.

Results

PATIENT	AGE	DEGREE OF PSORIASIS	INITIAL PLATELET COUNT ($x10^9/l$)	EXPERIMENTAL TEMPERATURE	%SPA	TIME FOR MAX. SPA (mins.)
DLS	60	Mild	187	22°	5.9	60
BW	64	Mild	204	22°	22.0	135
CP	38	Severe	270	22°	33.0	105
WW	58	Severe	277	22°	30.7	75

Table 7.10. ; Whole blood SPA in psoriasis patients.

7.3.3.5. ; Discussion.

All three groups of patients investigated have previously been reported to show enhanced platelet activation both *in vivo* and *in vitro*[45,132,133]. Considerable differences between individuals exists in both platelet number and SPA. Owing to the infrequency of patient's visits to out-patient clinics, it was not possible to study any of the patients on more than one occassion and so it was not possible to establish whether, for example, diabetic H8 consistently shows over 60% SPA, or if there were any increases in SPA with progression of vascular disease.

The mean results for each group of patients were as follows;

Diabetics Age = 58.0 +/- 15.2 years (n=17)

Platelet no. = 242.9 +/- 95.9 $x10^9/l$ (n=22)

$SPA_{max(22)}$ = 24.9 +/- 11.3 % (n=12)

$SPA_{max(37)}$ = 35.7 +/- 17.6 % (n=10)

Warfarin Age = 53.9 +/- 19.0 years (n=7)

Platelet no. = 281.5 +/- 63.8 $x10^9/l$ (n=8)

$SPA_{max(22)}$ = 41.5 +/- 20.6 % (n=6)

$SPA_{max(37)}$ = 34.2 +/- 0.2 % (n=2)

Psoriasis Age = 55.0 +/- 11.6 years (n=4)

Platelet no. = 234.5 +/- 45.6 $x10^9/l$ (n=4)

$SPA_{max(22)}$ = 22.9 +/- 12.3 % (n=4)

The equivalent results from multiple assays of two healthy controls are given in section 7.3.3.1.

7.3.4. ; The effects of PGE_1 on whole blood SPA.

7.3.4.1. A longtitudinal study to determine variations in platelet microaggregates.

Citrated whole blood samples were obtained from donors AJS and BEPS on numerous occasions over a two month period. The degree of platelet disaggregation in the presence of 150nM PGE_1 was established and the number of platelets present as microaggregates expressed as;

$$\frac{\text{Maximum platelet no. - Initial platelet no.}}{\text{Maximum Platelet no.}}$$

Results

Donor BEPS; age 50.

DATE	EXPERIMENTAL TEMPERATURE	INITIAL PLATELET COUNT ($x10^9/l$)	MAXIMUM PLATELET COUNT ($x10^9/l$)	%MICRO-AGGREGATES	TIME FOR MAXIMUM DISAGG-REGATION (minutes)
26.09.	37°	152	191	20.4	180
03.10.	37°	142	183	22.4	15
18.10.	37°	199	205	3.0	160
01.11.	37°	196	234	16.2	15
01.11.	22°	196	221	11.3	50

Table 7.11. ; Microaggregates, individual variations in donor BEPS.

Donor AJS ; age 23.

DATE	EXPERIMENTAL TEMPERATURE	INITIAL PLATELET COUNT ($x10^9/l$)	MAXIMUM PLATELET COUNT ($x10^9/l$)	%MICRO-AGGREGATES	TIME FOR MAXIMUM DISAGG-REGATION (minutes)
31.09.	37°	142	146	2.7	15
07.10.	37°	106	142	25.3	45
14.10.	37°	157	174	9.8	45
31.10.	37°	147	153	3.9	15
14.11.	37°	154	169	8.9	105
31.10.	22°	147	150	2.0	15

Table 7.12. ; Microaggregates, individual variations in donor AJS.

7.3.4.2. Microaggregates in diabetic patients.

Method as for section 7.3.4.1.

Results

PATIENT	AGE	DEGREE OF MICRO-ANGIOPATHY	EXPT. TEMP.	INITIAL PLATELET COUNT ($x10^9/l$)	MAXIMUM PLATELET COUNT ($x10^9/l$)	%MICRO-AGGREGATES
H3	71	++	37^o	162	253	36.0
H4	46	+++	37^o	221	221	0
H5	46	+	37^o	308	308	0
H6	61	++	37^o	146	257	43.2
H7	78	+	37^o	162	185	12.4
H10*	71	++	37^o	342	342	0
H11*	56	+++	37^o	398	420	5.2
H6	61	++	22^o	198	239	17.1
H7	78	+	22^o	162	165	1.8

Table 7.13. : Microaggregates from diabetic donors.

* = Patients taking Adalat

7.3.4.3. Microaggregates in patients at risk of thrombosis (taking Warfarin).

Method as in section 7.3.4.1.

Results

PATIENT	AGE	EXPT. TEMP.	INITIAL PLATELET COUNT ($x10^9/l$)	MAXIMUM PLATELET COUNT ($x10^9/l$)	%MICRO-AGGREGATES
LO	32	37^o	365	384	4.9
JS	72	37^o	190	211	11.0
AMF	24	37^o	220	239	7.9
JM	59	37^o	308	308	0

Table 7.14. ; Microaggregates from warfarin-taking patients.

7.3.4.4. ; Discussion.

Platelet microaggregates, formed either *in vivo*, or during venupuncture, will not be counted by the Technicon H*1. However, their presence may be an important indicator towards the possibility of thrombotic episodes.

Splawinska *et al*[138,139] have previously reported that prostacyclin was able to increase platelet numbers in whole blood. The experiments reported here employed the cheaper, more stable analogue, PGE_1 to dissociate aggregates. Mean results at 22^o and 37^o were as follows;

Control AJS: Age = 23

$\%\ Microaggregates_{(22)} = 2.0\ (n=1)$

$\%\ Microaggregates_{(37)} = 10.1 +/- 9.0\ (n=5)$

Control BEPS: Age = 50

% Microaggregates$_{(22)}$ = 11.3 (n=1)

% Microaggregates$_{(37)}$ = 15.5 +/- 8.7 (n=4)

Diabetics: Age = 61.3 +/- 12.7 (n=7)

% Microaggregates$_{(22)}$ = 9.45 +/- 10.8 (n=2)

% Microaggregates$_{(37)}$ = 13.8 +/- 18.3 (n=7)

Warfarin: Age = 46.8 +/- 22.5 (n=4)

% Microaggregates$_{(37)}$ = 5.95 +/- 4.7 (n=4)

The results appear to show little difference between the different groups of donors. However, the occurence of PGE_1-insensitive microaggregates must be considered. These microaggregates, possibly formed by ADP-independent SPA (see section 7.3.6), may be increased in number in the various pre-thrombotic states. Experiment 7.3.2. demonstrated that PGE_1 lacks the ability to dissociate all microaggregates since EDTA was more effective. Hence, a more complete study might include the time-dependent increase of platelet number in EDTA-anticoagulated blood.

7.3.5.: Experiment: The Effect of Bacterial Lipopolysaccharide on SPA.

Citrated whole blood was obtained from donor GMW. Samples were mixed at 22°C in the presence of increasing doses of LPS (0, 50, 500, 5000 ng/ml).

Results

TIME (mins.)	LIPOPOLYSACCHARIDE CONCENTRATION			
	0	50ng/ml	500ng/ml	5000ng/ml
15	185	156	161	164
55	167	143	124	133
80	152	158	127	130

Platelet Counts ($x10^9/l$)

Table 7.15.: Bacterial LPS: Effect on SPA.

The degree of SPA in each sample was as follows;

0ng/ml LPS = 17.8%

50ng/ml LPS = 22.7%

500ng/ml LPS = 33.0%

5000ng/ml LPS = 29.7%

(assuming initial platelet count to be 185 in all samples)

Discussion

Gram-negative bacteria produce an endotoxin that is a lipopolysaccharide (LPS) composed of three principal regions; the O-polysaccharide, the R-core oligosaccharide, and the lipid, A. The structure of the main toxic component, lipid A, is highly conserved among Gram-negative bacteria. In contrast, the O-side chains are highly diverse and also highly mutable, enabling the bacteria to stay one step ahead of the host's defense systems[6].

Sepsis due to Gram-negative bacteria is often complicated by disseminated intravascular coagulation and thrombocytopenia. Csako *et al*[92] have reported that purified endotoxin is able to cause a slowly developing platelet aggregation in citrated whole blood *in vitro*, but not in PRP, suggesting an indirect activation of platelets by endotoxin. This type of SPA is similar to the "non ADP-dependent" SPA described in section 7.3.6.

The paticular LPS employed in this experiment, from *E.coli* serotype 0111:B4, has been shown to initiate aggregation of platelets in plasma via the generation of thrombin at the surface of monocytes[92]. However, Davis and Johnstone[92] reported that *E.coli* 0111 LPS actually blocks aggregation in whole blood. 0111 LPS in this survey causes up to 15.2% additional SPA compared to untreated whole blood. This result may indicate that susceptibility to endotoxin varies from individual to individual. However, there seems little doubt that bacterial endotoxins can cause platelet activation *in vitro*. If these events are repeated *in vivo*, then bacterial infection might be expected to increase circulating microaggregates.

In view of the increased susceptibility of diabetic patients to infections, it is possible that the infectious agent itself could play an important role in the development of diabetic microangiopathies.

7.3.6. ; Experiment: Is ADP responsible for whole blood SPA?

7.3.6.1. The effect of potato apyrase on whole blood SPA.

(a) Two citrated blood samples were obtained from donor AJS. One sample was untreated. To the other was added apyrase to a final concentration of 0.27mg/ml blood. Both samples were stirred at 37°C.

Results

TIME (minutes)	PLATELET COUNT ($x10^9/l$)	
	-Apyrase	+Apyrase
15	165	174
30	168	155
45	160	151
60	157	158
90	143	150
120	120	143
180	120	143

Table 7.16. ; Donor AJS: The effect of Apyrase on SPA.

These results are expressed graphically in figure 7.5.

% SPA in absence of apyrase = 28.6%

% SPA in presence of apyrase = 17.8%

(b) Citrated blood samples were obtained from donpor BEPS on three occassions over a period of two weeks and the relative effect of apyrase at 37°C and 22°C was established.

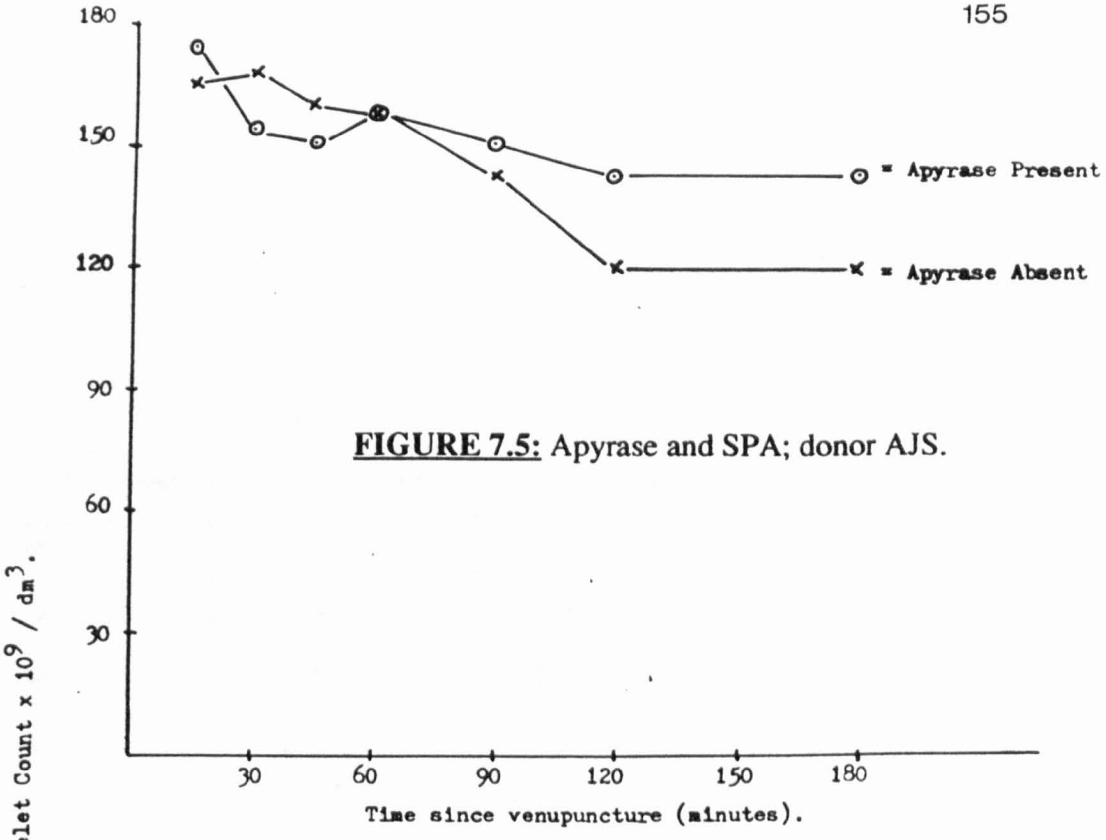


FIGURE 7.5: Apyrase and SPA; donor AJS.

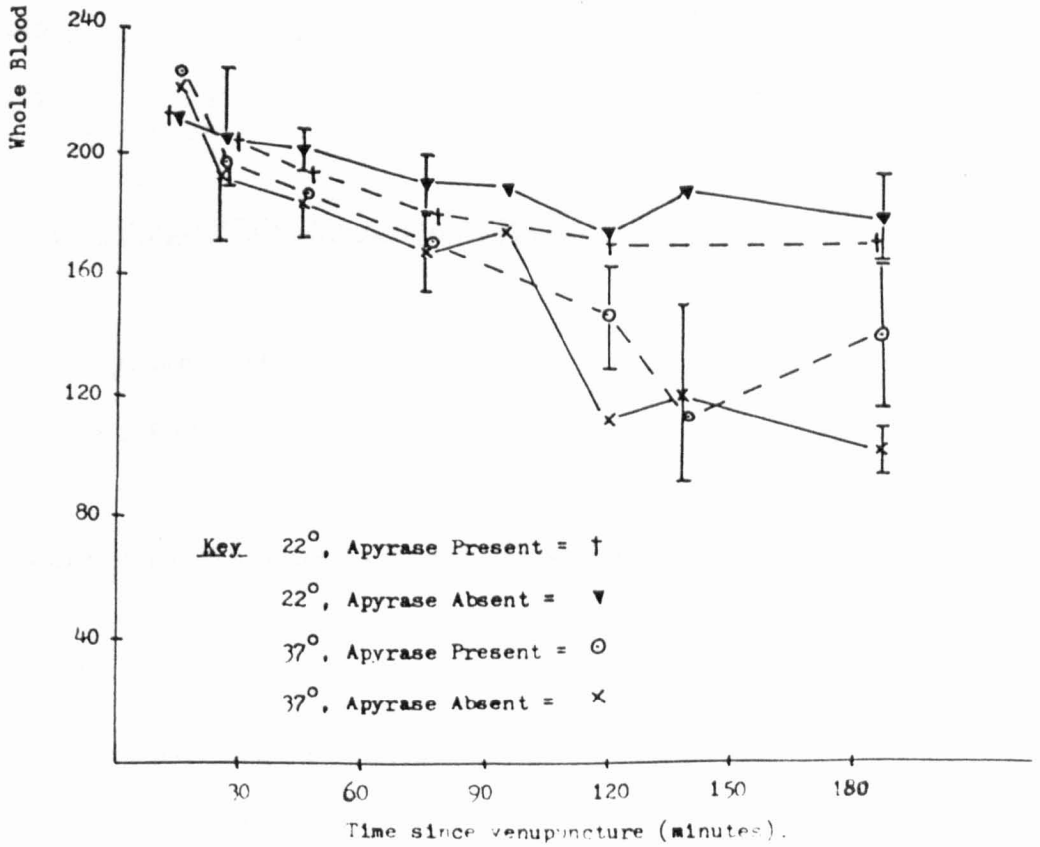


FIGURE 7.6: Apyrase and SPA; donor BEPS.

Results

(i)37^{o}C

TIME (mins.)	PLATELET COUNT (x10^{9}/l)			
	-A.(24.11.88)	-A.(1.12.88)	+A.(1.12.88)	+A.(6.12.88)
10-15	216	224	222	227
20-30	214	170	188	202
45-50	196	171	185	189
70-80	181	155	168	173
95	174	-	-	-
120	-	113	130	163
135-140	150	92	113	-
180-195	109	94	116	164

Table 7.17. : Donor BEPS: Apyrase and SPA at 37^{o}C.

Key: -A = Apyrase absent

+A = Apyrase present

% SPA in absence of apyrase (i) Date, 24.11.= 49.5%

(ii) Date, 01.12.= 58.9%

% SPA in presence of apyrase (i) Date, 01.12.= 47.7%

(ii) Date, 06.12.= 27.7%

(ii)22°C

TIME (mins.)	PLATELET COUNT ($x10^9/l$)			
	-A.(24.11.88)	-A(1.12.88)	-A(6.12.88)	+A(6.12.88)
10-15	215	222	209	212
20-30	227	197	180	204
45-50	208	175	195	193
70-80	199	147	181	180
95	189	-	-	-
120	-	145	174	169
135-140	187	145	-	-
180-195	192	143	164	168

Table 7.18. ; Donor BEPS: Apyrase and SPA at 22°C.

% SPA in absence of apyrase (i) Date, 24.11.= 13.0%

(ii) Date, 01.12.= 21.5%

(iii) Date, 06.12.= 35.6%

% SPA in presence of apyrase , Date, 06.12. =20.7%

See also figure 7.6.

Discussion

Potato apyrase has an ATPase activity as well as an ADPase activity. It catalyses the reaction;

$$ATP \longleftrightarrow ADP + P_i \longleftrightarrow AMP + 2P_i$$

Hence, any ATP or ADP released from platelets or erythrocytes will be rapidly converted to non-aggregatory AMP by the apyrase.

Figure 7.5 shows that apyrase is indeed capable of inhibiting SPA. In the absence of apyrase, a further 10.8% aggregation occurs. This may be termed **"ADP-dependent SPA"**, whereas the 17.8% aggregation, in the presence of apyrase is presumably **"ADP-independent SPA"**. Figure 7.6 shows the mean SPA in the presence and absence of apyrase at 37° and 22°C. These results, averaged out from three blood samples collected on different days appear to indicate very little inhibition of SPA by apyrase. However, the behaviour of the platelets varies from day to day. This is most clearly shown by a comparison of SPA in the absence of apyrase at 22°C on each day.

A comparison of samples collected with or without apyrase on the same day indicates that inhibition of SPA is occuring, but not to a significant degree. At 37°(1.12.88), ADP-dependent SPA is 47.7% and ADP-independent SPA 11.2%. In contrast, the SPA at 22°(6.12.88) appears to be almost entirely ADP-independent.

The use of potato apyrase to destroy ADP presented some unexpected problems. The experiment of 6.12.88 showed a considerable, time-dependent agglutination of erythrocytes in the presence of apyrase at both 37° and 22°C (see figure 7.7). This behaviour was clearly due to some contamination of the apyrase itself.

Matsumoto *et al*[140] have described and isolated a lectin from potato, solanum tuberosum agglutinin (STA) which recognises a specific sugar sequence, namely tri-(N-acetyl-glucosamine). The dimeric STA recognises this sequence and causes agglutination. "Pure" and "crude" STAs were purchased from Sigma and their ability to agglutinate a 2% suspension of erythrocytes (donor BEPS) was determined.

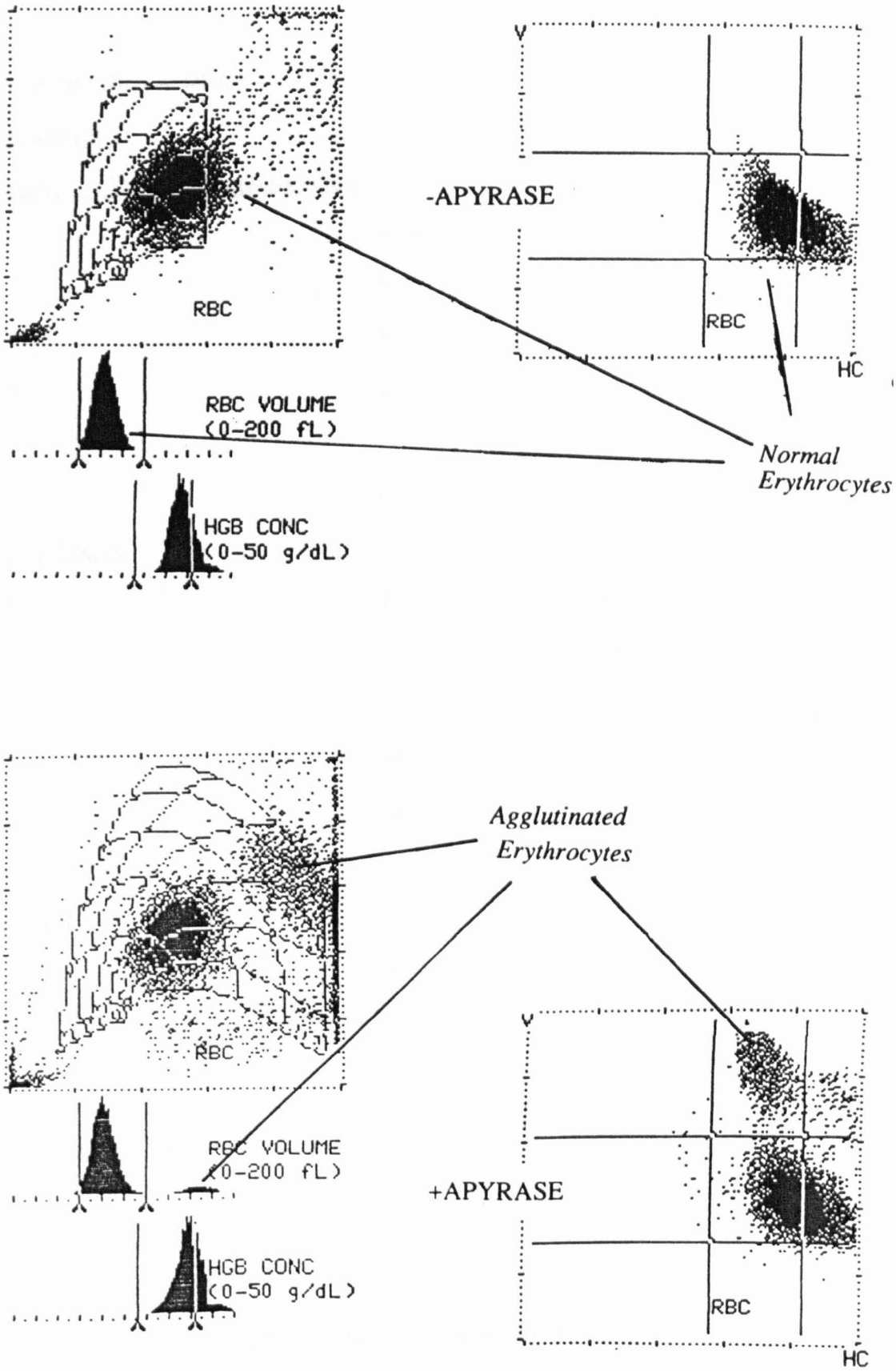


FIGURE 7.7: Technicon H*1 profiles showing abnormal red cell distribution in sample +A.(t=120) at 22°C.

Results are shown below;

Pure Lectin

Concentration	Degree of agglutination
10µg/ml	+++
5 µg/ml	++
2.5 µg/ml	+
1.25 µg/ml	+
1 µg/ml	+/-
0.1 µg/ml	-

Crude Lectin

100 µg/ml	++
50 µg/ml	+
40 µg/ml	+
30 µg/ml	+/-
25 µg/ml	+/-
10 µg/ml	-

Apyrase

500 µg/ml	+++
50 µg/ml	++
10 µg/ml	+
5 µg/ml	+
2.5 µg/ml	+/-
1 µg/ml	-

These results clearly show that potato apyrase is contaminated with STA to such a level that the agglutinating capacity of apyrase is actually greater than that of the "crude" commercial preparation.

This contamination of apyrase could potentially pose problems for the study of SPA since the agglutinin could also recognise and bind platelet membrane glycoproteins, causing

an agglutination of platelets, as opposed to a true aggregation, masking apyrase's ADP-destroying effects.

A second potential problem in the use of apyrase for preventing ADP-dependent SPA is that ATP acts as a substrate for the enzyme. Both platelets and erythrocytes contain 5 to 10 times as much stored ATP as ADP. Any release of ADP will be accompanied by a concommitant release of ATP. This ATP will be rapidly converted to AMP, but there will be a transient ADP formation during this process which could activate platelets.

For these reasons, a second ADP-destroying system was investigated.

7.3.6.2.: The effects of Pyruvate kinase and Phospho(enol)pyruvate on whole blood SPA.

(a) Citrated whole blood was obtained on two occassions, 7 days apart, from the same donor (AJS). Samples were mixed at either 22° or 37°C in the presence or absence of 20 units/ml pyruvate kinase (PK) and 100μM phospho(enol)pyruvate (PEP).

After two hours mixing, 5 or 10μM ADP was added to test the effectiveness of the ADP-destroying system.

Results

TIME	28.4.89(22°C)		5.5.89(37°C)		
(mins.)	-PK/PEP	+PK/PEP	+PK/PEP	-PK/PEP	+PK/PEP
15	153	147	136	138	136
35	145	159	152	100	137
50-55	139	149	148	105	138
80-85	149	148	150	96	140
120	142	146	143	95	142
ADP added	5μM	5μM	10μM	5μM	5μM
130	7	144	133	39	124

Table 7.19. The effect of PK/PEP on SPA: Donor AJS.

These results are expressed graphically in figure 7.8.

% SPA -$PK/PEP_{(22)}$ = 9.1%

+$PK/PEP_{(22)}$ (i) = 2.7%

+$PK/PEP_{(22)}$ (ii)= 5.9%

-$PK/PEP_{(37)}$ = 31.1%

+$PK/PEP_{(37)}$ = 0

Discussion

As expected, the 37° sample shows considerably more SPA than the 22° sample. Pyruvate kinase, which converts ADP to ATP;

$$ADP + PEP \xrightarrow{\text{pyruvate kinase}} ATP + Pyruvate$$

almost totally abolishes SPA. The effectiveness of the ADP-destroying system is shown by the lack of response to 5 or 10μM ADP in the samples treated with PK/PEP. These results indicate that the SPA seen in this donor's blood is almost totally of the ADP-dependent type.

(b) Citrated whole blood was obtained from donor BEPS. Samples were mixed at either 22° or 37°C in the presence or absence of 20 units/ml PK and 100μM PEP. After two hours of mixing, 2μM ADP was added to test the effectiveness of the ADP-destroying system.

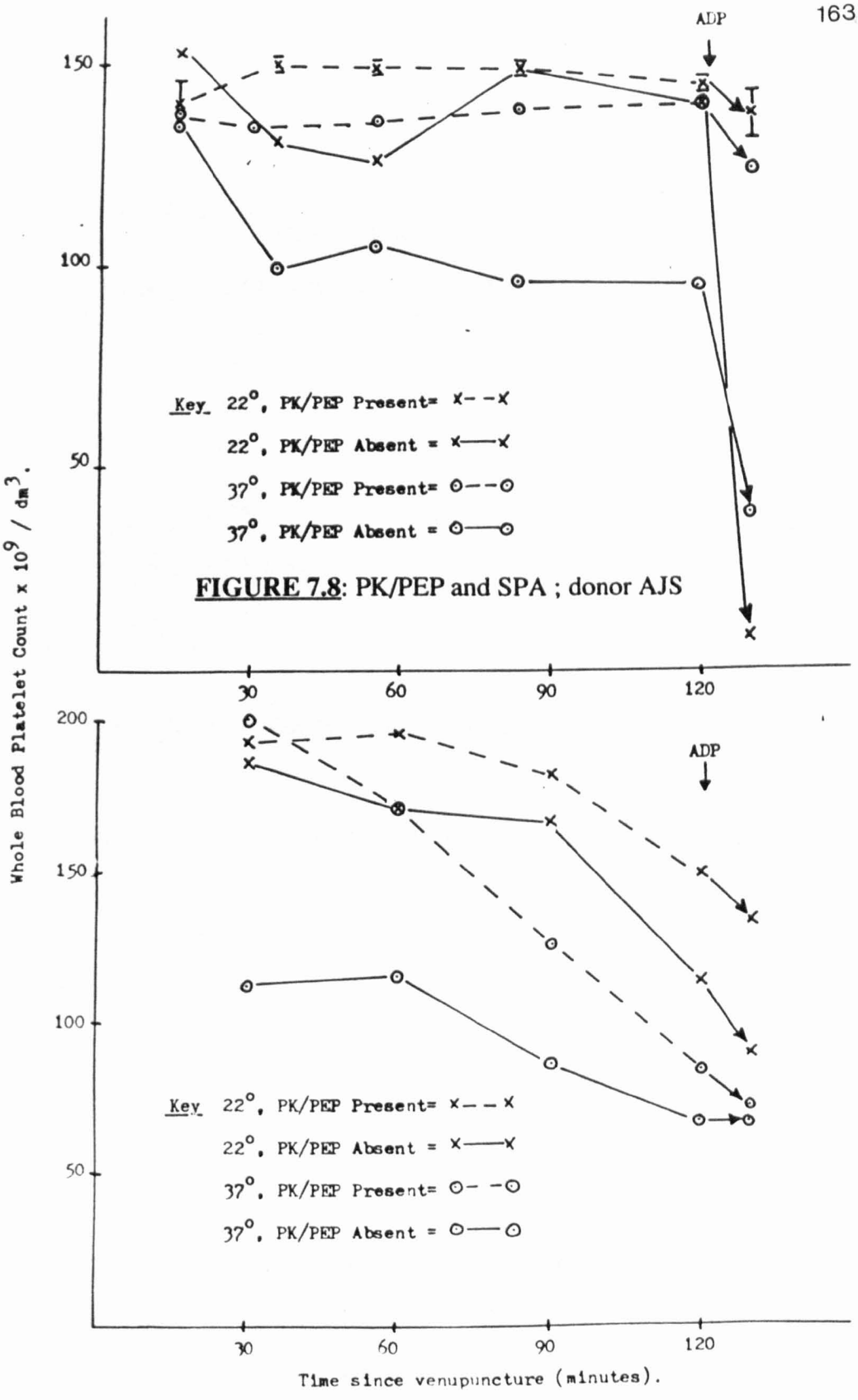


FIGURE 7.8: PK/PEP and SPA ; donor AJS

FIGURE 7.9: PK/PEP and SPA; donor BEPS

Results

TIME (mins.)	PLATELET COUNT ($x10^9/l$)			
	-PK/PEP(22^o)	+PK/PEP(22^o)	-PK/PEP(37^o)	+PK/PEP(37^o)
30	186	192	112	200
60	172	195	115	172
90	166	181	87	126
120	113	149	67	84
2μM ADP added to each sample.				
130	90	133	67	71

Table 7.20. ; The effect of PK/PEP on SPA: Donor BEPS.

These results are expressed graphically in figure 7.9.

% SPA -$PK/PEP_{(22)}$= 43.5%

+$PK/PEP_{(22)}$= 25.5%

-$PK/PEP_{(37)}$= 66.5%

+$PK/PEP_{(37)}$= 58.0%

Discussion

The lower dose (2μM) of ADP employed to test the PK/PEP efficiency failed to cause any additional platelet aggregation in this experiment. Presumably, the 2μM ADP is insufficient to cause irreversible aggregation in this donor.

In contrast to the previous experiment, a major proportion of the SPA observed was ADP-dependent. The degree of ADP-independent SPA increased with temperature.

The lack of response to 2μM ADP poses an interesting question. How much ADP needs to be released from erythrocytes or platelets to cause the ADP-dependent SPA?

7.3.6.3. The effects of PK and PEP on SPA in diabetic patients.

Citrated blood samples were obtained from two diabetic patients. Samples were mixed at 22^{o} or 37^{o}C in the presence or absence of PK and PEP.

Patient Data

Diabetic R1 : Male, NIDDM, age 65, no microvascular complications.

Diabetic J8 : Female, IDDM, age 30, retinopathy.

Results

TIME	PLATELET COUNT (x10^{9}/l)			
(mins)	-PK/PEP(22^{o})	+PK/PEP(22^{o})	-PK/PEP(37^{o})	+PK/PEP(37^{o})
25	207	206	192	204
40	156	199	158	188
60	177	207	142	191
90	170	209	162	180
120	176	185	117	182
135	167	180	110	176

Table 7.21. : The effect of PK/PEP on SPA : Donor, diabetic R1.

The sample from diabetic J8 was split into two, and PRP prepared from one half. The effect of PK/PEP on platelet aggregation in whole blood and PRP was then compared at 22^{o}C.

TIME (mins.)	PLATELET COUNT ($x10^9/l$)			
	WHOLE BLOOD		PRP	
	-PK/PEP(22^o)	+PK/PEP(22^o)	-PK/PEP(37^o)	+PK/PEP(37^o)
10	166	168	262	261
30	162	167	260	259
55	147	162	244	271
85	124	151	243	273
110	149	148	238	268

Table 7.22. : The effect of PK/PEP on SPA : Donor diabetic J8.

These results are expressed graphically in figure 7.10.

Discussion

Patient R1 shows fairly typical behaviour, the degree of SPA under each set of conditions being as follows;

22^oC : ADP-dependent SPA = 12.0%

ADP-independent SPA = 12.6%

37^oC : ADP-dependent SPA = 29.0%

ADP-independent SPA = 13.7%

Thus, in this patient, only the ADP-dependent SPA increases at 37^oC relative to 22^oC.

The second patient, J8, shows similar behaviour at 22^oC using whole blood;

22^oC WHOLE BLOOD: ADP-dependent SPA = 13.4%

ADP-independent SPA = 11.9%

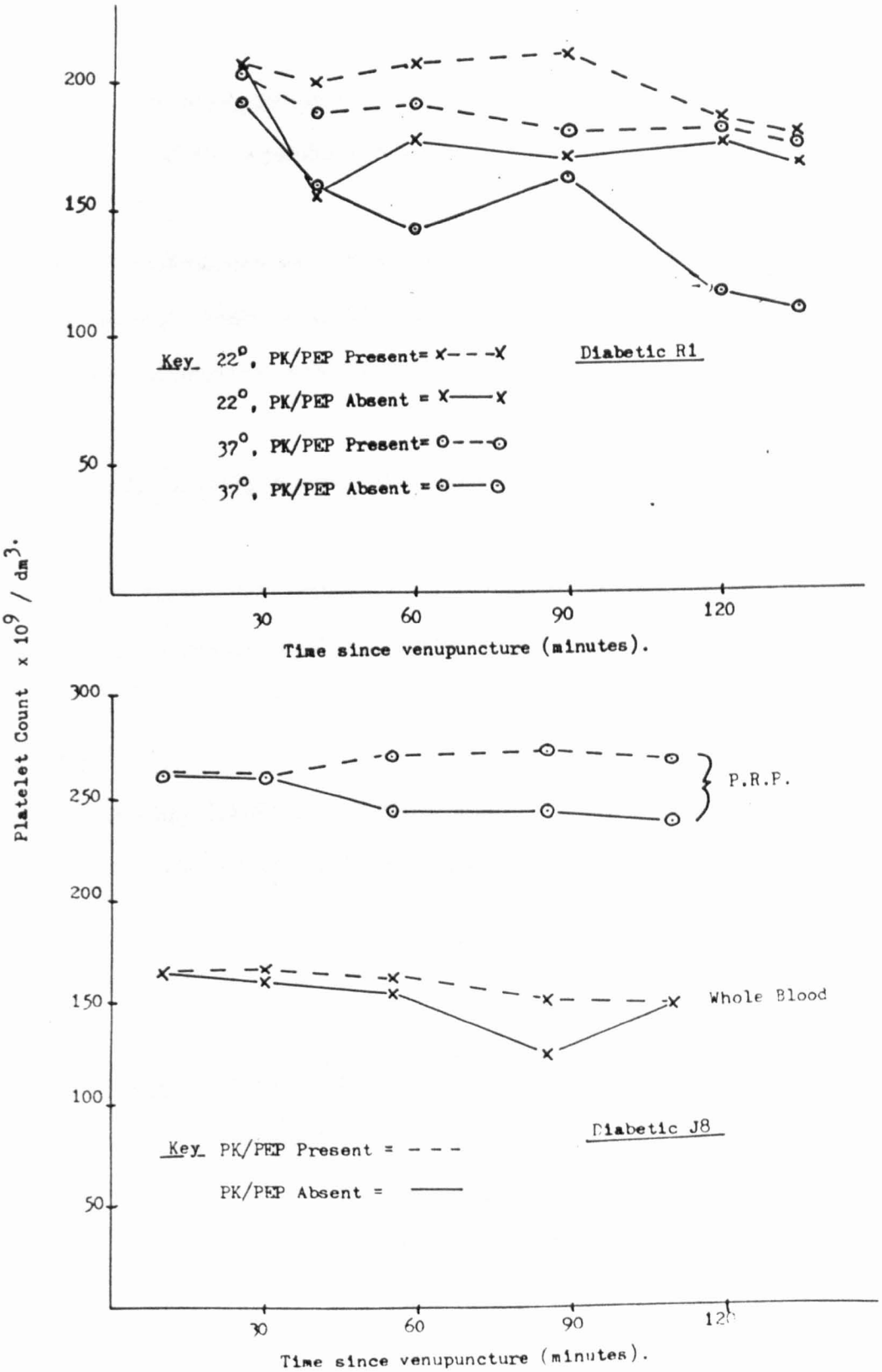


FIGURE 7.10: PK/PEP and SPA in two diabetic donors

22°C PRP : ADP-dependent SPA = 9.2%

ADP-independent SPA = 0

The equivalent PRP sample demonstrates that in the absence of erythrocytes, SPA is reduced, and in addition, is totally of the ADP-independent type. This strongly suggests that "ADP-dependent" SPA requires the presence of erythrocytes.

7.3.6.4. Is Insulin involved in whole blood SPA in diabetic patients?

Citrated blood samples were obtained from two diabetic donors. Samples were mixed at 22°C in the presence or absence of 20 units/ml PK, 100μM PEP and 1μM insulin.

Patient Data.

Diabetic J1 : Female, IDDM, age 80, retinopathy.

Diabetic J2 ; Female, IDDM, age 63, retinopathy.

Results

TIME (mins.)	PLATELET COUNT ($x10^9/l$)			
	-PK/PEP	+PK/PEP	-PK/PEP +Insulin	+PK/PEP +Insulin
20	198	221	183	198
35	181	188	179	199
60	159	186	159	195
100	157	180	159	184
120	151	164	158	184
5μM ADP added to each sample				
130	10	111	13	109

Table 7.23. ; Whole blood SPA ; Diabetic J1.

TIME (mins.)	PLATELET COUNT ($x10^9/l$)			
	-PK/PEP	+PK/PEP	-PK/PEP +Insulin	+PK/PEP +Insulin
10	251	237	239	230
30	228	236	248	250
60	223	247	227	247
75	214	244	232	241
115	169	217	225	245
5μM ADP added to each sample				
125	20	201	12	219

Table 7.24. ; Whole blood SPA ; Diabetic J2.

These results are expressed graphically in figure 7.11.

Discussion

Following the interesting effects of insulin on ADP-induced aggregation in PRP reported in chapter 6, it was decided to test the effects of high dose insulin on two IDDM patients.

J1 ADP-dependent SPA = 5.9%

ADP-independent SPA =25.8%

ADP-dependent SPA + insulin =11.8%

ADP-independent SPA +insulin =16.7%

J2 ADP-dependent SPA =20.5%

ADP-independent SPA =12.1%

ADP-dependent SPA + insulin = 5.7%

ADP-independent SPA + insulin = 3.6%

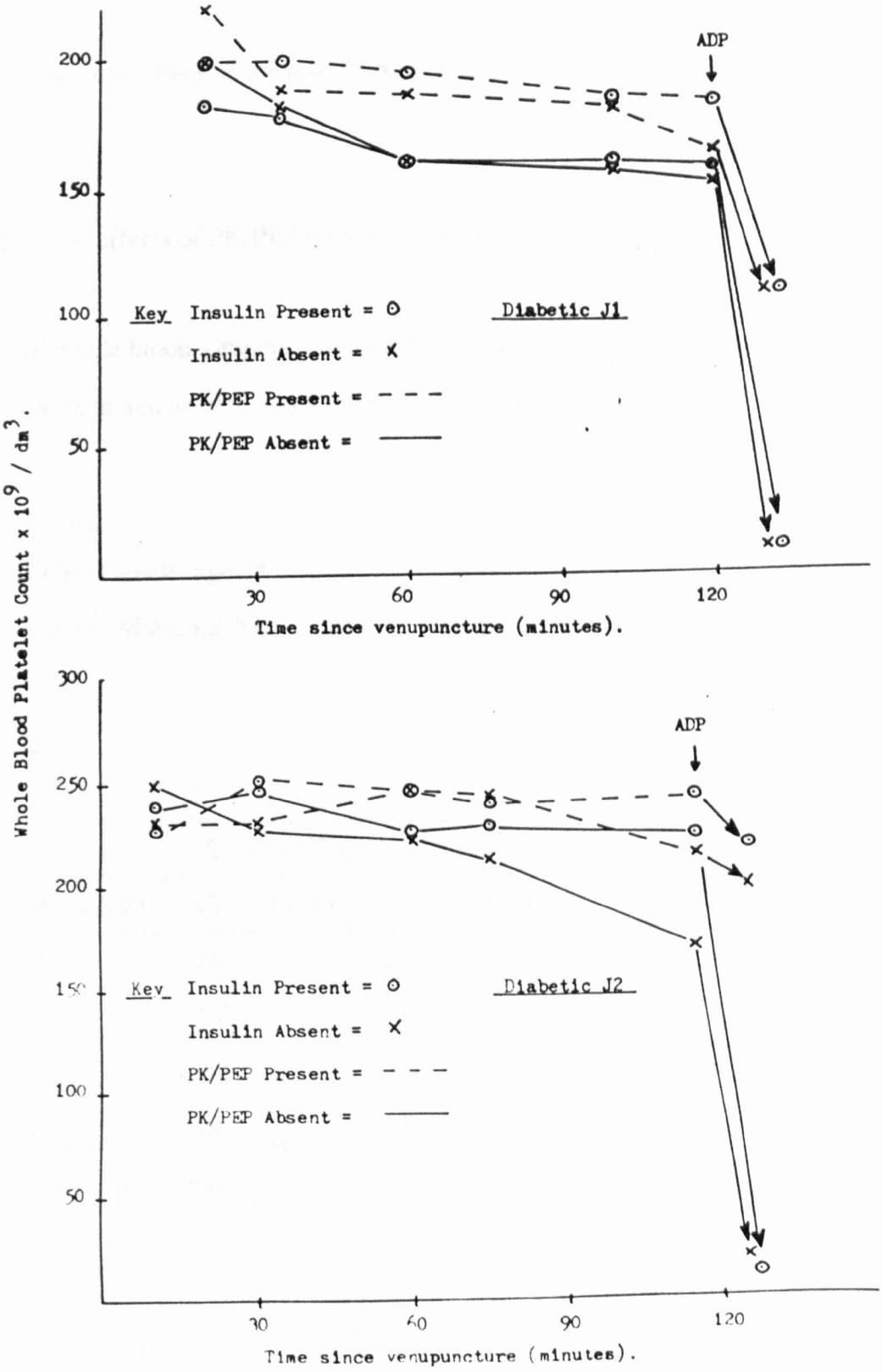


FIGURE 7.11: The effect of insulin on whole blood SPA

Insulin has little effect on the total SPA seen in donor J1, but clearly inhibits SPA from donor J2.

7.3.6.5. The effects of PK/PEP on SPA in psoriasis patients.

Citrated whole blood samples were obtained from two patients with severe psoriasis. Both samples were mixed at 22°C in the presence or absence of PK and PEP.

Patient Data.

Patient CP : Female, age 38.

Patient WW : Male, age 57.

Results

TIME (mins.)	PLATELET COUNT ($x10^9/l$)			
	CP:-PK/PEP	CP:+PK/PEP	WW:-PK/PEP	WW:+PK/PEP
45	270	285	277	250
75	216	197	192	234
105	181	215	201	205
135	220	224	193	173
5μM ADP added to each sample				
145	7	205	42	147

Table 7.25. ; Whole blood SPA ;Psoriasis patients.

See also figure 7.12.

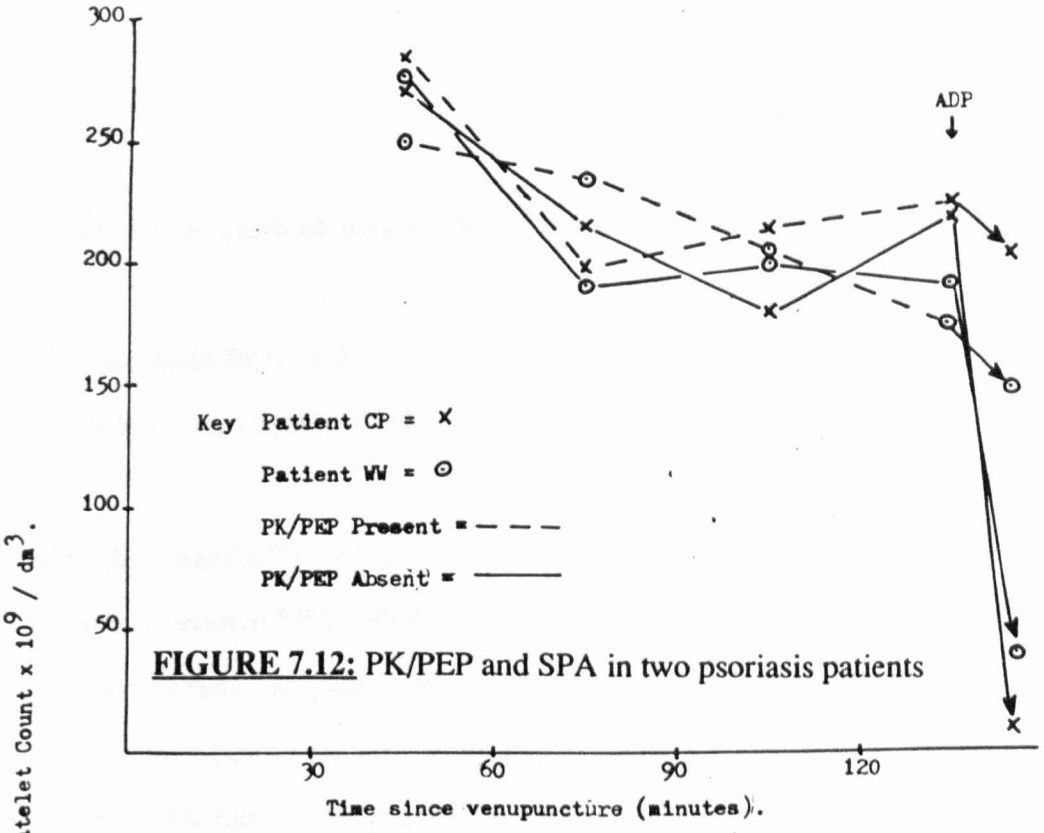


FIGURE 7.12: PK/PEP and SPA in two psoriasis patients

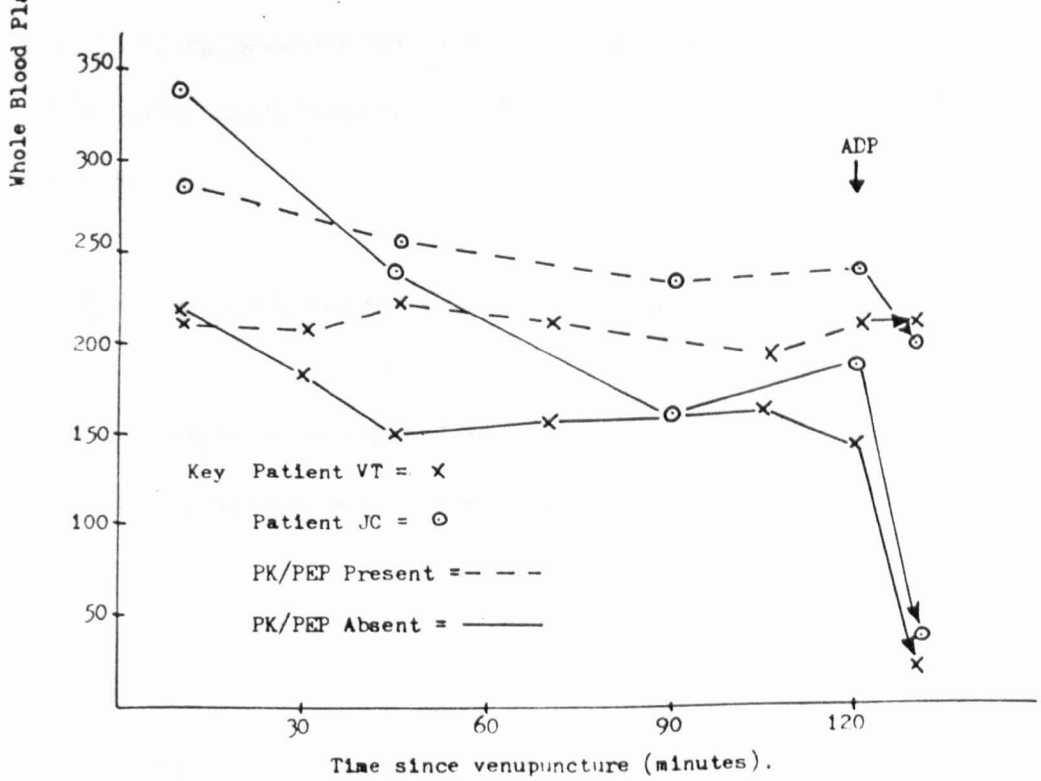


FIGURE 7.13: PK/PEP and SPA in two Warfarin -taking patients

Discussion

Interestingly the SPA of both patients is almost totally of the ADP-dependent type;

CP ADP-dependent SPA = 2.1%

ADP-independent SPA =30.9%

WW ADP-dependent SPA = 0.1%

ADP-independent SPA =30.8%

This behaviour is rather atypical. Berrettini *et al*[132] reported enhanced spontaneous aggregation in PRP from psoriasis patients compared to controls. These results suggest that although the SPA in whole blood from psoriasis patients may not be greater in total than the general population's, the mecanisms underlying the development of the SPA are different. Both patients studied here would be expected to show similarly exaggerated SPA in PRP as Berrettini's patients did.

7.3.6.6. : The effect of PK and PEP on SPA in two patients taking warfarin.

Citrated blood samples were obtained from two non-diabetic patients taking the anti-thrombotic drug, warfarin. Samples were mixed at 22^{o}C in the presence or absence of PK/PEP as before.

Patient Data.

Patient VT ; Female, age 74, femoral embolectomy (deep vein thrombosis). On warfarin for 10 months.

Patient JC ; Female, age 56.

Results

TIME (mins.)	PLATELET COUNT ($x10^9/l$)			
	VT:-PK/PEP	VT:+PK/PEP	JC:-PK/PEP	JC:+PK/PEP
10	218	214	341	286
30	183	209	-	-
45	150	223	239	257
70	157	212	-	-
90	-	-	160	233
105	163	194	-	-
120	143	209	188	239
5μM ADP added to each sample				
130	19	210	36	198

Table 7.26. ; Whole blood SPA : Patients at risk of thrombosis.

See also figure 7.13.

Discussion

In contrast to the psoriasis patients, the potential thrombosis patients show both ADP-dependent and independent SPA;

VT : ADP-dependent SPA = 21.4%

ADP-independent SPA = 13.0%

JC : ADP-dependent SPA = 34.6%

ADP-independent SPA = 18.5%

The degree of SPA is not extreme, considering the age of the donors.

7.3.6.7. : Conclusions.

Evidently, ADP plays a major role in the formation of platelet aggregates in whole blood. This observation is in agreement with Saniabadi's group[128-131] and others[125,126], who reported the effects of red blood cells and ADP-destroying systems on SPA.

The inhibitory effects of two ADP-destroying systems on whole blood SPA and the varying degrees of ADP-dependent and ADP-independent SPA from numerous donors are reported here. The mechanisms of both types of SPA are still uncertain. ADP-dependent SPA appears to be dependent on the presence of erythrocytes. Recent work by a Canadian group[125,126] has indicated that erythrocytes release ADP sub-lytically even when only gently agitated, and has shown that this ADP is able to activate platelets.

The mechanism of ADP-independent SPA is rather more difficult to explain. Even in the presence of ADP-destroying systems capable of preventing aggregation induced by up to 10$_{\mu}$ M ADP, a considerable degree of aggregation can still take place. There are numerous candidates for inducing this form of SPA including thrombin, platelet-activating factor, immune complexes[95], bacterial endotoxins[92] or any combination of these. Alternatively, the non-ADP dependent SPA may be caused by platelets that are already pre-activated or "poised" to aggregate, either prior to venupuncture , or are activated by the process of venupuncture itself.

7.3.7. The effects of "Treated" erythrocytes on SPA in healthy controls and diabetics.

Key to tables

Crbc = "control" red cells

Nrbc = "neuraminidase" red cells

Trbc = "trypsin" red cells

Plt.no. = Platelet number $x10^9$/l blood

RBC no. = Red cell number $x10^{12}$/l blood

7.3.7.1. Treated erythrocytes and SPA in a healthy control.

Citrated blood was obtained from donor AJS. Whole blood and reconstituted samples (see 7.2.4) using each of the three types of fixed cells were rolled at 22°C.

Results

TIME (mins.)	VARIABLE	DATE 21.6.89.		
		Whole Blood	PRP+Crbc	PRP+Nrbc
30	Plt.no.	158	119	190
	RBC no.	4.52	2.28	2.06
60	Plt.no.	148	121	11
	RBC no.	4.48	2.37	1.99
90	Plt.no.	144	115	15
	RBC no.	4.53	2.31	2.10

Table 7.27(a) ; Treated erythrocytes and SPA; Donor AJS.

TIME (mins)	VARIABLE	DATE 4.7.89.			
		Whole Blood	PRP+Crbc	PRP+Trbc	PRP+Trbc
30	Plt.no.	165	159	147	146
	RBC no.	4.45	2.24	2.30	2.19
60	Plt.no.	144	169	154	156
	RBC no.	4.43	2.35	2.37	2.32
90	Plt.no.	155	171	154	150
	RBC no.	4.40	2.29	2.39	2.33

Table 7.27(b) : Treated erythrocytes and SPA: Donor AJS.

See also figure 7.14.

The degree of SPA at t=90 minutes in each sample was as follows;

%SPA, Whole blood (21.6.89) = 8.9%

PRP+Crbc (21.6.89) = 4.9%

PRP+Nrbc (21.6.89) = 94.2%

Whole blood (4.7.89) = 12.7%

PRP+Crbc (4.7.89) = 0

PRP+Trbc(i) (4.7.89) = 0

PRP+Trbc(ii)(4.7.89) = 3.8%

Discussion

Untreated whole blood shows only a small degree of SPA under the conditions of this experiment. Similarly, the reconstituted samples comprising PRP and "control" erythrocytes or "trypsinised" erythrocytes show only minimal SPA.

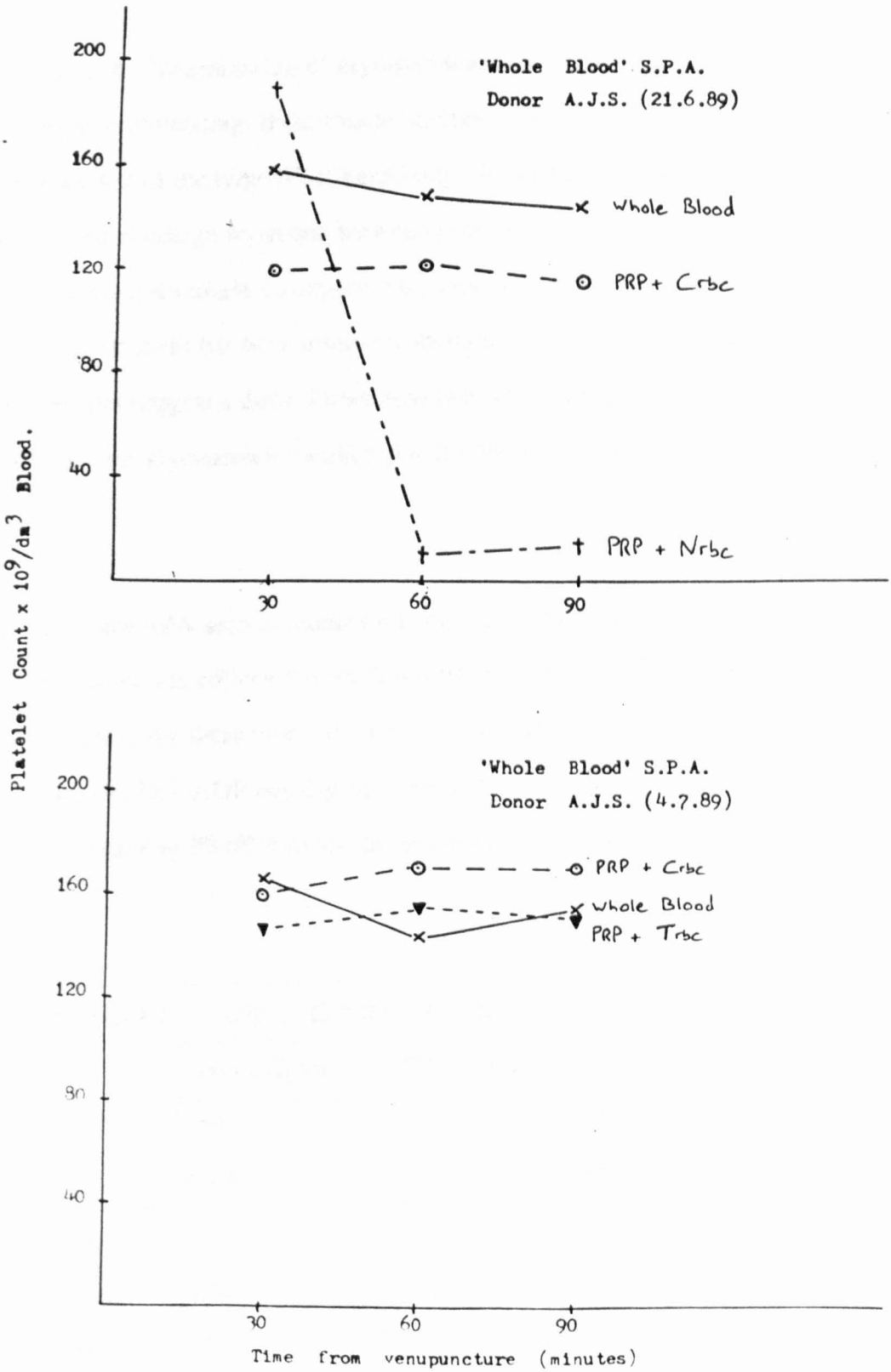


FIGURE 7.14: "Whole Blood" SPA; donor AJS

In contrast, the "neuraminidase" erythrocytes cause almost total SPA within 30 minutes of mixing commencing. If the interaction between platelets and erythrocytes was merely a consequence of the removal of negatively-charged sialate from the erythrocytes preventing the normal charge-repulsion between platelets and erythrocytes, then the "trypsinised" erythrocytes might be expected to cause a similar result. In these cells, the entire surface glycoprotein has been removed, including the terminal sialate.

These results suggest a direct interaction between normal, healthy platelets and desialated erythrocyte glycoproteins and/or glycolipids, rather than a charge-dependent interaction.

7.3.7.2. Is the SPA seen in reconstituted samples ADP-dependent?

Citrated blood was collected from donor BEPS. Whole blood and reconstituted samples using each of the three types of fixed red cells were stirred at 37°C in the presence or absence of the PK/PEP ADP-destroying system. After 120 minutes, 5μM ADP was added to the samples containing PK/PEP to test the effectiveness of the system.

Results

TIME (mins.)	VARIABLE	DATE 22.5.89 (ADP-destroying system absent)			
		Whole Blood	PRP+Crbc	PRP+Trbc	PRP+Nrbc
20-25	Plt.no.	164	202	218	169
	RBC no.	4.20	1.92	1.57	1.55
45	Plt.no.	119	110	182	31
	RBC no.	4.34	1.95	1.58	1.56
80-90	Plt.no.	131	156	84	81
	RBC no.	4.25	1.90	1.53	1.54
120	Plt.no.	118	278	141	130
	RBC no.	4.28	1.92	1.58	1.51

Table 7.28(a) : Treated erythrocytes and SPA: Donor BEPS. PK/PEP absent.

TIME (mins.)	VARIABLE	DATE 23.5.89(ADP-destroying system present)			
		Whole Blood	PRP+Crbc	PRP+Nrbc	PRP+Nrbc
20-25	Plt.no.	215	195	175	187
	RBC no.	4.31	2.14	2.00	2.03
45	Plt.no.	210	163	81	35
	RBC no.	4.33	2.16	1.95	2.01
65	Plt.no.	192	180	44	45
	RBC no.	4.35	2.14	1.99	2.01
80-90	Plt.no.	183	257	120	125
	RBC no.	4.31	2.14	1.97	1.99
120	Plt.no.	189	270	176	205
	RBC no.	4.31	2.17	1.95	1.98
5μM ADP added to each sample					
130	Plt.no.	161	281	168	205
	RBC no.	4.09	2.12	1.91	1.92

Table 7.28(b) : Treated erythrocytes and SPA: Donor BEPS, PK/PEP present.

See also figure 7.15.

The maximum degree of SPA in each sample was as follows;

Whole blood = 28.0%

PRP+Crbc = 45.5%

PRP+Trbc = 61.7%

PRP+Nrbc = 81.7%

Whole blood + PK/PEP = 14.9%

PRP+Crbc + PK/PEP = 16.4%

PRP+Nrbc + PK/PEP(i) = 74.9%

PRP+Nrbc + PK/PEP(ii) = 81.3%

78.1% = mean SPA, PRP+Nrbc + PK/PEP

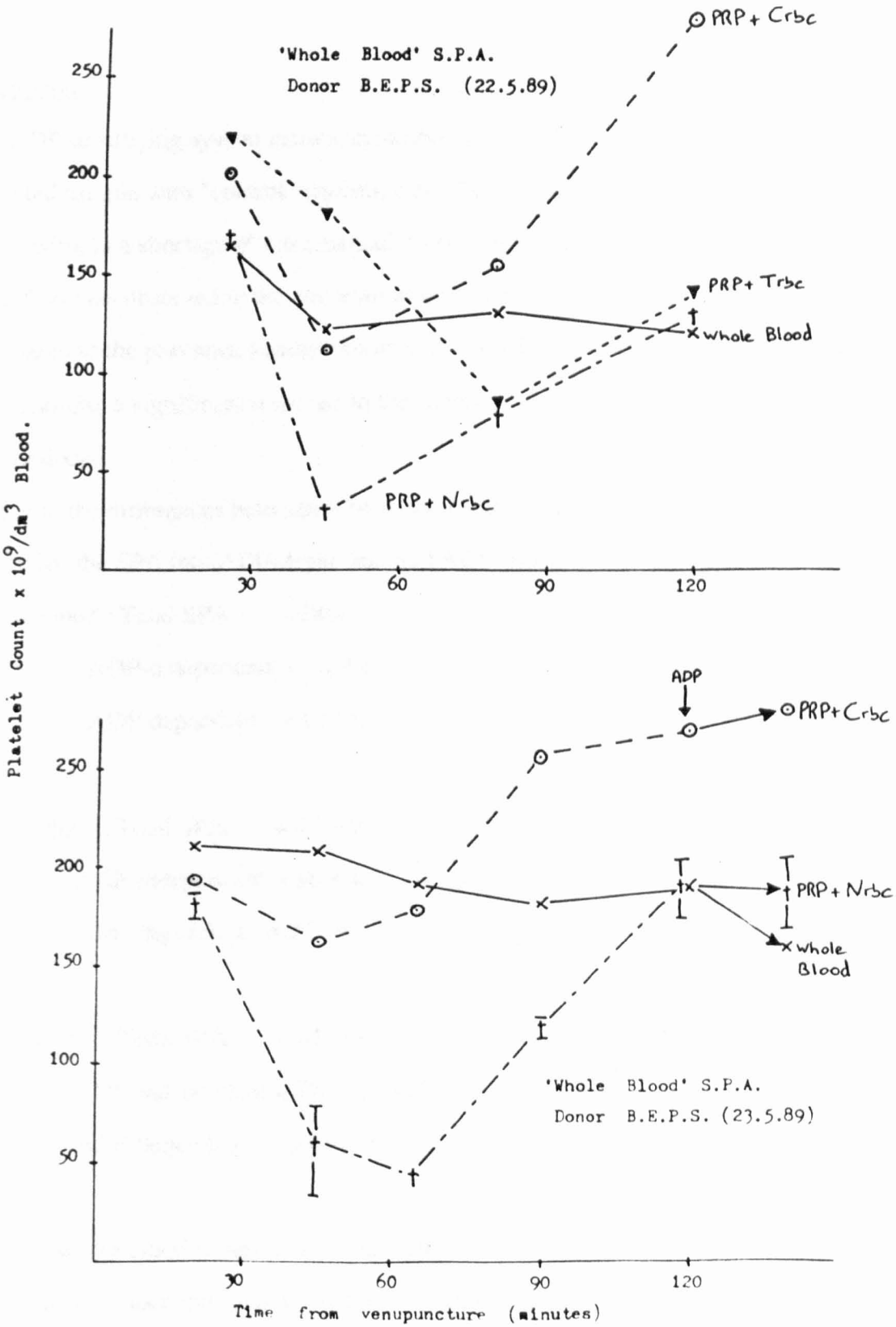


FIGURE 7.15: "Whole Blood" SPA; donor BEPS

Discussion

The ADP-destroying system causes an inhibition of SPA in whole blood and also in the reconstituted sample with "control" erythrocytes. The "trypsin" red cell experiment was not repeated owing to a shortage of these paticular cells on the day of the experiment. No inhibition of SPA was observed in the neuraminidase-treated samples.

Compared to the previous, younger control, donor BEPS shows an enhanced SPA in whole blood and also a significant response to the "control" and "trypsin" erythrocytes absent in the younger donor.

Owing to the differences between SPA in the presence or absence of PK/PEP, it is possible to divide the SPA into ADP-dependent and ADP-independent aggregation;

Whole blood : Total SPA = 28%
ADP-independent = 14.9%
ADP-dependent = 13.1%

PRP+Crbc : Total SPA = 45.5%
ADP-independent = 16.4%
ADP-dependent = 29.1%

PRP+Nrbc : Total SPA = 81.7%
ADP-independent = 78.1 +/- 3.2%
ADP-dependent = 3.6 +/- 3.2%

Like the whole blood samples, it appears that SPA in the "control" erythrocyte experiments can be divided into two discrete types, ADP-dependent and independent.In contrast, the neuraminidase-treated erythrocytes are able to cause almost total disappearance of platelets even in the absence of ADP.

All the treated erythrocyte samples show a large degree of disaggregation after maximal SPA has occured. This long-term reversible SPA is paticularly evident in the "control" red cell samples where it proceeds to such an extent that the final platelet count is

higher than the initial, pre-mixing count. Since the phenomenon is not seen in whole blood, the "extra" platelets are unlikely to be due to microaggregates present in the blood at venupuncture. A more likely explanation is that some SPA had occured in the "control" erythrocyte sample between sample preparation and the first (t=30) count.

7.3.7.3. Do high concentrations of sugars inhibit the interaction between platelets and neuraminidase-treated red cells?

Citrated blood was collected from donor BEPS. The spontaneous aggregation of platelets in PRP, whole blood and in reconstituted samples using neuraminidase-treated erythrocytes in the presence or absence of 35mM glucose, 10mM galactose or 10mM N-acetyl-glucosamine was investigated by stirring samples at 37°C.

Results

TIME (mins.)	VARIABLE	DATE 9.6.89		
		Whole Blood	PRP+Nrbc	PRP+Nrbc+Glucose
20-30	Plt.no.	208	194	211
	RBC no.	4.25	0.45	0.47
60	Plt.no.	172	27	81
	RBC no.	4.36	0.40	0.37
85-90	Plt.no.	144	18	13
	RBC no.	4.43	0.38	0.39
110-120	Plt.no.	130	23	19
	RBC no.	4.44	0.35	0.35

Table 7.29(a) : Treated erythrocytes and SPA; Donor BEPS, the effect of glucose.

TIME (mins.)	VARIABLE	DATE 22.6.89.			
		PRP+Crbc	PRP+Nrbc	PRP+Nrbc +Galactose	PRP+Nrbc +N-A-G
20-30	Plt.no.	235	282	287	288
	RBC no.	2.36	1.95	1.93	1.91
60	Plt.no.	152	12	10	13
	RBC no.	2.39	1.95	1.92	1.91
85-90	Plt.no.	110	15	15	17
	RBC no.	2.30	1.95	1.87	1.93
110-120	Plt.no.	125	25	35	15
	RBC no.	2.41	1.96	1.90	1.91

Table 7.29(b) ; Treated erythrocytes and SPA: Donor BEPS, the effects of galactose and N-acetyl-glucosamine.

The equivalent PRP sample showed no SPA, platelet number increasing from 342 to $370x10^9$/l plasma during the assay.

See also figure 7.16.

The maximum degree of SPA in each sample was as follows;

Whole blood = 37.5%

PRP+Nrbc(9.6.89) = 90.7%

PRP+Nrbc + 35mM Glucose = 93.8%

PRP+Crbc = 52.8%

PRP+Nrbc(22.6.89) = 95.7%

PRP+Nrbc + 10mM Galactose = 96.5%

PRP+Nrbc + 10mM N-acetyl-Glucosamine = 95.5%

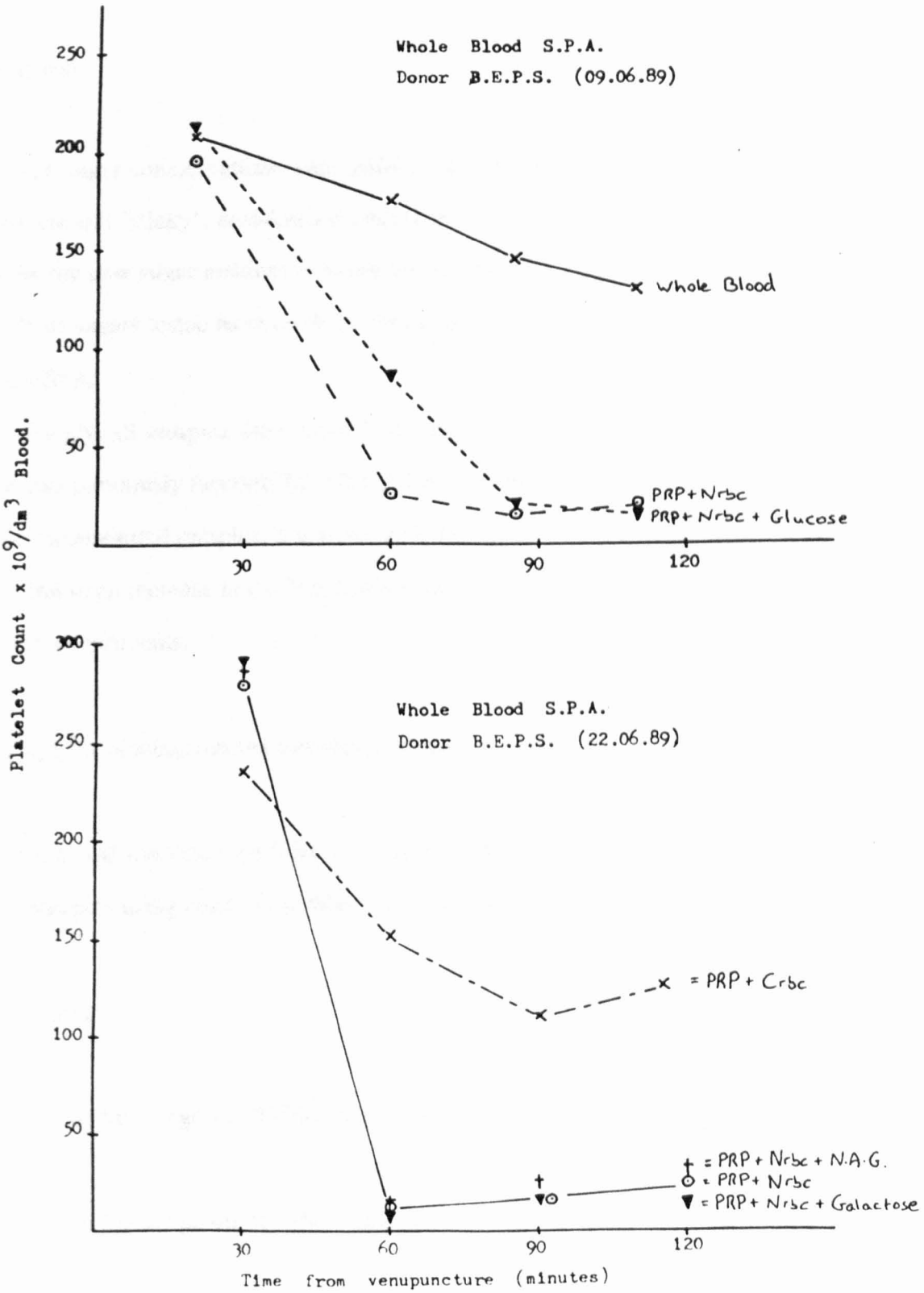


FIGURE 7.16: The effects of sugars on "whole blood" SPA.

Discussion

The high sugar concentrations were added in an attempt to inhibit the "interaction" between platelets and "sticky", desialated erythrocytes by occupying a putative receptor site on platelets for the new sugar residues exposed on the desialated glycoproteins of the red cell. Clearly, the three sugars tested have no effect on the potential of neuraminidase-treated red cells to induce SPA.

Interestingly, all samples show more SPA than the equivalent samples from the same donor two weeks previously (section 7.3.7.2.). Since this phenomenon is seen in whole blood as well as the reconstituted samples, it is unlikely to be due to a deterioration of the fixed erythrocytes, but to an increase in the "sensitivity" of the platelets from this donor in the period between experiments.

7.3.7.4. SPA in reconstituted samples with diabetic PRP.

Citrated blood was obtained from two diabetic patients. PRP, whole blood and reconstituted samples using each of the three types of fixed red cell were rolled at 22°C.

Patient Data

Diabetic J4: Male, age 72, IDDM, retinopathy.

Diabetic J6: Male, age 46, IDDM, retinopathy.

Results

TIME (mins.)	VARIABLE	Whole Blood	PRP+Crbc	PRP+Nrbc	PRP
30	Plt.no.	180	187	236	353
	RBC no.	5.00	2.20	1.69	0
55-60	Plt.no.	154	98	35	352
	RBC no.	5.13	2.13	1.81	0
90-100	Plt.no.	135	64	75	334
	RBC no.	5.04	2.24	1.68	0
120	Plt.no.	114	65	80	321
	RBC no.	4.81	2.05	1.53	0

Table 7.30. ; Treated erythrocytes and SPA; Donor, diabetic J4.

TIME (mins.)	VARIABLE	Whole Blood	PRP+Crbc	PRP+Trbc	PRP+Nrbc
30	Plt.no.	168	123	87	92
	RBC no.	3.87	2.32	2.33	1.64
55-60	Plt.no.	137	75	48	19
	RBC no.	3.80	2.26	2.33	1.52
90-100	Plt.no.	128	47	19	33
	RBC no.	3.77	2.24	2.30	1.40
120	Plt.no.	125	40	19	33
	RBC no.	3.79	2.25	2.31	1.43

Table 7.31. Treated erythrocytes and SPA; Donor, diabetic J6.

See also figure 7.17.

The maximum degree of SPA in each sample was as follows;

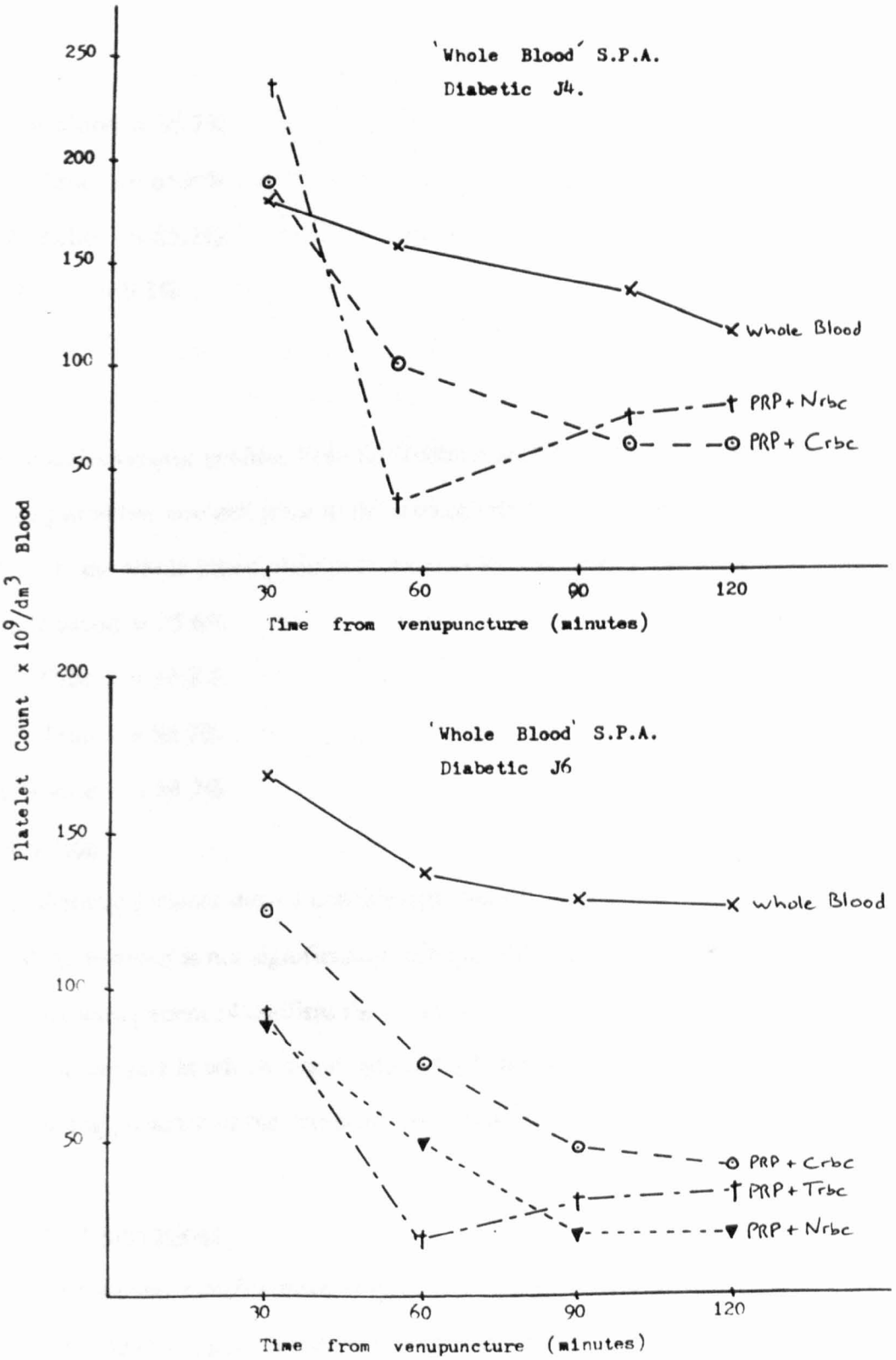


FIGURE 7.17: "Whole Blood" SPA in two diabetic donors

J4

Whole blood = 36.7%

PRP+Crbc = 65.8%

PRP+Nrbc = 85.2%

PRP = 9.1%

J6

The platelet volume profiles from the Technicon H*1 clearly showed that some platelet aggregation had occured prior to the start of mixing at t=30, in the reconstituted samples. Hence, the whole blood platelet count at t=30 was used to calculate total SPA.

Whole blood = 25.6%

PRP+Crbc = 76.2%

PRP+Trbc = 88.7%

PRP+Nrbc = 88.7%

Discussion

Both diabetic patients show a considerable degree of SPA in whole blood. The amount of SPA, however is not significantly different from that seen in controls of a similar age. The results with patient J4 confirm the observation that a greater proportion of platelets spontaneously aggregate in whole blood than in PRP. Interestingly, both diabetics show marked SPA in the presence of the "control" erythrocytes.

7.3.7.5. ; Conclusions

The reconstituted samples show very similar initial platelet counts to that seen in the equivalent whole blood sample. However, erythrocyte numbers are usually only around 50% of the number seen in whole blood. This suggests that the red cell pellet obtained by low speed centrifugation of the 4% cell suspensions does not contain truly "packed" cells. This may be due to a decrease in the deformability of gluteraldehyde-fixed red cells compared to fresh erythrocytes. The relatively low red cell numbers do not seem to hinder SPA. This is paticularly evident from experiment 7.3.7.3. On the first day of testing, the neuraminidase-

treated samples contained only 10% of the whole blood erythrocyte numbers owing to a shortage of these paticular cells. In contrast, the second day of testing provided samples with almost 50% of the whole blood erythrocyte number. Despite this discrepancy, the response of platelets on each occassion was almost identical.

There appears to be considerable difference between individuals in their response to the various types of treated red cells. However, the one consistent observation is that the neuraminidase-treated erythrocytes cause almost total aggregation of platelets in all donors examined. This aggregation occurs relatively quickly, and is not dependent on ADP released sub-lytically from erythrocytes or secreted from platelet granules (see section 7.3.7.2.).

Treatment of cells with neuraminidase cleaves the terminal sialic acid residue from glycoproteins and glycolipids. Human erythrocytes possess four distinct membrane sialoglycoproteins designated α, β, χ and δ[141-144]. It has been suggested that several of these proteins associate with membrane skeletal proteins and appear to be important for the maintainance of the biconcave shape of the normal erythrocyte[142,143]. Loss of sialic acid by a neuraminidase-like activity and of glycoproteins by a proteolytic activity has been reported during red cell aging or nutrient depletion *in vivo*[141]. Sialic acid depletion has been proposed as one method by which old red cells are recognised and removed from the circulation by the spleen, liver and bone marrow[135]. Similarly, removal of as little as 8-10% of platelet sialic acid results in their recognition as "foreign" *in vivo*[145].

The results described here indicate that desialated erythrocytes cause massive SPA of apparently normal platelets. This phenomenon is not dependent on ADP, which suggests a more direct form of platelet activation by the treated erythrocytes. This activation could take the form of platelets binding to "sticky" desialated red cells or may be due to the "activation" of a plasma factor by the treated red cells, which then initiates platelet aggregation.

If desialated erythrocytes are able to cause platelet loss *in vivo*, then people with an increased turnover of red cells might be expected to be prone to increased *in vivo* platelet activation. Erythrocyte lifespan and deformability are generally recognised to be reduced in diabetes[3,135] and thus desialation may play a major role in the development of platelet-erythrocyte interactions in diabetes.

Chapter 8

GENERAL CONCLUSIONS

8.1 : Platelets and Microvascular Disease

Microangiopathy, the disease of small blood vessels, is rare in the general population, but seriously affects many sufferers of diabetes mellitus, leading to blindness, kidney failure and disorders of the nervous system (see section 1.8). Inappropriate *in vivo* platelet activation with microaggregate formation and subsequent capillary occlusion has been postulated to play a major role in the development of these complications[37,44-53].

Evidence for *in vivo* platelet activation is given by the presence of platelet activation and secretion markers in the plasma. β-thromboglobulin[48,49], thromboxane B_2[50] and MDA[51] have all been reported to be raised in diabetic plasma. The results reported in chapter 3 show that, in general, the capacity of diabetic platelets to produce MDA, and hence TxA_2, is no different from age-matched controls. This would appear to indicate that elevated plasma (as opposed to serum) TxB_2 levels in diabetics[45,50] are not due to an intrinsic fault in the diabetic platelets, but rather to an "over-activation" (or "under-suppression") of TxA_2 synthesis by external agonists.

Two of the 63 diabetics tested showed significantly raised serum MDA levels compared to the other diabetics and controls. These observations may point to a transient malfunction in platelet metabolism, possibly induced by a period of poor diabetic control. Potentially, the platelets from these donors could cause excessive *in vivo* thrombosis. A stimulus that normally would cause only limited platelet activation might be expected to trigger more widespread aggregation in the presence of these "super-TxA_2 producing" platelets.

Further evidence for similarities in diabetic and control platelets comes from the responses of platelets to ADP (tables 4.3 and 6.4) and the Ca^{2+}-antagonist, verapamil (table 4.3). There is no real difference in response to either agent, suggesting similar resting Ca^{2+} levels in platelets from both diabetics and controls. However, calcium-channel blockers may have a role to play in the treatment or prevention of diabetic microangiopathy. The tendency

for platelets to aggregate in response to a given stimulus would be diminished, and in addition, the chances of capillary occlusion would be reduced by the calcium-channel blocker's vasodilatory action.

The fact that platelet aggregation tends to increase with age has long been recognised[3,4]. The results shown in figure 3.3 indicate that the capcity of platelets, from both diabetics and controls, to produce MDA does not increase significantly with donor age. Thus, the observed increase in sensitivity to agonists of platelets from older controls and diabetics may be due to an, as yet, unidentified plasma platelet aggregation-enhancing factor (chapter 5) rather than age-dependent changes in the platelets themselves.

The resonse of diabetic platelets to PGE_1 is significantly reduced compared to non-diabetic platelets (table 6.4). PGE_1 and its physiological equivalent, prostacyclin, share a common receptor on platelets[4]. Hence, if this situation is repeated *in vivo*, the anti-thrombotic capability of vascular endothelium will be reduced. To make matters worse, there have been frequent reports that diabetics synthesize less PGI_2 than controls (review; ref. 45). A possible reason for this is the inhibition of PGI_2 production by high levels of glucose[43]. The results reported in chapter 6 show that the decreased anti-aggregatory potential of PGE_1 towards diabetic platelets is made worse by the presence of high levels of insulin. Thus, the actual act of infusing insulin may promote localised platelet activation in diabetics. Platelet microaggregates formed in this way would pass through the circulation until they were either dissociated, or caused occlusion of a minor blood vessel.

Instances of poor diabetic control, with accompanying hyperglycaemia may provide a mechanism for transient platelet activation. Diabetic platelets need only be exposed to pro-aggregatory conditions for a short period of time for serious microvascular consequences to occur, and non-enzymatic glycosylation provides several mechanisms by which thrombogenesis can be accelerated (see section 1.9).

The importance of interactions between platelets and other blood cells should not be underestimated. Two distinct types of interactions between platelets and red cells are described in chapter 7. ADP-dependent spontaneous platelet aggregation can be inhibited by ADP-destroying enzymes and is presumably due to ADP, released either from platelet

granules or, sub-lytically, from erythrocytes[125,126]. The trigger for ADP-release is probably mechanical. Cell-cell collisions induced by blood sample stirring or rolling *in vitro* are the equivalent of cellular interactions in flowing blood. Deformations of red cells, passing through capillaries *in vivo* might be expected to cause similar effects.

ADP-dependent spontaneous platelet aggregation appears to be at least partially due to direct interactions between platelets and red cells. Desialated red cells cause a massive, rapid decrease in platelet number. Old red cells become desialated in the circulation prior to removal by the liver and spleen, and these cells could be "sticky" towards platelets, paticularly pre-activated or "poised" platelets such as may occur during diabetic hyperglycaemia. Red-cell platelet aggregates are potentially even more damaging to the microvasculature than platelet microaggregates.

The various mechanisms potentially involved in the development of capillary occlusion are summarised in figure 8.1.

8.2.: Future Research

A series of long-term studies, involving a relatively small number of newly diagnosed diabetics and regular blood samplings with tests for such variables as platelet response to PGE_1 and ADP, plasma MDA and β-TG levels, and haemoglobin glycosylation could be carried out. This may establish whether there are gradual, progressive changes in platelet function, or sudden, transient effects that might be missed by single or infrequent samplings during the development of diabetic microangiopathy.

In addition, clinical studies involving the incidence of vascular complications in two groups of diabetic patients, one taking a suitable Ca^{2+}-antagonist such as verapamil and the other taking a placebo, could be undertaken to see if the drug prevents or retards the development of diabetic complications.

A new avenue of research involves flow-cytometry of platelets. Using fluorescent-labelled antibodies to platelet proteins, pre-activated or abnormal platelets could be detected in whole blood. Potential markers for platelet activation should be proteins that are only

expressed or bound at the platelet surface following activation. Such a technique has been used to identify abnormal platelets lacking the fibrinogen receptor in the bleeding disorder Glanzmann's Thrombosthania[151]. Conversely, the presence of the fibrinogen receptor on "resting" platelets would indicate that inappropriate platelet activation had taken place. Shattil *et al*[149] have used a monoclonal antibody, PAC-1, to the $GPII_b/III_a$ complex to characterise the expression of the complex on the surface of activated platelets. Potentially more useful in the search for pre-activated platelets may be antibodies to fibrinogen or other adhesive proteins such as von Willebrand's factor and fibronectin. Pre-activated platelets formed *in vivo* should already have fibrinogen bound, and this could interfere with the binding of anti-$GPII_b/III_a$ to the receptor[148]. McEver and Martin[150] have developed an antibody, S12, to a protein, normally found in the platelet alpha-granules, that becomes surface-expressed following thrombin activation of platelets. This protein presumably appears at the platelet plasma membrane following the release reaction and fusion of granules with the external membrane. Its presence on the surface of "resting" platelets would be indicative of serious *in vivo* platelet activation.

Flow-cytometry techniques can be performed with very small volumes of blood and could give an indication as to whether all platelets become equally "activated" in the pre-thrombotic state, or only an abnormal population of "hypersensitive" platelets.

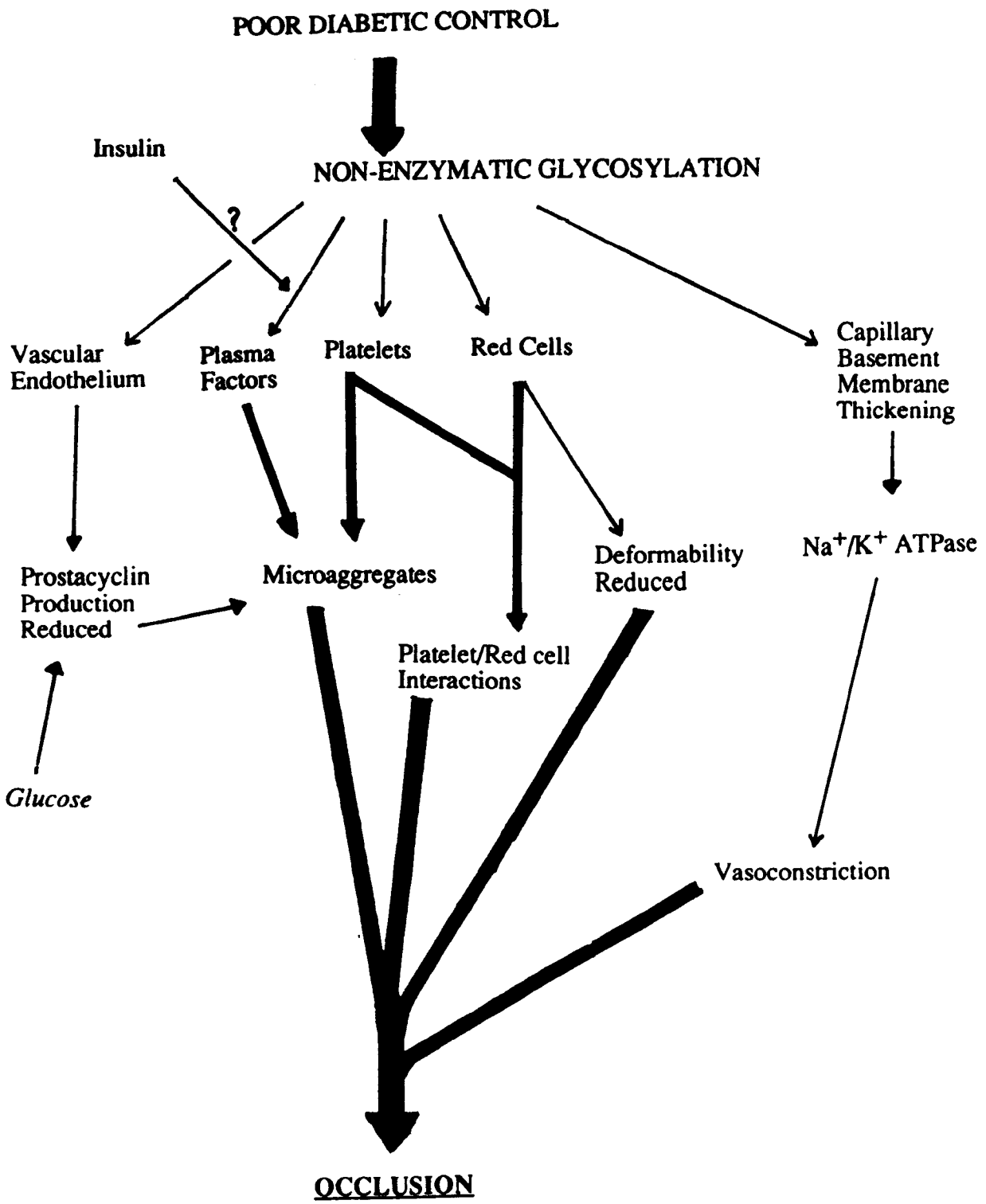


FIGURE 8.1: Postulated events leading to diabetic microvascular disease.

Appendix 1: Platelet Aggregometry

(a) Preparation of PRP and PPP

Whole blood is collected by venepuncture, either into a special "Vacutainer", or, via a syringe, into a 12.5ml plastic centrifuge tube containing an appropriate anticoagulant.

All glass and plastic-ware is siliconised using "Sigmacote" (Sigma) prior to use to prevent interactions between platelets and their container.

PRP is prepared by centifuging whole blood at approximately 100g for 10-20 minutes at room temperature. Older controls and diabetics require a shorter centrifugation period than young controls. Typically, 10ml of whole blood yields 3-5ml of PRP. PRP is carefully removed from above the sedimented red and white cells using a plastic disposable pipette, and transferred to a second container.

PPP is prepared by centrifuging the remaining red and white cells at 1000g for 10 minutes.

(b) Aggregometry

The platelet aggregometer is a specially adapted spectrophotometer, fitted with a magnetic stirrer base, chart recorder, and usually a 37°C thermo-stat. Aggregometry works on the principle that a large number of small particles (platelets) scatters more light than a small number of large particles (platelet aggregates). Hence, as platelets aggregate there is a gradual decrease in optical density.

1.0ml PRP samples are transferred to 3.0ml aggregometry cuvettes (LIP Ltd.). The aggregometer "zero" is set using PPP as a blank, and adjusting the OD (600nm) to 0.1 on the chart recorder scale. A PRP sample is used to set the starting OD to 0.9 on the chart scale.

Mixing is commenced by adding a small, siliconised magnetic stirrer bar to the cuvette and switching on the stirrer at a speed of 500-1200rpm.

After following the progress of any spontaneous aggregation for a few minutes, a small volume (~10µl) of a platelet agonist is added.

Initially, there is a small increase in OD, corresponding to the platelet shape change, followed by a rapid decrease in OD due to platelet aggregation.

Three types of platelet aggregation may be obtained with ADP; reversible aggregation occurs at low agonist concentration and coincides with primary platelet aggregation without large scale granule release and "tight" aggregate formation. These 1^{o} agregates spontaneously dissociate to give individual platelets. The shape change, however, is irreversible. Irreversible aggregtion is seen at higher agonist concentrations, sufficient to cause rapid and complete granule release and 2^{o} aggregation. Biphasic aggregation occurs at a concentration of agonist that is only just capable of causing granule release, hence the two phases of aggregation are visually separate. (See figure A1)

Appendix 2: Platelet Density Centrifugation

(Personal communication, G.M.Wilkins)

PRP is obtained as described in appendix 1, and a 2.0ml sample layered onto a column of "Percoll" of density 1.04g/ml to 1.09g/ml.

The column is created as a discontinuous gradient with 2ml steps of "Percoll" of densities; 1.09, 1.08, 1.07, 1.065, 1.06, 1.05 and 1.04g/ml. Each gradient step is prepared containing 150nm PGE_1 to prevent platelet activation during centrifugation.

Gradients are centrifuged at 30,000g for 30 minutes in a swing-out rotor, resulting in the formation of a pseudo-linear density gradient, and up to 15 discrete bands of platelets in the density range 1.055-1.068 g/ml. (see figure A2).

On aggregation, platelets secrete their granule contents and decrease in density. Hence, any platelet aggregates present are visible at a higher position on the gradient.

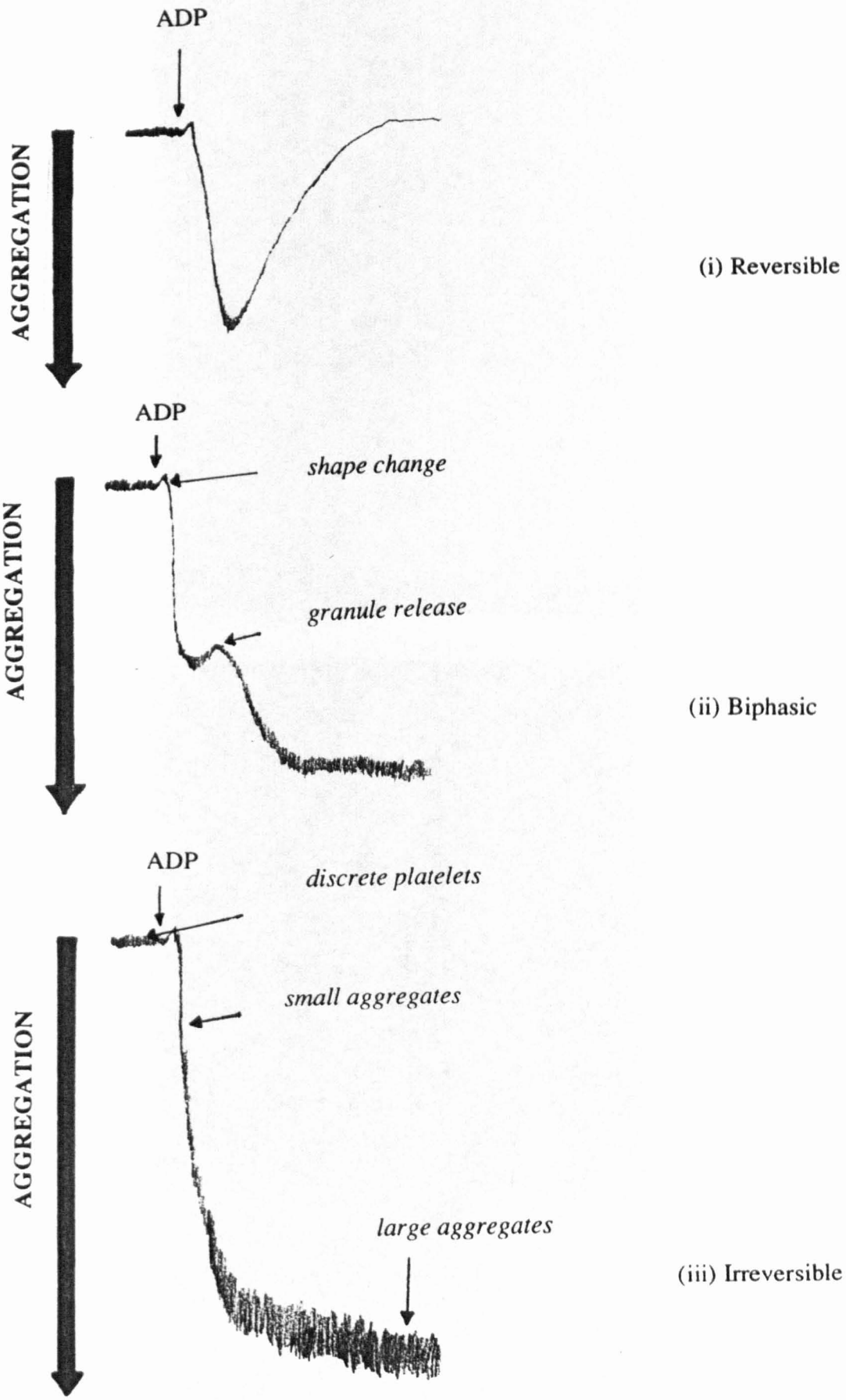
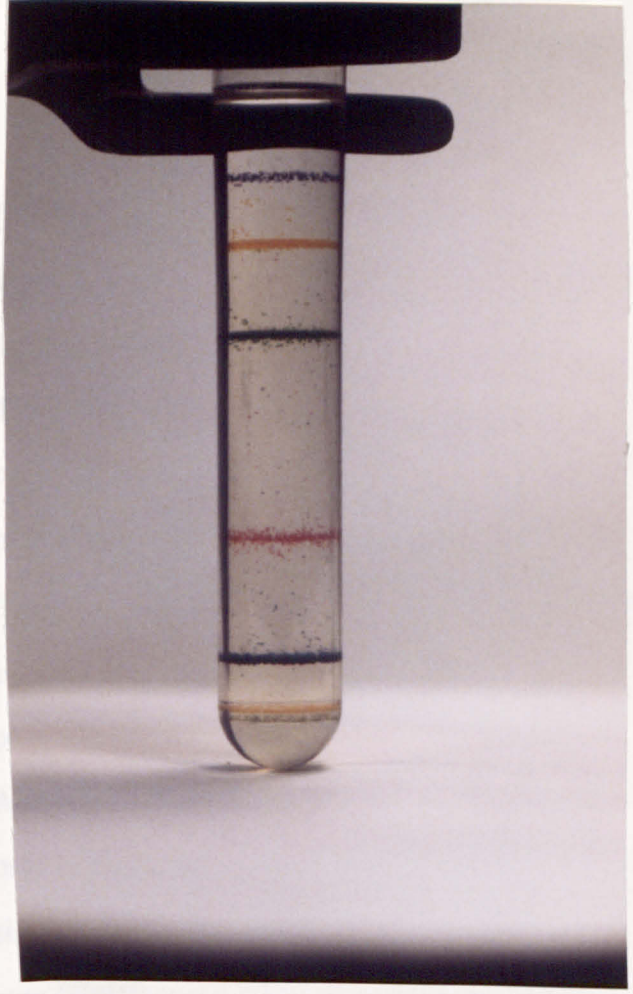


FIGURE A1: Typical ADP-induced Platelet Aggregometry Traces



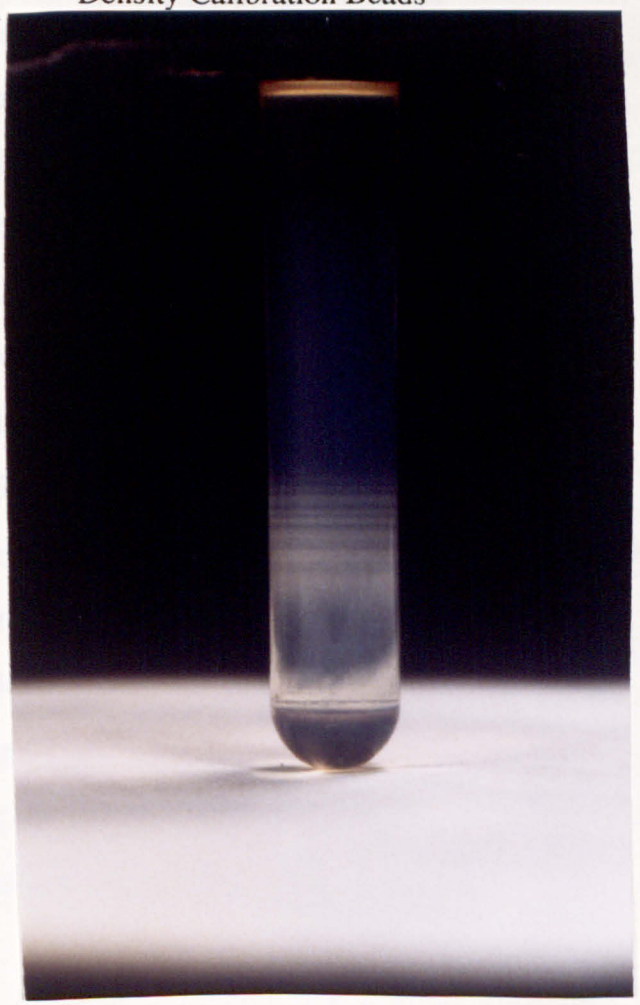


FIGURE A2: Separation of Platelet Populations on a "Percoll" density Gradient

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