FACTORS INVOLVED IN THE INDUCTION OF PLATELET AGGREGATION

DURING THE COLLECTION AND STORAGE OF HUMAN BLOOD

A thesis submitted for the degree of

Doctor of Philosophy

in July, 1979

by Roger Anthony Oakes M.I. BIOL., University of Keele,

Staffordshire.

The following was redacted from this digital copy of the original thesis at the request of the awarding university:

The published paper from "The Lancet" Aug 5, 1978

CONTENTS

ACKNOWLEDGEMENTS AND DECLARATION OF PARTICIPATION

ABSTRACT

In this thesis, some of the causes and mechanisms of platelet aggregation have been discussed. Platelet/granulocyte aggregates occur in stored blood and have been implicated in the development of respiratory distress syndrome following massive blood transfusions. Some of the factors that may be responsible for platelet aggregation in CPD anticoagulated blood have been examined.

Catecholamine concentrations were measured in blood plasma. During blood collection, the concentration of plasma noradrenaline increased, but there seemed to be no relationship between the final noradrenaline concentration and the onset or extent of platelet aggregation.

It was found that platelet aggregation measured by the screen filtration pressure technique, occurred very rapidly in blood stored at 1°C, more gradually at 4°C, and not at all at ambient room temperature.

The measurement of plasma catecholamines, 5-hydroxytryptamine and adenosine di-phosphate concentrations during blood storage did not demonstrate that any one of these factors was responsible for the onset of platelet aggregation. However, it is suggested that these, and other factors, might act individually or synergistically to extend platelet aggregation during blood storage. It was found that adenosine di-phosphate released from erythrocytes might accelerate platelet aggregation in blood stored at 1°C.

It has been shown that potential platelet aggregating agents are released from platelets in blood stored at room temperature. It is suggested that the apparent lack of platelet aggregation at this

temperature was due to a rapidly reduced pH, and the rapid degradation of adenosine nucleotides to aggregate inhibitory products.

The evidence suggests that platelet aggregation in stored blood is triggered by the cold, and that it may involve an energy dependent mechanism, possibly independent of the platelet release reaction. It is suggested that blood storage at room temperature would reduce or prevent platelet aggregation.

FACTORS INVOLVED IN THE INDUCTION OF PLATELET

AGGREGATION DURING THE COLLECTION AND STORAGE OF HUMAN BLOOD

CHAPTER ONE

INTRODUCTION

1.1 A BRIEF HISTORY OF BLOOD TRANSFUSION

The concept of blood transfusion is very old, though it was not until 1667 that the first recorded blood transfusion into man took place. The problem of finding a volunteer was overcome when a French mathematician, Denis, assisted by a surgeon, 'seized' a 'madman' outside Paris and transfused 8 ounzes of fresh calf's blood into his veins. Remarkably, the recipient not only recovered but his madness was apparently cured. Denis became a celebrity, though short lived because during a second transfusion, the recipient died. As a consequence of this and several similar incidents, blood transfusion fell into disrepute and was made criminal (in France) in 1668 (Lewes, 1859).

It was only in 1818 that blood transfusion was revived when it was suggested that, when transfusing blood into humans, human blood must be employed (Blundell, 1818). However, only at the turn of the 20th Century did the development of suitable anticoagulants and recognition of antigenic differences within a species, make blood transfusion relatively safe. The resultant upsurge in the demand for blood for transfusion paved the way for a blood banking system, which involved the storage of blood.

Blood platelets were first described by Donne in 1842 (cited - Nour-Eldin, 1967) but it was only in 1938 that platelet aggregation during blood storage was documented (Fantus, 1938). Although improvements, such as filtration and better storage conditions have partly alleviated the problems associated with transfusing cellular aggregates, the underlying cause of aggregation during blood storage has yet to be resolved.

The work described in this thesis is concerned with examinations of certain chemical and physical parameters that may relate human blood collection and storage to platelet aggregation.

1.2 THE BLOOD PLATELET

The following is a brief review of platelet research with particular reference to aggregation.

1.2.1 PLATELET PRODUCTION

Platelets develop in the bone marrow from megakaryocytes by a process of compartmentation of the cytoplasm. The platelets are 'pinched off', mainly into the marrow sinuses, and enter the circulatory system where they survive for 8 - 10 days. Mature megakaryocytes also reach the lung, where up to 17% of the platelet population may be released (Vane, 1969). Platelet half life has been suggested as an index of platelet survival as opposed to platelet turnover (about $5 \times 10"$ platelets per day in man) (Jamieson, 1977), which is a better measure of platelet economy. This is because platelets are destroyed not just as a result of age, but also indiscriminately as a consequence of the effects of certain factors which may influence platelet membranes, and subsequently survival (Mustard et al. 1966).

The absolute number of platelets in the circulation is controlled
by a negative feedback system involving a humoral factor, thrombo-
poietic stimulating factor (TSF) which is capable of stimulating
megakaryocyte production. A reduction in the number of platelets
results in raised TSF levels and hence a rise in platelet production.
(Sanderson and Goldstone, 1977). Because megakaryocytes take some
time to mature, platelet production may be regulated to the negative
feedback containing a time delay and therefore may be oscillatory
rather than constant, that is, a platelet cycle may occur (Morley,
1969). The normal platelet count is 150 - 400 x $10^9 L^{-1}$.

1.2.2 PLATELET STRUCTURE (Figure 1)

Platelets are non-nucleated cells, and in the blood circulation,
they are disc-shaped and 2 - 3 μ in diameter (Mustard and Packham, 1970).
The platelet has been divided into three regions: the peripheral
zone, the sol-gel zone and the organelle zone (Sanderson and Gold-
stone, 1977).

1.2.2.1 THE PERIPHERAL ZONE

This includes the exterior coat, or the glycocalyx, which is rich in
glycoproteins and is a layer unique to the platelet. Mucopolysaccharides
are often associated with the glycocalyx though it is now thought
that they are not actual components (Jamieson, 1977). The glycocalyx
is important in platelet function and in normal conditions, is
surrounded by a nimbus of proteins called the plasmatic atmosphere.
It is not clear if the glycocalyx is an integral part of the plasma
membrane, which makes up the remainder of the peripheral zone.

Figure 1   NORMAL PLATELET STRUCTURE

(Platelet from a sample of citrated platelet rich plasma fixed at 37°C in glutaraldehyde and osmic acid. The discoid cell has been sectioned in the equatorial plane, x 33,200)

Key:

DTS-(Dense tubular structures) and MT - microtubules in the sol-gel zone. The circumferential microtubules are involved in maintaining the platelet discoid form.

OCS-Open canicular system. This extends from the peripheral zone into the organelle zone.

G - Lysosomal granules in the organelle zone.

DB - Dense bodies ("bulls-eye" granules) in the organelle zone.

GLY-Glycogen particles in the organelle zone.

M - Mitochondria in the organelle zone.

(after White, 1974)
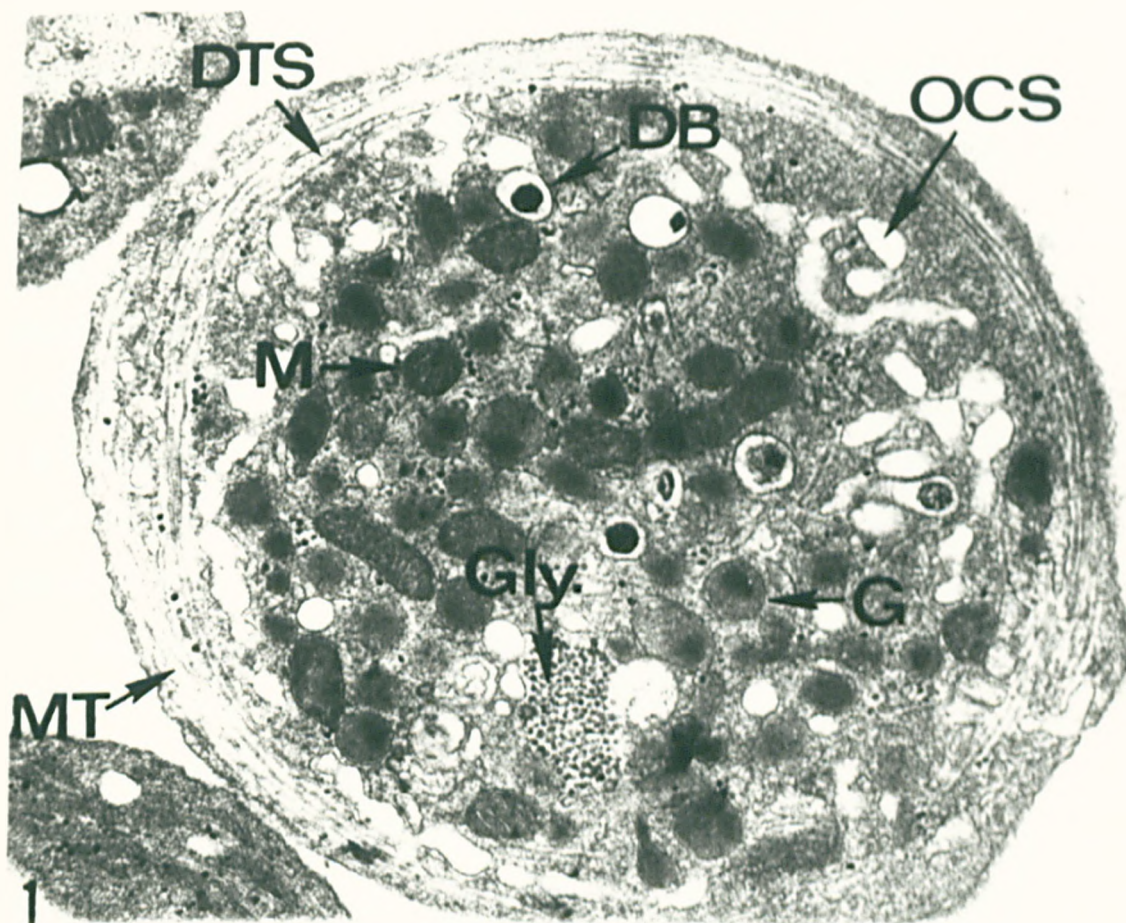
Figure 1   NORMAL PLATELET STRUCTURE

This membrane is of the usual trilaminar structure and is rich in phospholipids. It has deep invaginations into the platelet interior, the canalicular system, which may assist in the transport of various platelet factors. Platelets also provide an active lipoprotein surface which interacts with plasma coagulation factors. The term platelet factor 3 (PF3) has been used to describe this lipoprotein, though it is not an identifiable biochemical substance. (Jamieson, 1977). The location of platelet membrane phospholipid has been probed with 2,4,6, trinitrobenzenesulphonate, and it has been shown that exposure of phosphatidylethanolamine may have a critical role in platelet haemostatic function, that phospholipid asymmetry exists, and that this may have physiological significance (Schick et al., 1976). A number of blood clotting factors I, V, VIII and XIII are present in platelet preparations and most of the other factors, with the possible exception of factor XIII, are reported to be associated with platelets (Bennet and Ratnoff, 1972).

The platelet plasma membrane is 7% carbohydrate, 36% protein and 52% lipid. Enzymes associated with the plasma membrane include adenosine triphosphatase, adenal cyclase, phosphodiesterase, cholinesterase, acid phosphatase and several glycosyltransferases. Sialic acid residues, mainly contained within the glycoprotein, glycophorin, are very dense on platelet membrane surfaces and partly contribute to the platelet charge or zeta potential (Nurden and Caen, 1976). Platelets normally have a very high negative charge with an isoelectric point at 3.6 (Jamieson, 1977). The nature of the cell surface reflects the environment in which the cell is found and the molecular composition of the plasma membrane reflects any specialised function placed on it. Isolation of glycoproteins on the platelet

membrane surface have revealed several polypeptides, though it is generally agreed that 3 major glycoproteins, I, II and III, exist (Phillips, 1973; Steiner, 1974; Nurden and Caen, 1976; Jamieson, 1977). These contain heterosaccharide units significantly different from those in red blood cell membranes (Steiner, 1974). The platelet membrane is asymmetrical with the carbohydrate moieties of the glycoproteins, most of which span the membrane, in a prominent position on the external surface. The molecular weights of the 3 glycoproteins seem to vary depending upon the investigator and the separation method, though glycoproteins I and II, with approximate molecular weights of 150,000 and 118,000 daltons respectively, contribute mainly to platelet adhesion and aggregation. The third surface glycoprotein is associated with pore formation and transport (Nurden and Caen, 1976). Sialic acid is found particularly in glycoprotein I which is 70% carbohydrate, having galactose as its sole neutral sugar and equimolar amounts of galactosamine and glucosamine as N-acetyl derivatives (Jamieson, 1977).

1.2.2.2 THE SOL-GEL ZONE

This consists principally of microtubules and microfilaments composed of a contractile protein, thrombosthenin, which is similar in character to actin and myosin. These structures play an important role in providing a cytoskeleton active in platelet shape changes and in clot retraction (Jamieson, 1977) A marginal bundle of fine tubules can be seen lying along the membrane in the equatorial plane of platelets (Mustard and Packham, 1970).

1.2.2.3 THE ORGANELLE ZONE

This is the remainder of the cytoplasmic area in which are found
a few primitive mitochondria, glycogen granules, vacuoles, possibly
ribosomes, lysosomal granules and so-called dense (osmophilic) or
'bulls eye' granules (Mustard and Packham, 1970). Platelet
fractionation and subsequent enzyme assay have shown that acid phos-
phatase has the highest specific activity of all the acid hydrolases
found in platelets, and that it is mostly firmly bound in the lysosomal
granules (Polasek, 1968; Zucher and Borrelli, 1959).

Human platelets are very rich in most lysosomal enzymes, including
acid phosphatase, β-glucuronidase, β-galactosidase, the cathepsins
(Day et al., 1969) and collagenase (Chesney et al., 1972). Lysosomal
granules have also been associated with fibrinogen and platelet
factor 4 (PF4) (Holmsen et al., 1969a). However, better separation
procedures have demonstrated that fibrinogen is contained within
the dense granules (Broekman et al., 1975), associated with 5-hydroxy-
hyptamine (5HT), adenosine diphosphate (ADP), adenosine triphosphate
(ATP), catecholamines, calcium and potassium (Born, 1956; Holmsen et
al., 1969a; Jamieson, 1977). More recently, evidence has been
produced that another storage granule exists, separate from the
dense granules and lysosomes, in which it is suggested are stored
PF4, fibrinogen and a platelet specific protein, β-thromboglobulin
(Fukami and Salganicoff, 1977; Dawes et al., 1978).

The results of ^{32}P radiolabelling of platelets suggest that
three pools of adenine nucleotides exist, two being metabolic and
the third (the largest, containing about 60% of the total platelet
ATP content) contained in the dense granules. One of the metabolic
pools is described as a release energy pool, which provides energy

for the platelet release reaction (described later) and the other,
which is retained, is probably involved in platelet energy requirements
distinct from the release reaction. The concentration of ATP in
the dense granules is large in comparison with that of 5HT, the ratio
being about 5:1 in human platelets. The ratio of ATP to ADP has been
estimated to be 0.6:1.1 (Holmsen et al., 1969b).

1.2.3 PLATELET METABOLISM

Platelets require energy to carry out all their functions, but the
study of platelet metabolic requirements during their resting state is
difficult due to stimulation of the release reaction during platelet
isolation. It is possible that anaerobic glycolysis is the main
pathway for energy production (Maynert and Isaac, 1968) though there
may be more complete glucose oxidation via oxidative phosphorylation
depending upon mitochondrial efficiency. Platelets can metabolise a
variety of hexoses including glucose, glycogen, fructose and galactose.
A large proportion of platelet ATP provides energy for the release
reaction and probably, takes no part in platelet metabolism. Like
other cells, platelets are sensitive to changes in adenine nucleotide
levels so that reduced ATP concentration result in increased phospho-
rylase activity and hence glycogenolysis. (Mustard and Packham, 1970).

1.2.4 PLATELET FUNCTION

Platelets have four main functions, storage and transport;
defence; haemostasis; and blood coagulation (Mustard et al., 1966).

1.2.4.1 STORAGE AND TRANSPORT

Platelets contain most of the blood 5HT, estimated to be between 200 and 1300 $ng/10^9$ platelets (Franzen and Eysell, 1969); and a large proportion of histamine and catecholamines. It is not clear how far this acts as a transport mechanism; perhaps as a means of providing vaso-active compounds at a local point of injury (Mustard et al., 1966). However, it is widely recognised that platelets are able to take up both 5HT (Maynert and Isaac, 1968; Pletscher, 1968; Baumgartner, 1970) and catecholamines (Barthel and Markwardt, 1974; Born et al., 1967a). The platelet surface has specific binding sites for both 5HT and catecholamines.

1.2.4.2 DEFENCE

Platelets are capable of phagocytosis and therefore may contribute to a defence mechanism (Mustard et al., 1966). Indeed, it has been suggested that the primary role of platelets is defence against foreign organisms (Copley and Witte, 1976). Many observations have been made of platelet clumping and adherence to bacteria, and it has been demonstrated that the body's first reaction to bacterial invasion is platelet agglutination and adherence of platelets to bacteria, jointly termed conglutination. This may act together with platelet phagocytosis ability in general body surveillance against bacteria, viruses and even tumor cells (though ingested viruses in platelets may effectively be 'harboured'), resulting in elimination perhaps by local monocytic phagocytosing systems engulfing the entire platelet clump. Aggregated platelets are capable of neutrophil chemotaxis possibly via release of a lipo-oxygenase, 12, hydroxy,5,8,

10,14 eicosatetraenoic acid (HETE) (Turner et al., 1975). HETE may be the main chemotactic material for monocytes, since evidence suggests that the complement fraction C5a, which is widely held to possess powerful chemotactic properties, is not chemotactic for monocytes (Turner et al., 1975). However, other evidence suggests that a 'platelet protein fraction' released during aggregation, and itself lacking chemotactic activity, generates such activity by acting directly on C5 (Weksler, 1974; Niewlarowski, 1977). The two activities may be the same, but since one is reported to be lipid and the other protein, there are possibly two separate mechanisms.

1.2.4.3 BLOOD COAGULATION

Platelets are important in blood coagulation for three reasons. Firstly, many blood coagulation factors and PF3 are associated with platelets. Secondly, platelets are responsible for clot retraction (Bennet and Ratnoff, 1972). Finally, stimulated platelets may be able to promote in-vivo blood clotting (Mustard et al., 1966; Ehrman et al., 1978) via activation of factor XII in the intrinsic coagulation pathway (Walsh, 1973).

There is evidence for multiple different functions of platelets in intrinsic coagulation including 'contact product forming activity' where platelet surface properties may be altered by aggregating agents to activate factor XII (Walsh, 1973; Ardlie and Han, 1974). Another function is 'factor Xa forming activity', which is the capacity of platelets to enhance the reactions of factors XIa, VIII, IX and X to form Xa. (Walsh, 1973). Also, platelets are rich in fibrinogen,

which is released during the release reaction (see later).

PF3, often erroneously used synonymously with platelet pro-coagulant activity (Ehrman et al., 1978) is important in intrinsic blood coagulation. The mechanism of its conversion to an active form is unknown, though activity seems to depend on the presence of at least one phospholipid dependent protein (Wu and McCoy, 1977). PF3 is almost certainly the 'platelet like activity of serum' described by O'Brien (1955) and Alagille and Soulier (1957) and also the 'platelet dust' described by Wolf (1967).

PF3 activity, which catalyses particularly the reactions of factor Xa and V to activate prothrombin in the presence of calcium ions (Walsh, 1973), has been postulated as being the initiator of the intrinsic coagulation pathway (Mammen et al, 1960 - cited Wu and McCoy, 1977).

Clot retraction is associated with certain changes in platelets and may be linked to the aggregating activity of thrombin. (See later). The aggregates become linked by pseudopodia, contraction of which (dependent on thrombosthenin) produces the contraction of the clot (Bennet and Ratnoff, 1972).

Platelet shape change, the first visible event in aggregation, is itself associated with the development of platelet procoagulant activity. This is the binding of soluble activated coagulant prot-eins to the platelet surface prior to coagulation, and it is possibly due to exposure of phospholipid on the platelet membrane (Ehrman et al., 1978).

1.2.4.4 HAEMOSTASIS

Possibly the most widely documented activity of platelets is

their ability to aggregate under the influence of a variety of aggreg-
ation inducers. The role of platelets in blood haemostasis is to adhere
to exposed collagen to form a platelet plug when the vascular integrity
is lost. (Mustard et al., 1966; Jamieson, 1974; Wang et al., 1975).
The plug formation is a consequence both of platelet aggregation and
of the release reaction which facilitates the interaction of coagulation
factors leading to fibrin formation and clot retraction (Ardlie and
Han, 1974). It is widely believed that in the haemostatic process,
the early stages of platelet aggregation occur prior to, and independent
of coagulation reactions. However, in-vitro studies have suggested
that small amounts of thrombin present, or formed prior to clotting,
may serve as the initial stimulus to platelet changes in haemostasis,
and that platelet aggregation and blood coagulation are interdependent
and can be regarded as one phenomenon (Ardlie and Han, 1974, Akbar
and Ardlie, 1978).

There is evidence that platelets may also affect the fibrino-
lytic, plasminogen-plasmin system thought to be an important haema-
static regulatory mechanism to prevent inappropriate extension of the
plug, which, if uncontrolled, may lead to total vessel occlusion
(Joist, 1977).

Although platelet counts vary from one person to another, they
have been estimated to be about four times greater than necessary
for haemostasis. This leads to the alternate hypothesis, either
that platelet function is homogeneous, large numbers being required
to meet all demands, or that platelet function is heterogeneous with
a subpopulation of platelets being responsible for normal haemostasis.
Evidence favours the latter hypothesis and suggests that a subpopulation
of megathrombocytes (large platelets) are the more important functioning

platelets in haemostasis (Karpatkin, 1978).

1.2.4.5 PLATELETS AND THROMBOSIS, AND THE USE OF ANTI-PLATELET DRUGS

In addition to playing a vital role in preventing blood loss from vessels damaged by injury, platelets are involved in the occlusion of arteries damaged by atherosclerotic disease, a condition known as arterial thrombosis. Platelets are also thought to play a role in venous thromboembolism though veins do not become atherosclerotic (Lancet, 1977). Venous thrombi originate as "white heads" composed of platelets, leucocytes and fibrin (Walsh, 1977) and usually begin in valve pockets where rheological conditions favour the formation of relatively stagnant pools of blood (Weiss, 1976).

Considerable attention has been paid towards drugs and agents that might prevent thrombus formation. Heparin is used successfully in protecting against deep vein thrombosis and pulmonary embolisms, perhaps because of the importance of blood coagulation in venous thrombosis. Platelet aggregation may be a more important event in the initiation of an arterial thrombus, and a growing body of clinical evidence supports the use of antiplatelet drugs as the approach to pre-vention of arterial thrombosis. The roles of ADP, prostaglandins and cyclic adenosine monophosphate (cAMP) in platelet aggregation have been important in evaluating the potential effectiveness of various drugs. However, though many compounds have been shown to inhibit platelet function in-vitro (Mustard and Packham, 1970), only a few have been tested for their antithrombotic effects in man (Hirsch, 1977). These include acetyl salicylic acid (aspirin) which inhibits the platelet release reaction by inhibiting the synthesis of prost-aglandin cyclic peroxide intermediates. Sulphinpyrazone, a

uricosuric agent closely related to phenylbutazone, inhibits platelet function in an unknown manner (Weiss, 1976). Dipyridamiole is one of a number of pyrimido-pyrimidine compounds which inhibit platelet function, probably by increasing cAMP levels by inhibiting phosphodiesterase activity. Hydroxychloroquine, often used as an anti-inflammatory agent, weakly inhibits ADP induced platelet aggregation. Finally, clofibrate is a hypolipidemic agent which appears to reduce platelet adhesiveness, though like hydroxychloroquine, no convincing evidence exists that it is an effective antithrombotic drug. (Hirsch, 1977). Recent evidence for a role of prostaglandins and prostaglandin intermediates in the control of thrombosis (see prostaglandins) has lead to new approaches in the search for antithrombotic agents. Lipid peroxidation has been implicated in atherosclerosis, and one possibility is that inhibition of prostacyclin synthesis by lipid peroxides could be a reason for the increased tendency to thrombotic complications in atherosclerosis. Antioxidants, by protecting prostacyclin synthetase would therefore form a basis for antithrombotic therapy (Lancet, 1977a).

1.2.5. PLATELET AGGREGATION - THE EVENTS

The chain of events that occurs on platelet stimulation follows a well defined pattern, involving shape change, primary aggregation, release and secondary aggregation. It is not clear if these stages are separate and distinct, but the extent of the reaction is related to the type and strength of the stimulus. Under certain conditions, platelet aggregation is completely reversible and does not impair platelet survival, or their involvement in further platelet functions. Reversible aggregation is probably better defined as

incomplete aggregation, the cells not having had a strong enough
stimulus to completely aggregate (Holmsen, 1974).

A wide variety of biological agents are known to be capable of
inducing platelet aggregation, including ADP, adrenaline, noradren-
aline, 5HT, long chain fatty acids, collagen and thrombin (Mustard
and Packham, 1970), calcium (Born, 1962) and vasopressin (Haslam
and Rosson, 1971).

1.2.5.1 ENERGY REQUIREMENTS FOR AGGREGATION

The majority of platelet functions studied depend upon a cont-
inuous cellular production of ATP. Those that do not, include plate-
let agglutination by, for example, polylysine, though these activities
are often distinguished from aggregation because of the lack of ATP
dependence (Solum, 1968 - cited Holmsen, 1977a). ATP is the source
of energy during the whole aggregation chain and is associated with
increased glycolysis, oxidative phosphorylation and glycogenolysis
(Deisseroth et al., 1970; Schneider, 1974). Interpretation of some
results should be treated with caution, however, as several studies
relating platelet function and metabolism have used washed,
resuspended platelets in EDTA containing media. Such platelets
have altered morphology with pseudopodia and large vacuoles, in
contrast to resting platelets in whole blood, or citrated platelet
rich plasma (PRP)(Holmsen, 1977a). However, glycogen is an import-
ant source for increased ATP production during platelet activation
probably arising from direct mobilisation of glycogen. The process,
glycogenolysis, normally depends upon cyclic adenosine monophosphate
(cAMP) activating a cAMP dependent protein kinase. This, in turn,
begins a series of enzyme activations resulting in the breaking of

the -1,4- linkages of glycogen to yield glucose 1-phosphate. This type of mechanism allows amplification of the original signal at each activation step, and hence a small change in cAMP can cause a large change in the activity of the final enzyme, phosphophosphorylase. The process probably explains how increased platelet aggregation is associated with a decrease in cAMP (Schneider, 1974), though other evidence suggests that control of platelet phosphorylase activity may be modulated by non-cyclic adenine nucleotides (Deisseroth et al., 1970).

Protein kinase catalysed phosphorylation reactions are involved in contractile protein functions as well as membrane permeability control. High levels of membrane phosphorylation appear to maintain the platelet in a non-aggregating state and it has been proposed that the rapid dephosphorylation of the platelet surface is the common reaction that leads to membrane changes and subsequent platelet aggregation (Booyse et al., 1973).

1.2.5.2 MEASUREMENT OF PLATELET AGGREGATION

The stages of aggregation have been widely assayed using the aggregometer (Born, 1962), in which light transmission changes as a consequence of shape change and aggregation in stirred PRP or resuspended platelets are recorded. Characteristic patterns of aggregation have been observed. It is thought that adrenaline does not induce a shape change (Holmsen, 1974; MacFarlane and Mills, 1975), though this is controversial (Mustard and Packham, 1970), and the primary aggregation phase is not distinguishable from secondary agg-regation induced by collagen (Weiss, 1976) (Figure 2).

Figure 2
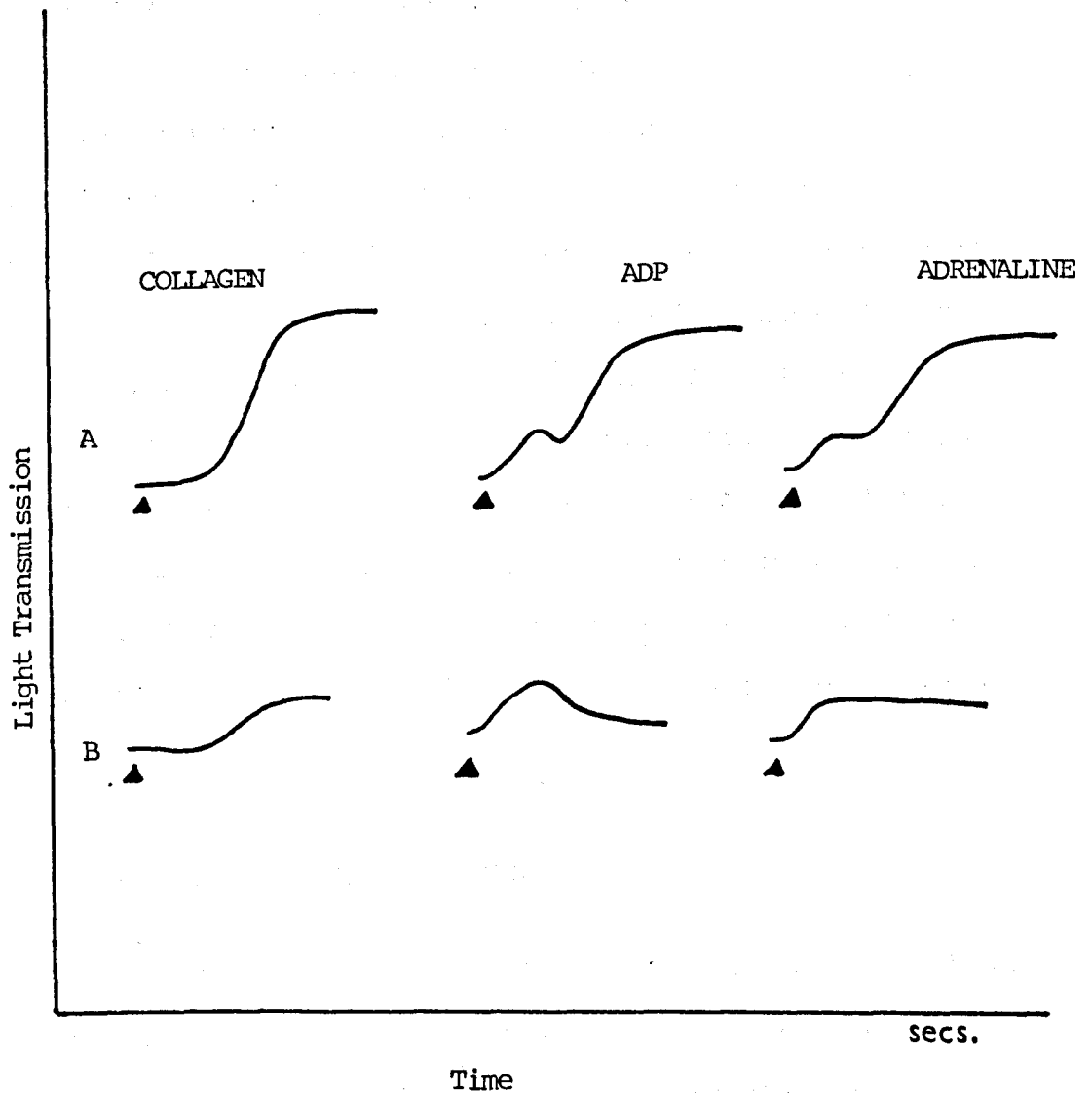
Platelet aggregation responses (using PRP in an aggregometer)

A   —   Normal patterns

B   —   Response on exposure to drugs that inhibit the release
           reaction (e.g. aspirin and indomethacin).

(From Weiss, 1976)

1.2.5.3 PLATELET SHAPE CHANGE AND FIRST STAGE AGGREGATION

Platelet shape change, the discocyte – echinocyte transformation
(Ehrman, 1978), is the earliest manifestation of platelet stimulation
(Lüscher, 1977), when platelets swell from their normal disc shape
to a sphere (Holmsen, 1974) sometimes with pseudopodia (Hovig, 1974).
Unlike the other events in aggregation, the shape change process is
thought to be independent of extracellular divalent cations (Massini,
1977). It is characterised by the disappearance of the peripheral
ring of microtubules, and by the morphologically discernable contract-
ile activity involving thrombosthenin. These are both dependent
upon intracellular calcium in the same way as actin and myosin in
muscle contraction (Lüscher, 1977). The shape change results in
'sticky' platelets which adhere to form loose reversible aggregates
(Barrer and Ellison, 1977). This primary aggregation depends upon
the presence of certain extracellular factors (discussed in aggreg-
ation mechanisms).

1.2.5.4 THE PLATELET RELEASE REACTION

If the aggregating stimulus is above a specific threshold level,
a second phase of aggregation is observed, and this is associated
with the release reaction. This reaction is characterised by the
discharge of various substances. The process is very rapid and is
morphologically associated with centralisation of the dense granules
(observed in vitro). Centralisation has been termed, the "contractile
wave", and probably involves polymerized cytoplasmic thrombosthenin
rather than any organised microtubule system (Hovig, 1974). This is
followed by fusion of many of these granules with the membranes of

the platelet canalicular system, and discharge of the contents of the granules into the system. The substances released include non-metabolic ADP and ATP, 5-HT, adrenaline, noradrenaline, platelet factor 4, fibrinogen, potassium and calcium ions, PF3, β-glucuronidase, and β-thromboglobulin (Holmsen, 1969a; Detwiler and Feinman, 1973; Barrer and Ellison, 1977; Niewiarowski, 1977). The amounts released seem to depend upon the type of aggregating agent and the concentration of viable platelets. For example, 0.5 μM adrenaline will induce the release of up to 55% of the total intracellular ADP from the platelets in citrated human PRP (Mills et al., 1968). This corresponds to approx-imately 2.5 μM per 10" platelets (D'Souza and Glueck, 1977). 1 - 2 uM ADP will induce a similar release of intracellular ADP. Adrenaline, thrombin, collagen and ADP are each capable of inducing the release of 30% to 40% of platelet ATP (Mills et al., 1968; D'Souza and Glueck, 1977). It has been estimated that collagen is capable of inducing the release of up to 80% of the 5-HT, 60% of the ADP, 40% of the ATP and 50% of the potassium ion content of platelets (Holmsen et al., 1969a). Thrombin is thought to be the most powerful release reaction stimulating agent, followed equally by collagen, and ADP, and then by adrenaline (D'Souza and Glueck, 1977).

Most of the released substances are associated with the "bulls eye" granules, or like PF3, with the plasma membrane (Barrer and Ellison, 1977). However, some aggregating agents may provoke other changes including lysosomal activation. For example, thrombin and collagen cause a significantly greater release of β-glucuronidase (Mills et al., 1968). This type of activation is sometimes referred to as release II. Thrombin and collagen induce both release I and

release II, and includes lysosomal acid hydrolases such as β-glucuron-idase, β-galactosidase, and the cathepsins. Only release I occurs with aggregation induced by adrenaline and ADP, causing the release of the contents of the dense granules (Holmsen et al., 1969a).

The release process may be divided into three steps (Holmsen et al., 1969a):

a) Induction, an interaction between the aggregating agent and the plasma membrane, during which a "release impulse" is created. Primary aggregation takes place during this step.

b) Intracellular transmission, the transmission of the "impulse" from the plasma membrane to the α-granules. Calcium ions have been suggested as this intracellular transmitter (Holmsen, 1974).

c) Extrusion, the process triggered by the "impulse" which leads to the specific emptying of the granular content extracellularly. Many of the extruded substances are of biological importance and platelets may be regarded as being immediate homeostatic secretory cells.

Acid phosphatase is not released during the release reaction, and the detection of traces of acid phosphatase in the plasma following the induction of platelet aggregation is probably due to cellular damage (Mills et al., 1968).

Extracellular calcium ions may be important for the release reaction, though both collagen and thrombin are able to induce release in their absence. (Mueller-Eckhardt and Lüscher, 1968 - cited Massini, 1977).

1.2.5.5 SECOND STAGE PLATELET AGGREGATION

This stage of aggregation is associated particularly with the

release of 5HT, ADP, calcium ions, and catecholamines during the release reaction, and it can be regarded as release linked aggregation. It is widely considered that this stage is irreversible (Born, 1962; Mustard and Packham, 1970; White et al., 1973; Ardlie and Han, 1974; Barrer and Ellison, 1977), though it is also thought that platelets can go through the entire cycle of activation and still be capable of returning to their resting state, in spite of having shed the contents of their storage granules (Lüscher, 1977). The latter may occur under controlled in-vitro conditions, but it seems unlikely that it occurs during normal in-vivo haemostasis and coagulation.

1.2.5.6 VISCOUS METAMORPHOSIS

Viscous metamorphosis is the term reserved for the fusion of a large number of platelets into an irreversible amorphous mass. During this stage, platelet lysis occurs and acid phosphatase and lactic dehydrogenase are released. Acid phosphatase release has been used as a marker of platelet destruction (Brown et al., 1975). Viscous metamorphosis is observed with thrombus formation, blood coagulation (Zucker and Borrelli, 1959; Sharp, 1958) and blood storage (Polasek, 1968). These metamorphosed aggregates are often associated with fibrin strands, which are thought to stabilise the aggregate (Mustard et al., 1966). The release of acid phosphatase during platelet lysis is sometimes referred to as the second phase release reaction (Mustard and Packham, 1970), but this term can be confused with the term release II. Release II is probably strictly an in-vitro term to distinguish between the effects of collagen and thrombin and other aggregating agents. Second phase release is a continuation of release II associated with platelet lysis. However,

in-vitro release I could also result in second phase release, depending upon the strength of the stimulus and the state of the platelets.

1.2.6 THE MECHANISMS OF PLATELET AGGREGATION

The mechanisms by which platelets adhere to thrombogenic surfaces, change shape, aggregate, and release their granular contents are not fully understood, though evidence is available for the involvement of many factors including surface charge, glycosyltransferases, prostaglandins, calcium ions, ADP and fibrinogen.

1.2.6.1 ENDOGENOUS ADP

Endogenous ADP is thought to play a central role as the mediating agent between the aggregation agent and the platelet response (Haslam, 1964; Benner and Brunner, 1973; Jamieson, 1977). This role is based upon ADP clearing studies using for example, phosphoenolpyruvate/pyruvate kinase (Haslam, 1964) and creatine phosphate/creatine phosphokinase (Izrael et al., 1974) in which platelet aggregation was very much reduced. However, there is evidence against a role for released ADP in primary aggregation, since the release of ADP has not been detected during first phase aggregation by adrenaline, thrombin, 5HT or vasopressin (MacFarlane and Mills, 1975) and studies, again with creatine phosphate/creatine phosphokinase, have shown that platelet adhesion to collagen fibrils is not mediated via endogenous ADP (Tschopp and Baumgartner, 1976).

Therefore, it is probable that endogenous ADP is not involved in adhesion, shape change or primary aggregation, but that these result as direct responses to the aggregating agent. It seems that

- 20 -

ADP is involved only in the second stage of aggregation as a consequence of its release in the release reaction; its possible mechanism being discussed later.

1.2.6.2 THE ROLE OF CALCIUM IONS IN PLATELET AGGREGATION

Platelets contain large amounts of calcium, most of which is thought to be in the dense granules and released during the platelet release reaction, along with ATP, ADP and 5HT (Massini, 1977). However, a recent comparison of the release of ATP and calcium ions showed that the release of calcium preceded the release of ATP and by a kinetically distinct reaction (Detwiler and Feinman, 1973). Vesicles similar to the sarcoplasmic reticulum of muscle cells are another calcium ion storage compartment, as are the cytoplasm, the mitochondria and even the platelet surface. Platelet activation is triggered by a rise in cytoplasmic calcium resulting in increased contractile activity. Shape change requires the breakdown of the peripheral ring of microtubules which hold platelets in their discoid shape. This is most probably due to intracellular calcium ions, which are known to depolymerise microtubules. Platelet aggregation and release require calcium ions involved in the contractile system (Massini, 1977), though thrombosthenin contraction may also be controlled by ADP, indicating the existence of two independent systems that control aggregation and release in intact platelets (Aledort et al., 1973). It has been proposed that the role of calcium ions in the mechanism of the release reaction is in altering the membranes of the storage organelles and cytoplasmic membranes in such a way that fusion is induced resulting in the extrusion of the contents

of the organelles (Lucy, 1970; Massini, 1977).

Holmsen (1974) suggested an alternative scheme for platelet aggregation in which, instead of regarding the stages as separate (Jamieson, 1974), the functions are manifestations of one basic cellular reaction. The aggregating agent interacts with the platelet membrane resulting in the release of an intracellular transmitter which activates ATP requiring systems. He postulates that this transmitter is intracellular calcium and that the basic reaction is cellular contraction. The stage reached in the sequence depends upon the type and strength of the stimulus and the responsiveness of the platelets.

Extracellular calcium ions have been demonstrated to be necessary for platelet aggregation by, ADP, 5HT, adrenaline, thrombin and vasopressin (Massini, 1977), although agents such as thrombin, collagen and antibody/antigen complexes are thought to be able to induce release in the absence of extracellular calcium.

This requirement for calcium or other divalent cations raises the question of how aggregation can take place in citrated PRP or whole blood. It has been found that ADP at even 5×10^{-7}M can induce aggregation in citrated human PRP (Born and Cross, 1963), even though it was found that, working with unwashed resuspended pig platelets, the optimal calcium ion concentration was about the normal physiological one (Born and Cross, 1964).

More recently, however, it has been found that the optimum calcium ion concentration for aggregation is below the physiological level (Heptinstall, 1976), and that the addition of citrate could favour, at least, secondary aggregation, by reducing the calcium concentration (Akbar and Ardlie, 1978). Anticoagulation

with citrate containing anticoagulants is designed to prevent blood

coagulation, but the fact that sufficient calcium ions remain free

to allow platelet aggregation is suggested by the action of EDTA,

which chelates all divalent cations and effectively inhibits ADP

induced aggregation (Born and Cross, 1963). Magnesium ions are

complexed to a lesser extent than calcium ions by citrate and may

act as an alternative to calcium ions in platelet aggregation in

anticoagulated plasma or blood. However, magnesium ions have

been shown to be less effective than calcium ions in platelet

aggregation (Born and Cross, 1964) and perhaps it is a combination

of available divalent cations that functions in platelet aggreg-

ation in citrated blood.

1.2.6.3 THE ROLE OF PROSTAGLANDINS IN PLATELET AGGREGATION

The second phase of platelet aggregation is inhibited by, for

example, acetyl salicylic acid (aspirin) and indomethacin. These

drugs also inhibit prostaglandin synthesis, and this suggests a

role for prostaglandins, or their intermediates, in platelet

aggregation (Smith et al., 1974a). This is supported by the obser-

vation that platelets produce prostaglandins when stimulated by

aggregation inducers (Smith and Willis, 1970; Smith et al., 1973).

Although primary platelet aggregation can be induced by exogenous

arachidonic acid, a prostaglandin precursor (Smith et al., 1974a),

and certain exogenous prostaglandins themselves (Smith et al., 1977),

the formation of endogenous prostaglandins does not take place until

the onset of the release reaction (Smith et al., 1973). Hence

primary aggregation probably only involves the direct effect of

the aggregated agent with calcium ions and fibrinogen as cofactors

(Holmsen, 1977b). Activation of prostaglandin synthesis is probably responsible for the platelet release reaction (Gerrard et al., 1975; Weiss et al., 1976), and the release reaction may be regarded as the link between prostaglandin synthesis and platelet aggregation (Smith et al., 1974b).

One prostaglandin precursor is arachidonic acid, and its liberation from membrane phospholipid is the crucial step. This may occur by hydrolytic cleavage of the ester bond by phospholipase A_2, a membrane bound enzyme, that requires calcium ions for activity. Hence its stimulation within the intact platelet may require mobilisation of calcium from an intracellular store (Weiss et al., 1976; Holmsen, 1977b). Whether calcium mobilisation is the primary mechanism by which aggregating agents initiate prostaglandin synthesis or not, is not known. The next step in prostaglandin synthesis is oxidative cyclization of the precursor for which a coenzyme is required to provide the necessary reducing equivalents. It has been suggested that catecholamines and 5HT may be the natural coenzymes in regulating the in-vivo biosynthesis of prostaglandins (Sun et al., 1970); and platelets store both.

Recent evidence suggests that the interaction of an aggregating agent with the platelet membrane activates a unique precursor serine protease on the platelet outer membrane surface. This activated protease then activates phospholipase A_2 (Aoki et al., 1978) presumably by hydrolytic cleavage in the presence of calcium ions. However, inhibition of this protease with, for example, aprotinin, inhibited only the second phase of aggregation, thus providing further evidence for the role of prostaglandin synthesis in release linked aggregation only.

It is known that both prostaglandins E_2 and $F_{2\alpha}$ (PGE$_2$ and PGF$_{2\alpha}$) are formed during platelet aggregation (Smith et al., 1974b) and PGE$_2$ is thought to be a potential aggregation inducer. However, it has been demonstrated that endoperoxide intermediates formed during prostaglandin synthesis are more powerful aggregating agents. These endoperoxide intermediates, PGH$_2$ and PGG$_2$ are formed by cyclic oxidation of arachidonic acid and have jointly been termed "labile aggregating stimulating substance (LASS)" (Willis, 1974; Gerrard et al., 1975). More recent work has further elucidated the prostaglandin biosynthetic pathway and has shown that a further intermediate, formed from PGH$_2$, is produced, thromboxane A$_2$, which is a very powerful aggregating agent (Sun et al., 1977). (Figure 3)

Arachidonic Acid

cyclooxygenase

PGG$_2$

peroxidase

prostacyclin synthetase

Prostacyclin ← PGH$_2$ → PGF$_{2\alpha}$

PGE$_2$

thromboxane synthetase

6-keto PGF$_{1\alpha}$

Thromboxane A$_2$

Thromboxane B$_2$
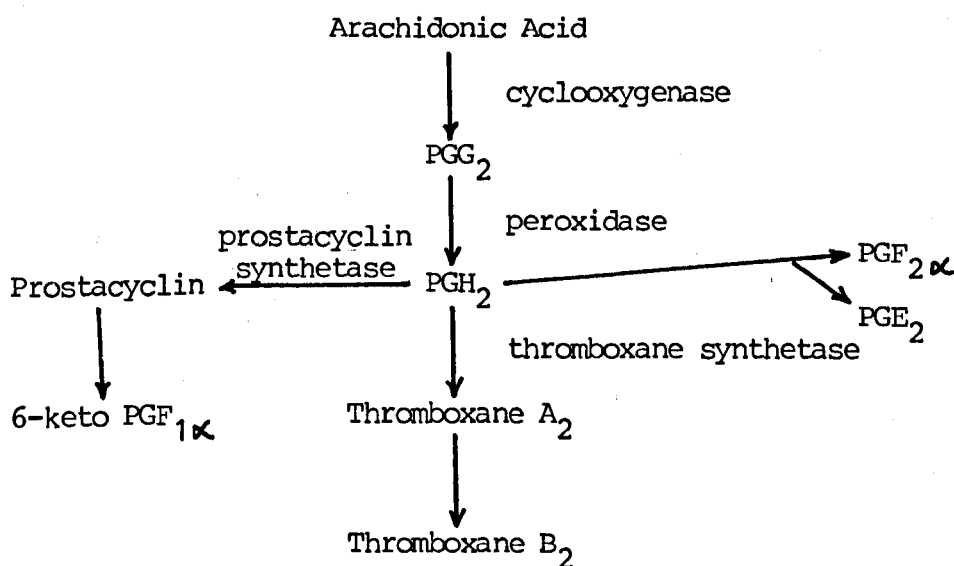
Figure 3. SUMMARY OF PROSTAGLANDIN SYNTHESIS FROM ARACHIDONIC ACID

The mechanism of prostaglandin induced aggregation is unknown, though synthesis is associated with reduced cAMP levels (similar to

platelet aggregation). However, it may be that prostaglandin and
cAMP levels are in a cause and effect relationship (Salzman et al.,
1973). ADP, prostaglandin endoperoxides and thromboxane A_2 initiate
a similar sequence of internal transformations in platelets that
lead to release and aggregation, though the changes triggered by ADP
are less pronounced than those triggered by endoperoxides and throm-
boxane A_2. ADP also places a greater dependence on external factors
such as fibrinogen and calcium ions. The mechanism of changes induced
by ADP and prostaglandins are independent of each other. During
collagen induced aggregation, both endogenous prostaglandins and
released ADP become available simultaneously and platelet aggreg-
ation is probably induced by both (Kinlough-Rathbone et al., 1977).
The response is probably greater than would be produced by either
ADP or prostaglandins alone (Smith et al., 1977).

An important role for prostaglandins has been proposed in
the regulation of thrombus formation, whereby a powerful anti-
aggregating agent is present to help control thrombus formation
as a result of thromboxane A_2 release during haemostasis. This
agent, prostaglandin I_2 (PGI_2) (also called prostaglandin X, or
prostacyclin), is formed in another pathway from PGH_2 (Figure 3)
and is found in endethelial cell walls. It is thought that a balance
exists between the levels of thromboxane A_2 formed in platelets,
and PGI_2 in the vessel walls and that this balance prevents thrombus
formation on undamaged vessel walls (Lancet, 1976). Recent evidence
suggests that PGI_2 may actually be a circulating hormone, generated
in the lungs and functioning as an active control of platelet
aggregation in-vivo (Moncada et al., 1978). It is interesting that
hyperventilation may increase the output of PGI_2, whereas tobacco

smoke may diminish it (Gryglewski et al., 1978).

1.2.6.4 THE CONTROVERSIAL ROLE OF CYCLIC GUANOSINE MONOPHOSPHATE (cGMP) IN PLATELET AGGREGATION

Many compounds that inhibit platelet aggregation and release, for example adenine, act by increasing cAMP concentrations in platelets. It is known that a rise in cAMP in many systems is associated with a reduction in cGMP. Collagen, thrombin and adrenaline have been found to increase cGMP concentrations in platelets and this rise is slightly in advance of aggregation, proceeding rapidly as aggregation commences. This evidence suggests that the initiation of the release reaction requires an increase in cGMP (Haslam and McClenoghan, 1974; White et al., 1973). However, another study found that though induced release is associated with raised cGMP, it was unclear whether cGMP was necessary to mediate the reaction (Haslam et al., 1975). Recent evidence, using artificially increased cGMP levels has shown that cGMP does not modulate the release reaction (Weiss et al., 1978). Since a reduced cAMP level is associated with aggregation, it seems probable that the increased cGMP concentration is a consequence of this reduction but that it is not a mediator of platelet release.

1.2.6.5 THE MECHANISM OF PLATELET AGGREGATION IN HAEMOSTASIS .

Particular interest has been shown in the mechanism of collagen induced platelet aggregation because of the role of collagen and platelets in thrombus formation during haemostasis. Collagen constitutes a substantial part of the subendothelial basement

membrane of blood vessels, and it is its exposure, as a consequence of vascular damage, that provides the stimulus for platelet adhesion and aggregation resulting in a haemostatic plug. Collagen structure is important, a quaternary structure with a specific charge being essential for aggregation (Miyata et al., 1976; Wang et al., 1975). Platelet charge is also important. During aggregation, the normal surface negative charge decreases (Seaman, 1976) probably due to an orientation change in the platelet surface exposing more negatively charged groups, including the carboxyl groups of terminal sialic acid residues. When the vascular intima is damaged, the initial haemostatic reaction is the adherence of platelets to the exposed collagen and to each other. This latter phenomenon is known as platelet agglutination. It is not an energy requiring process and involves a plasma protein, "factor VIII related protein", binding to platelet surfaces (Solum et al., 1977). The mechanism of platelet agglutination is unknown but it is important in haemostasis, causing circulating platelets to clump together at an injury site. The mechanism for platelet collagen adhesion may involve an enzyme/acceptor complex between collagen glycosyl transferase, and collagen galactosyl transferase on the platelet surface, and incomplete heterosaccharide chains on the collagen fibrils. Galactose and glucose from the appropriate sugar nucleotides, for example uridine diphosphate glucose (UDP glucose) in platelet membranes, are transferred to the appropriate acceptors on collagen. The essential step in the theory is the interaction of the platelet enzyme and the collagen acceptor (Roseman, 1970). ADP released from erythrocytes has been shown to induce adhesiveness in platelets (Harrison and Mitchell, 1966)

and to be important in haemostasis (Gaarder et al., 1961). However, using creatine phosphate/creatine phosphokinase to rapidly remove any released ADP, Tschopp and Baumgartner (1976) showed that ADP played no part in platelet adhesion to subendothelium or fibrillar collagen. Perhaps the role for ADP may be, that together with uridine diphosphate (UDP), it increases the affinity of the enzyme for its acceptor (Jamieson, 1977).

Although collagen does not produce the primary aggregation phase (Weiss, 1976) seen in the aggregometer, during platelet aggregation by most other aggregating agents, the adhesion between collagen and platelets can be likened to a primary aggregation process. Since collagen is known to induce the release reaction with release of adenine nucleotides (Kobayashi and Didisheim, 1973), platelet aggregation induced by collagen probably involves ADP release and prostaglandin synthesis similar to other aggregating agents in secondary aggregation.

Collagen induced release is associated with the liberation of collagenase from lysosomal α granules, associated with β-glucuronidase (Mills et al., 1968). This collagenase may function in a negative feedback mechanism limiting thrombus formation (Chesney et al., 1972).

MODE OF ACTION OF SOME OTHER AGGREGATION INDUCERS

The information detailed so far on platelet function and aggregation mechanisms serves to demonstrate the diversity of platelet activity and the different mechanisms involved. The majority of work on platelets has involved separation techniques to produce either platelet rich plasma or platelet suspensions.

Though both are essential for examining platelets per se, they are "artificial" media for platelets, and it is conjectural whether platelets behave in the same manner in whole blood. However, before examining platelet aggregation in exsanguinated blood, it is worth considering the mode of action of several other aggregation inducers that may play a role in aggregation in stored blood.

1.2.6.6 EXOGENOUS ADP

ADP is a powerful aggregating agent, and if added to platelet rich plasma (PRP) in concentrations as low as 1×10^{-6}M, it will cause irreversible aggregation (Born, 1962; Seaman, 1976). ADP activity requires the presence of calcium ions (Born, 1962; Haslam, 1964) and fibrinogen (Mustard and Packham, 1970), the latter possibly being the "unknown plasma factor" (Haslam, 1964) or the "heat labile plasma protein" (Born and Cross, 1964) required for ADP induced aggregation. If calcium is absent then only the shape change occurs. Fibrinogen has been shown to become associated with platelet membranes immediately after the addition of ADP (Mustard et al., 1978). ADP receptor sites, possibly involving sialic acid, exist on platelets (Born and Cross, 1963) and there have been calculated to be as many as 2×10^5 receptors per platelet (Born, 1965). It has been suggested that ADP induced aggregation involves a stoichiometric interation between platelets and ADP (Born, 1962). Although it is not possible to demonstrate firm binding of ADP to platelets, there may be transient binding because a small degree of conversion of ADP to ATP has been observed (Mustard and Packham, 1970). This is possibly due to the presence of a membrane site

for phosphoryl groups that enables conversion of exogenous ADP to ATP (Feinberg et al., 1973).

Perhaps a complex of ADP, calcium ions and plasma fibrinogen forms bridges that interconnect the platelets. Another hypothesis is that bombardment by ADP molecules produce a configurational change in a protein on the platelet surface resulting in the formation of disulphide bonds between the platelets and plasma fibrinogen. Inhibition of platelet aggregation by substances which prevent sulphydryl groups from reacting, such as dithiothreitol, favour this hypothesis (Born, 1967). Recent evidence suggests that fibrinogen may contribute to the cross links, and that calcium ions may be involved in maintaining the optimal conformation of the fibrinogen molecule (Mustard et al., 1978). The configurational change in surface proteins may be due to the involvement of thrombosthenin (Izrael et al., 1974), which is directly affected by ADP (Puszkin et al., 1973).

Below a certain concentration, ADP induced aggregation is spontaneously reversible in-vitro (Born, 1962). ADP is rapidly broken down in plasma (Haslam and Mills, 1967) and the cause of the reversal of aggregation may be due to the action of some of the breakdown products of ADP (Born, 1962; Born and Cross, 1963; Born, 1967). It is not known if any one enzyme is specifically responsible for ADP breakdown in plasma, though an ADPase has been shown to be involved (Mills, 1966) but not adenylate kinase (Haslam and Mills, 1967). The products of ADP degradation may include adenosine and AMP, both of which interfere with platelet aggregation. AMP probably competes with ADP for aggregating sites on platelets (Born, 1962) and adenosine, at low concentrations,

activates adenylate cyclase, thus increasing the concentration of
cAMP which mediates the inhibition of platelet aggregation
(Haslam and Lynham, 1973).

1.2.6.7 THROMBIN

Thrombin in concentrations of 1 to 4 National Institute of Health
(NIH) units ml^{-1}, has been shown to induce second stage aggregation
in washed resuspended platelets. Released ADP is the mediator
of this action (Haslam, 1964). In PRP, only 0.25 NIH units ml^{-1} is
required to cause the release of up to 50% of the platelets' ADP
content, and this demonstrates that thrombin is a more powerful
releasing agent than collagen, exogenous ADP, or adrenaline
(D'Souza and Glueck. 1977).

The mechanism by which thrombin induces platelet shape change
and primary aggregation has been shown to involve a 120,000 dalton
glycoprotein, hydrolysis of which, results in a change in organisation
of membrane proteins so that additional proteins are exposed to the
membrane surface (Phillips, 1973). The platelet surface substrate
may be fibrinogen, and there is a requirement for calcium ions
(Mustard and Packham, 1970).

The interaction of platelets with ADP leads to the activation
of coagulation factor XII and PF3 (Walsh, 1973) which may initiate
thrombin generation (Ardlie and Han, 1974). It was noticed during
ADP induced aggregation in PRP, that the extent of platelet aggreg-
ation was related to the ADP concentration, and that the increase
in aggregation was related to increased PF3 availability. As a
result, it was suggested that second stage platelet aggregation may

be induced by thrombin generated through the intrinsic coagulation
pathway on the platelet membrane, and that the extent of the
platelet response to ADP may depend upon the extent of activation
of the intrinsic pathway (Ardlie and Han, 1974). Evidence to support
this hypothesis included the observation that an intact intrinsic
pathway is required for ADP induced second stage aggregation, and
that extending the activation of the pathway using Russell's viper ven-
om (to activate factor X) resulted in an increased response of plate-
lets to low ADP concentrations. It was suggested that thrombin
generated on the platelet membrane was responsible. Fibrinogen was
found to be necessary, and it was considered that an enzyme - substrate
complex between thrombin and fibrinogen may have been responsible
for the primary aggregation induced by ADP. The role of thrombin may
not be confined to release induced by ADP but may be extended to
explain, for example, collagen and adrenaline induced release. The
significance of the relationship is that coagulation and aggregation
may be interdependent and therefore, that they may be regarded as one
phenomenon (Ardlie and Han, 1974). An argument against this
hypothesis is the observed aggregation induced by exogenous ADP
when heparin is present (Born and Cross, 1963). However, this is
considered to be aggregation mediated by prostaglandin intermediates
which do not require ADP for their action and which are, therefore,
independent of the products of the release reaction (Akbar and
Ardlie, 1978). Thrombin is also capable of inducing aggregation
without ADP and prostaglandins, suggesting a third aggregating mech-
anism (Kinlough-Rathbone et al., 1977), which may be the activity
described above. Perhaps all three mechanisms play a role in thrombin
induced platelet aggregation in-vivo.

1.2.6.8   5-HYDROXYTRYPTAMINE

5HT induces the aggregation of human platelets in PRP, though
the extent of aggregation is small and the process reverses rapidly
without reaching the second phase (Baumgartner, 1970). However,
the platelets of most other animals are very responsive to 5HT
(Swank and Fellman, 1967). In human whole blood, the exposure of
platelets to 5HT concentrations as high as $1 \mu g.ml^{-1}$ rarely induces
aggregation (Swank et al, 1963). In contrast, 5HT very markedly
potentiates the ADP induced aggregation of human and other animals
platelets, both in blood (Swank and Fellman, 1967) and in PRP
(Mustard and Packham, 1970). This potentiation will be discussed
further in the section on synergism.

Human platelets contain large quantities of 5HT, about $50ng.10^8$
platelets. The 5HT is stored mainly in the bulls eye granules
(Baumgartner, 1970) where it is protected from degradation by
monoamine oxidase, to 5-hydroxyindole 3-acetic acid and 5 hydroxy-
tryptophol (Pletscher, 1968). The 5HT concentration may be increased
by up to 50% if exposed to raised plasma levels of 5HT (Baumgartner,
1969). Platelets may be able to synthesise small amounts of 5HT
intracellularly (Lovenberg et al, 1968), though this is controversial
(Maynert and Isaac, 1968), but most of the platelets'content of 5HT
is taken up during blood circulation (Pletscher, 1968). The uptake
process is both passive and active, the latter being able to occur
against a considerable concentration gradient (Baumgartner, 1970).
Specific receptor sites exist for 5HT on the platelet membrane
(Baumgartner and Born, 1969; Drummond and Gordon, 1975). Active
5HT uptake, which commences with the binding of 5HT, probably
involves an energy dependent carrier system (Pletscher, 1968), and

it has been proposed that aggregation of platelets induced by 5HT is mediated by ADP released from platelets incident to ATP utilisation in active 5HT uptake (Baumgartner, 1970). The presence of receptor sites helps to explain the concentration dependency of 5HT induced aggregation, since above certain concentrations, aggregation ceases, probably due to 5HT saturation of the receptor sites (Baumgartner and Born, 1969; Barthel and Markwardt, 1974). Presumably, this explains the limited aggregability of platelets by 5HT, since perhaps insufficient ADP is available to extend aggregation beyond the first phase. In rats, evidence has been obtained to suggest that more than one type of receptor site exists for 5HT on platelets; a specific site existing for 5HT uptake and another for 5HT induced shape change (Drummond and Gordon, 1975). If this is so, it suggests that two separate activities occur. In the first, the binding of 5HT to a specific site invokes a physiological response, namely shape change, and in the second, the binding of 5HT to another specific uptake site results in ADP production and in extremes, mild platelet aggregation.

Despite the weak aggregating ability of 5HT, a central role for 5HT in secondary platelet aggregation has been proposed. It was observed that adrenaline and noradrenaline induced aggregation in whole blood was inhibited in the presence of a 5HT inhibitor, UML 491 (a lysergic acid derivative). This lead to the suggestion that the catecholamines may act through 5HT, probably by releasing 5HT in the release reaction, which together with ADP produces secondary platelet aggregation (Swank and Fellman, 1967). No further evidence is available, though it is possible that UML 491 in fact inhibits the release reaction, perhaps by the inhibition of prostaglandin synthesis.

This seems likely. as another specific 5HT antagonist, methysergide, has been shown to have no effect on second phase aggregation (Thomas, 1967).

1.2.6.9 CATECHOLAMINES

Both adrenaline and noradrenaline have been shown to cause platelet aggregation in human PRP (O'Brien, 1964a), though noradrenaline is 50% (Born et al., 1967a) to 90% (O'Brien, 1964b) less effective than adrenaline. Dopamine probably does not induce platelet aggregation (Alexander et al., 1978). All three catecholamines are able to potentiate platelet aggregation induced by low concentrations of ADP, thrombin, collagen and, to a small extent, 5HT, though noradrenaline is only about 20% to 50%, and dopamine only 1% as active as adrenaline (Mills and Roberts, 1967). Considerable individual variation in susceptibility of human platelets to adrenaline and noradrenaline has been observed, which is not related to age, gender, time, exercise or any common variable (O'Brien, 1964a). Catechol-o-methyl transferase activity has been demonstrated in human platelets. This enzyme may convert the catecholamines to their methylated derivatives, which lack aggregating ability. Perhaps the variation in platelet responsiveness to adrenaline and noradrenaline could be explained by variations in platelet transferase activity (Stramentinoli, 1978).

Catecholamine induced aggregation is blocked by α-adrenergic antagonists such as phentolamine, and it has been suggested that the mechanism of catecholamine induced aggregation is via α-adrenergic receptors (Mills and Roberts, 1967; Thomas, 1967; Robison et al.,

1969; Barthel and Markwardt, 1974; Alexander et al., 1978).

Catecholamines compete for these receptors, estimated to be about

100 per platelet, in a stereospecific manner, with the potency

series of (-) adrenaline greater than (-) noradrenaline. This

may explain the more potent aggregating ability of adrenaline

(Alexander et al., 1978). There are probably no β-adrenergic

receptors on platelets (Robison et al, 1969). An increase in cAMP

activity in platelets is associated with aggregation inhibition

(Haslam and Lynham, 1973; Michel et al., 1976). Catecholamines

act upon the α-adrenergic receptors to produce a decrease in the

intracellular level of cAMP in platelets (Robison et al., 1969;

Moskowitz et al., 1971) probably due to their known inhibitory

effects on adenylate cyclase (Born et al., 1967a). The nature

of the supposed interaction between α-adrenergic receptors and

adenylate cyclase is not clear, though bound catecholamines may

inhibit adenyl cyclase activity via a direct link between the

α-receptor and the catalytic subunit of adenylate cyclase

(Alexander et al., 1978).

It has been reported that both the first and second phases of

aggregation involve ADP, since apparently, even the first phase is

much reduced by including an adenosine diphosphatase prepared from

viper venom (Haslam, 1967 – cited Mustard and Packham, 1970). How-

ever, this is controversial as adrenaline, without the mediation of

ADP, has been reported to induce primary aggregation in human

platelets (Mills and Roberts, 1967).

Blood platelets are carriers of catecholamines (Weil-Malherbe

and Bone, 1954) associated with 5HT, ADP and ATP in the bulls eye

granules. There is more noradrenaline than adrenaline (Weil-Malherbe

and Bone, 1954), and though the relative levels are disputed, the catecholamines have been calculated to total approximately $800pg.10^8$ platelets (Thomas, 1967). The catecholamines are taken up into platelets from plasma and it is possible that, rather like 5HT, uptake may be related to platelet aggregation (Born et al., 1967a; Barthel and Markwardt, 1974). Fibrinogen is thought to be necessary as a cofactor in catecholamine induced platelet aggregation. (Mustard and Packham, 1970).

1.2.6.10 SYNERGISM BETWEEN PLATELET AGGREGATION INDUCERS

Studies in-vitro have shown that ADP, thrombin and collagen can act synergistically in platelet aggregation and the release reaction. Low concentrations of ADP, insufficient to induce aggregation, combined with low concentrations of thrombin or collagen will cause extensive aggregation and release (Kinlough-Rathbone et al., 1977). Synergism has also been shown to occur between low concentrations of adrenaline and ADP; adrenaline and collagen; adrenaline and thrombin, 5HT and ADP, adrenaline and 5HT; ADP and 5HT; noradrenaline and ADP; and, noradrenaline and 5HT. (Swank and Fellman, 1967; Mills and Roberts, 1967; Thomas, 1967; Baumgartner and Born, 1969; Barthel and Markwardt, 1974; Takano, 1975). It seems probably that synergism would also occur between more than two inducing agents.

The extent to which one inducer will potentiate another is not clear. For example, both adrenaline and 5HT have been proposed as particularly powerful potentiating agents (Thomas, 1967; Swank and

Fellman, 1967), yet 5HT is thought to be particularly active with noradrenaline and that no combinations of 5HT with thrombin, or ADP show mutual potentiation, at least in dog PRP (Takano, 1975).

The mechanism of synergism remains to be elucidated, but it was found that prostaglandin synthesis often plays an important role. However, this role is very much reduced when thrombin is involved, probably due to the proposed third aggregating mechanism initiated only by thrombin (Kinlough-Rathbone et al., 1977). The role of prostaglandins is probably the reason for earlier observations that adrenaline particularly potentiated ADP and thrombin induced secondary aggregation (Mills and Roberts, 1967; Thomas, 1967).

Synergism also occurs on a more general basis, since the release reaction can be regarded as potentiation of platelet aggregation by the original stimulus. As the release reaction involves both release of granular contents and prostaglandin synthesis, the platelets seem to have two means of potentiating the response to external stimuli via two separate positive feedback loops (Holmsen, 1977b).

1.3 PLATELET AGGREGATION DURING BLOOD STORAGE

Platelet aggregation plays an essential role in haemostasis and blood coagulation and, to some extent, in defence mechanisms. The aggregation process depends upon an initiating stimulus. The mechanisms of some stimuli have been reviewed. The fact that plate-lets aggregate during the storage of blood was first recorded by Fantus in 1938. Since then, numerous publications have repeated the observations mainly in attempts to determine optimal blood

storage conditions, to examine the chemical and physical changes
occuring during blood storage, and to describe the physiological
effects of transfusing blood containing cellular aggregates.

1.3.1 THE DEVELOPMENT OF PLATELET AGGREGATES DURING BLOOD STORAGE

There are two anticoagulants in common use for long term blood
storage - acid: citrate: dextrose (ACD) and citrate: phosphate:
dextrose (CPD). During blood storage in both ACD and CPD at 4°C,
there is a steady reduction in the platelet count to about 50% of
its original level after 10 days (Limbird and Silver, 1974;
Marshall et al., 1975.). The decrease in platelet count is
inversely related to the increase in concentration of microaggreg-
ates during the storage of whole blood (Solis et al., 1974a, b).
Simple platelet aggregates appear within 24 hours, the platelets
becoming more densely packed and fused together after 3 days.
Platelet viability is markedly reduced after only 24 hours.
Degenerating granulocytes start to become part of the fused aggregate
after 3 to 4 days, possibly explaining why aggregates form to a
lesser extent in PRP (Marshall et al., 1975). The more rapid
aggregate formation seen in platelet concentrates is possibly due
to the close proximity of the cells and the effects of centrifugation
(Barrett et al., 1976). Fibrin is generally regarded as being imp-
ortant in stabilising aggregates, though there is some controversy
as to whether it is (Swank, 1961; Harp et al., 1974; Dawidson et
al., 1975) or is not (Dawidson, 1977) a component of blood storage
aggregates. However, experiments with ^{125}I labelled fibrinogen
showed that after about 10 days, fibrin did begin to accumulate on

pre-existing aggregates increasing the aggregate mass (Arrington and McNamara, 1974). Also, the use of urokinase and streptokinase as exogenous activators of fibrinolysis reduced the aggregate volume, suggesting that fibrin may be important as a cohesive element in formed microaggregates (Gervin et al., 1975).

Aggregates may reach up to 200 μm in diameter, although the majority are below 30 μm (Swank, 1971). It has been estimated that after 2 weeks storage, 90% of aggregates range between 10 and 40 μm in diameter and that at 3 weeks, the average number of aggregates is 5×10^7 per unit of blood (450 ml). (Solis and Gibbs, 1972). At least 40% are thought to be stable (Solis et al., 1974b).

Similar changes occur in both ACD and CPD solutions, the concentration of aggregates increasing alinearly in a sigmoid pattern. However, the extent of aggregation is different. In CPD, platelets tend to aggregate more readily during the first few days, than in ACD (Wright and Sanderson, 1974) but on subsequent days, the extent of aggregation is more rapid in ACD so that the concentration of aggregates in ACD and CPD become similar after about one week (Solis et al., 1974b; Marshall et al., 1975). Aggregation may be related to factors such as age and gender. Platelets from donors older than 35 years are reported to aggregate more readily during the first few days than those from younger donors. Similarly in blood donated by males, there is a greater tendency toward platelet aggregation during the first few days than there is in blood obtained from females (Harp et al., 1974).

1.3.2 THE PHYSIOLOGICAL EFFECTS OF TRANSFUSING PLATELET AGGREGATES

After severe injury, not involving the lungs, many patients die from respiratory failure, a syndrome known as post traumatic pulmonary insufficiency, congestive atelectasis, or respiratory distress syndrome. Factors which have been implicated in the pathogenesis of respiratory distress syndrome include fluid replacement such as massive blood transfusion (Bennett et al., 1972).

The structure of the pulmonary microvasculature is such that it is easily obstructed by emboli. Once an embolus has lodged in the microcirculation, its fate may depend on several factors. It may break down, remain unchanged, or act as a nidus from which in situ thrombosis propagates (Moseley and Doty, 1970). Small emboli may be removed by the reticulo-endothelial system (Swank and Porter, 1963), though even transient obstruction by a sufficient number of emboli could produce significant lung parenchymal damage (Moore, 1974). Because large numbers of aggregates are contained in stored blood, which could be filtered off in the pulmonary vascular bed, they are thought to be implicated in the development of respiratory distress syndrome following massive blood transfusions (Moseley and Doty, 1970; McNamara et al., 1972; Reul et al., 1974; Dawidson et al., 1975; Takaori et al., 1977; Barrett et al., 1978). Some of the evidence for this implication is as follows.

The transfusion of stored blood into dogs has been shown to be associated with an increase in pulmonary arteriovenous shunting of blood and a decreased diffusion capacity of the lung for oxygen. The causal factor was a large number of microemboli subsequently

found in the pulmonary microcirculation which has been found to be
dose related to the number of cellular aggregates in the trans-
fused blood (Reul et al., 1974; Barrett et al., 1975 - cited Barrett
et al., 1978). In another series of experiments, dogs' lungs were
examined following the transfusion of stored autologous blood. The
examination revealed extensive occlusion of the pulmonary micro-
circulation with platelet aggregates (McNamara et al., 1972;
Connell and Swank, 1973). Evidence of the effects of transfused
platelet aggregates in man were first based upon observations of
battle casualties who received massive blood transfusions. The
measurement of low arterial oxygen partial pressures following
transfusion, may be considered to support the idea of a role of
transfused cellular aggregates in respiratory distress syndrome
(Moseley and Doty, 1970). Objective data has only recently been
presented showing the pulmonary effects in humans of blood transfus-
ions containing microaggregates. Pulmonary function was measured
in man before and after intraoperative blood transfusion, and
demonstrated the development of pulmonary microembolisation after
only two units of blood had been transfused (Takaori et al., 1977).
This observation was more recently confirmed in patients transfused
with over 20% of their blood volumes, where an increase in
pulmonary arteriovenous shunting was observed in an amount directly
proportional to the quantity of microaggregates administered
(Barrett et al., 1978).

However, some disagreement exists as to the harm transfused
microaggregates may cause. In a series of experiments, dogs in
haemorrhagic shock were resuscitated by transfusion of stored auto-
logous blood into the left pulmonary artery. It was assumed that if

microaggregates affected vascular resistance there should be a
redistribution of pulmonary blood from the left to the right lung.
No distribution occured and vascular resistance rose in both lungs
with the same magnitude. This suggested that aggregates did not
play a major role in pulmonary changes associated with haemorrhagic
shock. (Giordano et al., 1976). It has been observed that, although
many blood vessels may become occluded as result of massive blood
transfusions, the number of cellular aggregates present in stored
blood is probably insufficient to occlude enough of the the pulmon-
ary vasculature to produce the observed respiratory symptoms
(Gervin et al., 1974a). Experimentally, large quantities of glass
beads have to be injected to cause in-vitro vascular occlusion in
dogs, and pulmonry compliance in man is such that cardiac output
can be increased from 4 to 30L.min with no change in pulmonary art-
erial pressure (Giordano et al., 1976). It has been reported that
after in situ lung perfusion studies using stored autologous
blood in baboons, lung biopsies revealed no traces of microemboli.
The observed, increased pulmonary vascular resistance was considered
to have been mainly due to underlying humoral factors with mechanical
obstruction by platelet aggregates   playing an additive role.
The primary mechanism may be pulmonary vasoconstriction in response
to high plasma concentrations of 5HT and ADP released from aggregated
platelets (Bennett et al., 1972). This may be added to by the
reduced pH of stored blood, increasing metabolic acidosis in the
recipient (Davidson et al., 1975) which is itself, a major deter-
minant in influencing pulmonary pressor responses such as pulmonary
vasoconstriction (Grand and Downing, 1970). Recipients of massive
transfusions are in a severe state of shock before transfusion and

there is little doubt that shock plays a major role in the development of respiratory distress syndrome. This is a consequence, not only of vasoconstriction and intravascular coagulation, but also stimulation of in situ platelet aggregation by 5HT, catecholamines and vasopressin, which may be sequestered in the lung (Swank, 1968; Kusajima et al., 1974; Giordano et al., 1976).

It seems, therefore, that the role of transfused platelet aggregates in the aetiology of respiratory distress syndrome is unclear. The fact that 65% of the lung microvasculature can be occluded before hypertension develops, could disguise the effects of transfusing aggregates (Hyland et al., 1963 - cited Swank and Edwards, 1968). However, in severely traumatised recipients of massive blood transfusions, it is difficult to determine the importance of any one contributory factor in the development of respiratory distress syndrome. Nevertheless, a role for platelet aggregates does seem to have been established, even though it may be a secondary one.

1.3.2.1 SECONDARY MICROEMBOLISM AFTER BLOOD TRANSFUSION

Many microemboli, particularly those below 15 µm in diameter may traverse the lung capillary bed after blood transfusion. An electron microscope study of the effects of these microaggregates on other organs has been reported using dogs as the transfusion recipients. Where biopsies were conducted immediately after transfusion, cellular aggregates were found in the liver and to a much lesser extent in the brain and kidneys. After a delay of 20 min. after the end of transfusion, few aggregates were to be found in these organs, but alterations in the fine structure of the kidneys,

brain and liver were clearly evident. It would appear that once microemboli enter the blood stream, they circulate from organ to organ. Their removal may not be the first capillary bed that they enter. More likely their removal is a slow process of attrition during which time, the aggregates circulate from one microvascular bed to another, causing repeated damage to each area (Connell et al., 1973).

During extracorporeal circulation in open heart surgery, the lungs are bypassed and stored blood added to the bypass apparatus would enter the recipient intra-arterially. In these cases, the capillary beds of the rest of the body can be regarded as the sites of primary embolization. It has been suggested that the use of blood containing aggregates to prime the extracorporeal circuit may cause embolic tissue damage (Swank and Porter, 1963; Swank, 1971). However, a study in dogs showed no histological evidence that intra-arterial blood transfusions containing aggregates, caused focal brain damage, or that there was any relationship between the occurrence of petechial haemorrhages and the presence of aggregates in the stored blood. Though it was observed that removal of the cellular aggregates appeared to reduce mortality in the experimental dogs, (Wright, 1975).

1.3.2.2 THE USE OF FILTERS TO PREVENT EMBOLIC DAMAGE

The damage caused by infusion of large quantities of micro-aggregates has been discussed. Numerous studies have shown that microfiltration of stored blood prevents the detremental changes described both from direct blood transfusion and via extracorporeal

circulation (Solis and Gibbs, 1972; Reul et al., 1973; Davidson
et al., 1975; Takaori et al., 1977; Kennedy et al., 1977). Stand-
ard blood pack filters with pore sizes of 170 μm are not suitable
for filtering microaggregates. The better microfilters will remove
the majority of particles greater than 15 μm in diameter (Dawidson,
1977), though the varying qualities of the available microfilters
will not be considered here.

The effect of microfiltration of stored blood could be considered
as evidence for a role for cellular aggregates in the mechanical
obstruction of the pulmonary vasculature. However, microfilters
may remove vasoactive amines such as 5HT (Davidson et al., 1975) and
therefore do not clarify what role transfused platelet aggregates
might play in respiratory distress syndrome.

1.3.2.3 METHODS TO REDUCE AGGREGATE FORMATION IN STORED BLOOD

Microfiltration of stored blood is an efficient method for the
removal of platelet aggregates. There are disadvantages, however,
including cost, their nuisance value if speed is essential, and the
reported possible alteration of erythrocyte survival (Gervin et al.,
1975). Some of the alternatives to filtration will be briefly
discussed.

1.3.2.3.1 PREVENTION OF AGGREGATION BY DRUGS AND AGITATION

Aspirin in concentrations of 100 to 200 μM will decrease plate-
let stickiness and if added to blood before storage, has been reported
to reduce aggregate formation by up to 50% (Gervin et al., 1975).
The addition of non-toxic concentrations of PGE, to blood stored at

4°C was also found to reduce platelet aggregation, increase their life span and did not impair the platelets'haemostatic effectiveness (Becker et al., 1974; Valeri et al., 1972). Adenosine was also found to minimise platelet aggregation (Mourad, 1968) and is also known to improve 2,3,diphosphoglycerate preservation in erythrocytes (Benesch and Benesch, 1969). Continuous gentle agitation of blood throughout storage has been observed to reduce platelet aggregation (Arrington and McNamara, 1975); though the type of agitation is considered important (Holme et al., 1978) and may even destroy platelets, giving the impression of reduced platelet aggregation (Gervin et al., 1978).

1.3.2.3.2 THE REMOVAL OF AGGREGATES AND SEPARATION OF BLOOD COMPONENTS

Microaggregates can be eliminated from stored blood by removing the buffy coat, where the largest number of aggregates have been found (Gervin et al., 1974b), by differential centrifugation (Gervin et al., 1975). However, this reduces the haemostatic effectiveness of transfused blood. To control bleeding, platelet viability is an essential prerequisite, and erythrocytes and leucocytes are unnecessary. For this purpose, platelets may be separated and stored for a short term as platelet concentrates in a small volume of plasma. A volume of 50ml has been considered most suitable for platelet viability (Murphy and Gardner, 1976; Holme et al., 1978). Platelet viability and reduced aggregation are thought to be best preserved by maintaining a pH between 6.8 and 7.2 (Murphy and Gardner, 1975), and by storage at 22°C which maintains

platelet viability for up to 96 hours (Murphy and Gardner, 1969).
Gentle agitation, as described for whole blood storage, is also
considered important (Holme et al., 1978). The use of cryoprot-
ective agents, such as dimethylsulphoxide has been successfully used
for freezing platelet concentrates with little loss of platelet
function (Lundberg, 1970; Baldini et al., 1976).

1.3.2.3.3 THE USE OF AGGREGATE FREE BLOOD

Only fresh whole blood is comparatively aggregate free
(Gervin et al., 1975) and is the ideal form of blood for transfusion.
This category could include the use of autotransfusion, in which
blood lost by a patient is collected and returned within a short
time, and is especially useful during major surgery where a large
blood loss might be expected (Wilson and Taswell, 1968). The use
of autotransfusion in which blood is collected from a donor to be
returned after blood storage, would be subjected to the same aggreg-
ation problems as discussed in whole blood platelet aggregation.

1.4 THE PLAN FOR THE INVESTIGATION OF POSSIBLE CAUSES OF PLATELET
      AGGREGATION IN WHOLE BLOOD

Evidence has been presented for the possible mechanisms of
platelet aggregation and for the probable role of these aggregates
in the development of respiratory distress syndrome in severely
traumatised patients. Although considerable attention has been paid
to the prevention of transfusing aggregates, little information is
available as to the cause of platelet aggregation during blood stor-
age.

1.4.1 THE MEASUREMENT OF PLATELET AGGREGATION DURING BLOOD STORAGE

The extent of platelet/granulocyte aggregation in stored blood can be assessed in several ways. Direct microscopy, measurement of the screen filtration pressure, and cell counting with the Coulter counter are the most popular. Both direct microscopy and the Coulter counter technique require sample manipulation, smearing or fixing for microscopy, and dilution for the counter. Although the latter is thought to be the most accurate (Solis et al., 1974a), its availability is an essential prerequisite. Screen filtration pressure (SFP) allows the measurement of aggregation to be made on the whole blood sample (Swank, 1961), thought it is reported not to be able to distinguish between platelet adhesion and platelet aggregation (Solis et al., 1974a). Changes in blood viscosity may also interfere with SFP measurements. However, increased viscosity probably parallels decreased erythrocyte deformability (Schmid-Schonbein et al., 1969) and occurs with much less magnitude than platelet aggregation (McNamara et al., 1971). Therefore, viscosity changes have a negligible effect on SFP, particularly during the first few days of storage.

1.4.2 THE BACKGROUND EVIDENCE FOR THE TYPE OF INVESTIGATION

During the investigation of tissue damage following extracorporeal circulation for open heart surgery, in the W. E. Dunn Unit of Cardiology at Keele University, several observations were made. Some of these were briefly as follows. Samples of human blood obtained from volunteers had normal SFP's of 12 - 21 mmHg immediately after collection and SFP's of 28 - 152 mmHg after 7 days storage in ACD at 4°C. Blood samples obtained at the

end of standard blood collection by the Blood Transfusion Service also had normal SFP's after collection, but after 7 days, the majority had SFP's of greater than 600 mmHg. This indicated that some alteration occurred in blood during standard donations, and that this alteration may be partly responsible for the formation of cellular aggregates during storage. The SFP of 8 dog arterial blood samples stored in ACD for 7 days at 4°C increased from a mean of about 38 mmHg to 50mmHg but 8 venous samples increased to a mean of 398mmHg. After the 7 days of storage, arterial blood samples contained cellular aggregates up to 40 μm in diameter, but 6 of the 8 venous blood samples contained aggregates up to 150 μm in diameter. The difference in aggregation may have been due to the slower rate of venous blood collection compared with arterial (Wright, 1975).

These observations suggested that the rate and extent of cellular aggregation in stored blood may have been dependent upon the rate of blood collection and the proportion of the total blood volume taken. The indications were that the processes of cellular aggregation were activated during blood collection, and that a biochemical mechanism was involved.

1.4.3 THE FACTORS THAT MAY BE RESPONSIBLE FOR PLATELET AGGREGATION
       DURING BLOOD COLLECTION AND STORAGE

Although many agents have been shown to be able to cause platelet aggregation in-vitro (Mustard and Packham, 1970), the agents that could be responsible for aggregation in blood during storage are limited to those factors that may be present during blood collection and storage. Blood collection may be a stressful

procedure, both psychologically (Callingham and Barrand, 1976) and physically, as a result of mild haemorrhage (Fowle, 1968). Because the indications were that platelet aggregation may be activated during blood collection, a suitable area of study was considered to be an examination of the known aggregation inducers that are released into the blood during stress. Only the catecholamines, 5HT and vasopressin fit into this category. Vasopressin is a powerful inducer of platelet aggregation in-vitro (Haslam and Rosson, 1971) and acute haemorrhage is a potent stimulus for its release (Moore, 1965). However, the only suitable assay for measuring plasma concentrations of vasopressin is radioimmunoassay. The necessary antiserum is not obtainable commercially and the facilities to raise one's own were not available. Therefore, the investigation into the possible causes of platelet aggregation in whole blood began with an examination of the roles of catecholamines and 5HT. Because of the weak aggregating nature of 5HT, particular emphasis was originally placed on catecholamine estimations during blood collection, with an investigation into the roles of catecholamines and 5HT during storage. Several other factors were also examined. ADP, because of its supposed mediatory role in platelet aggregation, and because of its high concentration in both platelets and erythrocytes making it a potential primary cause of aggregation during blood storage. ATP was examined because of the energy dependence of platelet aggregation. Cellular aggregation during storage was measured using the SFP technique. pH was measured during storage as an index of the metabolic changes resulting in a build-up of lactic and pyruvic acids. Plasma haemoglobin was measured to monitor erythrocyte integrity. Prothrombin time and partial thromboplastin

time were measured as tests of the efficiency of the intrinsic and extrinsic blood coagulated pathways which may be triggered during blood storage and contribute to an increased SFP.

Each of these factors will be discussed in detail with evidence for their possible involvement in platelet aggregation during blood storage, as separate chapters.

CHANGES IN PLASMA CATECHOLAMINE CONCENTRATIONS

DURING BLOOD COLLECTION AND STORAGE

2.1 CHANGES IN PLASMA CATECHOLAMINE CONCENTRATIONS DURING BLOOD COLLECTION

Voluntary blood donation at Regional Blood Transfusion Service collection centres, involves the rapid donation of 450 ml of venous blood; about 8% of the average total blood volume.

During haemorrhage, the sympathetic nervous system is activated and this results in the liberation of noradrenaline from the post-ganglionic nerve endings, and release of noradrenaline and adrenaline from the adrenal medulla (von Euler, 1946 - cited Chien, 1967). Dopamine release is probably independent of sympathetic activity (de Champlain et al., 1976). Although the extent of the liberation of adrenaline and noradrenaline is correlated with the severity of the haemorrhage, this stimulation is not necessarily a consequence of severe blood loss, but is a response even to mild haemorrhage (Fowle, 1968). With the exception of aldosterone release, adrenal cortical secretion is probably not stimulated by mild haemorrhage (Moore, 1965). Aldosterone has not been reported to be able to stimulate platelet aggregation.

Although plasma adrenaline and noradrenaline concentrations have recently been reported to rise in some anaesthetised patients during sodium nitroprusside induced hypotension (Rawlinson et al., 1978),

there is no reported work on the measurement of plasma catecholamine concentrations during acute haemorrhage in man. However, the rapid withdrawal of only 10% of the blood volume in dogs, resulted in immediate stimulation of the adrenal medulla, and in up to a 50 fold increase in catecholamines in the adrenal vein (Moore, 1965). Catecholamine concentrations have been measured in human fasting volunteers, in urine samples collected over 24 hours during which about 15% of their total blood volume was removed in a 15 minute period. A significant increase was observed in urine noradrenaline and adrenaline concentrations (Skillman et al., 1971).

Adrenal output during haemorrhage in dogs is also affected by emotional excitment (Chien, 1967) and fear and anxiety alone can stimulate adrenaline release in man (Britton et al., 1974; Carruthers, 1975). Blood collection is a mildly stressful procedure (Callingham and Barrand, 1976) in which even contact with the blood sampler has been shown to be effective stimulus for stress induced catecholamine elevation (Sauebier and von Mayersbach, 1977).

Adrenaline is more powerful than noradrenaline as an aggregating agent by a factor of two to ten times; dopamine is thought to be ineffective (see chapter one). Adrenaline, at only 10^{-7}M can induce in-vitro platelet aggregation (Born, 1967), but at 5×10^{-8}M may potentiate thrombin induced aggregation (Thomas, 1967), and at 10^{-8}M (about 1.8 ng.ml^{-1}), has been shown to potentiate ADP induced aggregation (Born, 1967). Mild stress has been reported to raise plasma catecholamine levels (Callingham, 1975), possibly to levels that might potentiate platelet aggregation (Born, 1967). 5HT release is usually associated with severe shock (Markley et al., 1975) and it is uncertain whether there is any significant rise in plasma 5HT

concentration due to mild haemorrhage and associated stress. However, it has been shown that as little as 250 ng.ml^{-1} of 5HT stimulated the medullae of isolated dog adrenal glands (Miller and Peskin, 1963), and so, if released, may potentiate catecholamine release in-vivo.

2.1.1 CATECHOLAMINE BIOCHEMISTRY AND PHYSIOLOGY

The three catecholamines found in man, noradrenaline, adrenaline and dopamine, are all related to 1,2 dihydroxybenzene, also known as catechol and hence the name catecholamines. Only the L-stereoisomer of catecholamines is physiologically active to any extent. They are mainly synthesised in the adrenal medulla and the sympathetic neurons, the initial step being the conversion of tyrosine to 3,4 dihydroxy-phenylalanine (DOPA) followed by conversion to dopamine by the non-specific, pyridoxal phosphate dependent, amino acid decarboxylase. Dopamine is then taken up into vesicles, the chromaffin granules, which contain dopamine β-hydroxylase for noradrenaline synthesis and, particularly in the adrenal medulla, phenylethanolamine N-methyltransferase for adrenaline synthesis. Catecholamines are secreted from the chromaffin granules upon stimulation. Secreted catecholamines are metabolised very quickly in the target tissue, or the liver, though noradrenaline may be retaken up into neuronal vesicles, and is thought to be an important mechanism for quickly terminating neurotransmitter activity. The first step in catabolism of catecholamines is, either methylation or oxidation depending upon the circumstances. For example, bound noradrenaline is initially oxidatively deaminated by the broadly specific mitochondrial enzyme, monoamine oxidase. The next step is a reversal of the first, so that

oxidation is followed by methylation and vice versa. The main

products for adrenaline and noradrenaline are metanephrine,

normetanephrine and vanilmandelic acid (VMA). Biologically, the

most important metabolic product for dopamine in noradrenaline, but

its main urinary product is homovanillic acid (Figure 4). The

concentration of catecholamines is controlled at all stages. All

three catecholamines are allosteric inhibitors of their own synthesis

by tyrosine hydroxylase, and for noradrenaline and adrenaline,

hydroxylation of dopamine by dopamine β-hydroxylase is also a rate

limiting step. During prolonged stress, the rates of synthesis of

all the enzymes for catecholamine synthesis are gradually increased

(Franzen and Eysell, 1969).

Major Pathways in the Metabolism of the Catecholamines

Figure 4
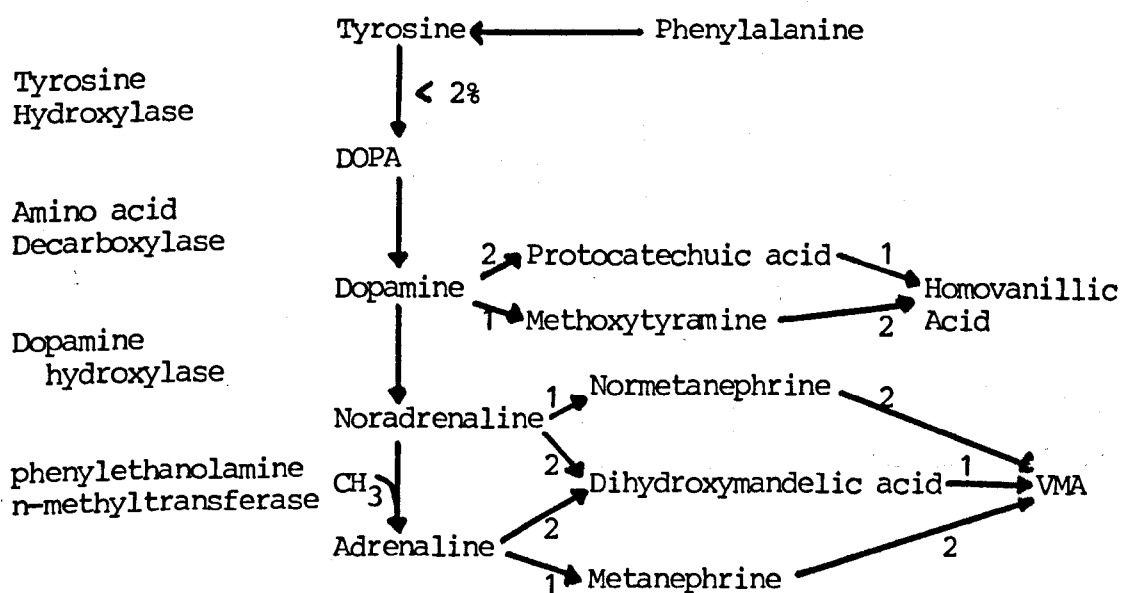
1 = catechol-o-methyl transferase (requires S-adenosylmethionine as
    methyl donor)

2 = monoamine oxidase

The primary function of adrenaline is as a hormone. Noradrenaline functions as the adrenergic nerve transmitter, though it also functions as a hormone. Dopamine is usually found with noradrenaline in nerve endings, but dopamine specific nerves and receptors are found. The adrenal medulla contains all three catecholamines, but mostly adrenaline, whereas comparatively little adrenaline is found in nerve endings. The response of tissues to catecholamine stimulation depends upon the presence of adrenergic receptor sites, the α and β sites. Generally, α-receptors are associated with smooth muscle contraction, and, with the exception of the intestine, β-receptors with relaxation. Dopamine and noradrenaline act mainly through α-receptors, and adrenaline through both α and β. Because of this, the effect of adrenaline depends upon quantity and/or the sensitivity of the site. Thus, for example, in the pancreas, the α response to adrenaline predominates, inhibiting insulin release. However, if an α-blocking drug, such as phentolamine, is given, then the β effect predominates, that is, increased insulin release.

The physiological effects of catecholamines are many and varied, the following is just a brief introduction to the better understood effects of adrenaline and noradrenaline in man.

Noradrenaline has a vasoconstrictor effect and increases blood pressure. It reduces the blood supply of the kidneys, the liver, the skin and the muscles, but the reaction of different vascular regions is varied. On the other hand, it dilates the coronary vessels and increases the coronary blood supply. Noradrenaline increases the rate and force of the heart beat and increases the oxidative metabolism of the heart, though its effect is substantially less than that

of adrenaline. The same applies to other metabolic effects. Noradrenaline is a mild broncho-dilator and increases the frequency and depth of respiration in man.

Depending upon the concentration, adrenaline may cause vaso-constriction or vasodilation. Like noradrenaline, it increases the rate and force of the heart beat. Adrenaline also increases the systolic blood pressure and causes a decrease in the peripheral res-istance. Adrenaline increases the blood supply to skeletal muscle, liver and brain, but causes vasoconstriction in the skin and the kidneys. The net result in a state of emergency, is that blood is diverted from skin and viscera to the organs which need it - such as skeletal muscle. Adrenaline stimulates respiration and dilates the bronchi. Compared with noradrenaline, adrenaline has more potent metabolic effects: the utilization of oxygen and the basal metabolic rate are increased, as is glycogenolysis.

Under conditions of stress, the adrenal medulla is stimulated with a resultant output. consisting mainly of adrenaline; hence its descriptive title of the "emergency hormone". Higher plasma concentrations of adrenaline increase sensual acuity and enhance the the ability to think and react quickly. all effects to prepare for "fight or flight".

2.1.2 CATECHOLAMINE STABILITY DURING BLOOD COLLECTION AND STORAGE

Although catecholamines are rapidly removed out of circulating blood (Vane, 1969), evidence suggests that both adrenaline and nor-adrenaline are quite stable in plasma removed from the blood circul-ation, even at 37°C (Cohen et al., 1958). This may be because only

very low levels of the main catabolising enzymes, monoamine oxidase and catechol-o-methyl transferase (COMT) have been found in human plasma. Dopamine may be catabolised due to the presence of benzylamine oxidase (Sharman, 1975). However, plasma catecholamines, may be reduced in exsanguinated blood quite rapidly (Carruthers et al., 1970), partly due to uptake into platelets and erythrocytes (Sharman, 1975). Though rapid uptake into erythrocytes has been refuted, particularly in the cold (Born et al., 1967b), and the rapid disappearance of adrenaline in dog blood, in-vitro, has not been observed (Mangan and Mason, 1958).

However, it seems probable that in exsanguinated blood, any catecholamines released during blood collection may have to act rapidly on platelets before being taken up into blood cells. Those catecholamines taken up into platelets are stored in the "bulls eye" granules. It has recently been observed that COMT is present in platelets, but it is associated with the mitochondria and is thought unlikely to affect the contents of the storage granules (Stramentinoli, 1978). Therefore, any catecholamines, even if in insufficient concentrations to induce platelet aggregation per se, may, as a consequence of stress during blood collection, be released from the adrenal medulla and taken up into platelets. The catecholamines may then be able to potentiate platelet aggregation when released during the release reaction, or platelet destruction.

2.2 THE MEASUREMENT OF PLASMA CATECHOLAMINES AS OPPOSED TO URINE CATECHOLAMINES

The measurement of urinary catecholamines is a comparatively simple procedure, though urine catecholamine concentrations indicate

the sum of events that have occurred over a long period of time, and hence cannot measure transitory changes of sympathetic activity (de Champlain et al., 1976). Therefore, to measure catecholamines liberated in proportion to the degree of sympathetic acitvity after physiological stimulus, requires the more difficult measurement of plasma catecholamines.

2.3 THE CONDITIONS FOR BLOOD COLLECTION

Ten blood samples for catecholamine estimation were obtained during routine blood collection sessions at West Midlands Regional Blood Transfusion Service Centres. The majority of samples were, however, collected from University staff volunteers within our own laboratories. The conditions were as near as possible to those at the Transfusion Service Centres, though more care was exercised in ensuring similar conditions from one donor to another. All volunteers were selected at random, but particular attention was payed to certain facts.

2.3.1 DONOR GENDER

Even though the basal plasma concentrations of circulating catecholamines are similar in males and females (Callingham, 1975; de Chaplain et al., 1976), it is possible that there might be a stress response difference and as it was easier to obtain male volunteers, it was decided to maintain one gender only.

2.3.2 MEDICATION

Many drugs have been shown to affect platelet function. Plate-
let concentrates prepared from blood from volunteers who had
previously ingested only 600 mg of aspirin have been shown to be
haemostatically less effective (Barrer and Ellison, 1977). Though
this effect was not observed in another study (Aster et al., 1976),
it was decided that to avoid any possible interference from drugs,
all donors were free from medication for at least the 7 days
immediately prior to blood collection.

2.3.3 TIME OF COLLECTION

All the laboratory donations were conducted between 9.00 am and
11.00 am, so as to avoid any possible diurnal variation which has
been observed in a minority of donors (Aronow et al., 1973). However,
despite the reported increase in adrenaline secretion   in winter
(Callingham, 1975), collections were made all the year round.

Other variables considered could not be excluded for logistical
reasons. These were:

2.3.4 DONOR'S AGE

Circulating catecholamines have been reported to be independent
of age (de Champlain et al., 1976), though there is contradictory
evidence, at least for noradrenaline, since basal levels in man have
been reported to increase with age. This increase is thought to
compensate for decreased adrenergic receptor sensitivity (Ziegler
et al., 1976; Sever et al., 1977).

2.3.5 SMOKING

Cigarette smoking has been associated with an increase in platelet aggregability (Strolin-Benedetti et al., 1976) which may be related to nicotine stimulation of adrenaline release (Raab, 1962 - cited Franzen and Eysell, 1969).

Other factors that influence catecholamine release include exercise and the movement from a supine to an erect position. Even gentle exercise will increase the plasma noradrenaline concentration by about 70%, though more severe work is necessary to induce adrenaline release (Callingham and Barrand, 1976). The effects of body tilting have been investigated and it was shown that tilting through only 30° can double the normal adrenaline and noradrenaline concentrations in the plasma. This was thought to be due to increased adrenergic nerve activity and to reflex stimulation of the adrenal medulla as a result of haemodynamic adjustments to an abrupt change in body position (Fluck and Salter, 1973). Similar effects were observed when moving from a supine to an erect position (de Champlain et al., 1976). Because of these considerations, all volunteers were asked not to take any exercise and the blood collections were performed with the donors in the supine position. According to Callingham (1975), the potential increase in plasma catecholamine concentrations due to the stress of blood collection itself, can be overcome by resting in a supine position for at least 30 minutes prior to collection. However, this period is rarely experienced by Transfusion Service volunteers, and was not experienced by our own laboratory volunteers. All of the latter were rested for a period of 10 minutes before blood collection.

Volunteers were accepted on the same medical grounds that would qualify them for the Transfusion Service. The majority of volunteers had, in fact, previously donated blood to the Transfusion Service (Table 1).

2.4 ANTICOAGULATION OF BLOOD FOR STORAGE

For long term blood storage, only two anticoagulants are commonly used, acid: citrate: dextrose (ACD) and citrate: phosphate: dextrose (CPD). Heparin, though probably the safest anticoagulant, is only short lived and results in widespread platelet aggregation after only 24 hours (Swank, 1971) and may even induce aggregation (Eika, 1973). EDTA is very effective as a Ca chelator, but results in rapid platelet damage (Mollison, 1972).

Mollison (1972) has given a short history of the development of anticoagulants. The safety of citrate for blood preservation was first observed in 1914. It acts as an anticoagulant by chelating calcium ions, though storage of blood with trisodium citrate alone is unsatisfactory because erythrocytes deteriorate rapidly. In 1941, Maizels found that the addition of dextrose prevented this rapid deterioration possibly by providing energy for ATP and 2,3 diphospho-glycerate (2,3DPG) synthesis. Acidification was initially invest-igated with the object of preventing caramelisation during auto-claving, but Loutit in 1943, found it gave even better preservation of erythrocytes. This completed the development of ACD which was only improved in 1957 when Gibson described an anticoagulant that was less acidic than ACD.

This anticoagulant, CPD, has been compared with ACD in several

TABLE 1.

DONOR DETAILS

a) TRANSFUSION SERVICE COLLECTIONS

EXP	DONOR	AGE (yrs)	DURATION (min)	PREVIOUS DONOR	SMOKER	BLOOD PRESSURE	
						BEFORE	AFTER (mmHg)
1	M.S.	21	6	YES	NO	...	...
"	A.B.	23	8	YES	NO	...	...
2	R.B.	32	7	YES	NO	...	...
"	J.D.	42	8	YES	YES	...	...
3	J.H.	24	17	YES	YES	...	...
"	D.B.	25	6	YES	NO	...	...
4	M.H.	23	15	YES	YES	...	...
"	F.G.	55	7	YES	NO	...	...
"	R.B.	23	5	YES	NO	...	...
"	K.G.	39	6	YES	NO	...	...

b) LABORATORY COLLECTIONS

EXP	DONOR	AGE (yrs)	DURATION (min)	PREVIOUS DONOR	SMOKER	BLOOD PRESSURE	
						BEFORE	AFTER (mmHg)
1	P.W.	48	18	YES	YES	...	...
2	D.N.	22	17	YES	YES	...	...
3	P.C.	33	14	YES	NO	...	...
4	C.H.	23	14	NO	YES	...	...
5	N.C.	34	9	YES	NO	...	...
6	S.J.	22	12	YES	NO	140/80	140/82
7	A.S.	28	7	YES	NO	150/90	134/90
8	N.B.	19	abandoned	NO	NO	130/70	110/65
9	D.B.	29	4	YES	YES	130/78	130/78

EXP	DONOR	AGE (yrs)	DURATION (min)	PREVIOUS DONOR	SMOKER	BLOOD PRESSURE BEFORE (mmHg)	AFTER (mmHg)
10	P.W.	48	21	YES	YES	126/78	120/70
11	N.C.	34	9	YES	NO	126/70	120/80
12	D.N.	22	8	YES	NO	110/72	110/72
13	G.H.	23	11	YES	NO	122/80	110/76
14	T.D.	29	12	NO	NO	140/90	126/82
15	R.G.	22	9	NO	YES	126/60	118/64
16	R.O.	30	7	NO	NO	126/72	127/73
17	G.W.	42	–	YES	NO	...	...
18	G.L.	23	8	YES	NO	120/80	110/74
19	A.S.	29	11	YES	NO	154/90	154/90
20	R.D.	51	8	YES	NO	...	...
21	K.W.	34	5	NO	NO	144/82	120/82
22	D.C.	29	9	NO	NO	...	...
23	P.M.	23	5	YES	NO	130/80	128/80
24	P.C.	34	7	YES	NO	122/78	110/76
25	G.W.	42	9	YES	NO	...	...
26	G.H.	23	5	YES	NO	120/80	104/70
27	R.G.	22	14	YES	YES	130/70	102/68
28	S.J.	23	4½	YES	NO	160/80	134/78
29	D.N.	23	5½	YES	YES	110/70	108/72
30	R.O.	31	6½	YES	NO	140/86	120/84
31	D.B.	30	6½	YES	YES	130/70	126/80
32	R.D.	51	5	YES	NO	128/74	116/70
33	P.W.	30	10	YES	NO	140/92	134/88
34	P.C.	34	4½	YES	NO	126/70	120/86
35	P.M.	23	5	YES	NO	130/80	116/84

investigations. Erythrocyte survival is better maintained in CPD, associated with lower plasma potassium and haemoglobin concentrations (Limbird and Silver, 1974). Altered haemoglobin is a very important problem associated with massive transfusions (Collins, 1974) and is correlated with 2,3DPG levels in erythrocytes. 2,3,DPG is an effective regulator of oxygen release by haemoglobin, a reduced 2,3,DPG level being associated with an increased oxygen affinity, thus affecting oxygen dissociation (Benesch and Benesch, 1969). 2,3 DPG levels are especially well maintained in CPD (Collins, 1974; Limbird and Silver, 1974; Marshall et al., 1975.), possibly due to the sodium dihydrogen phosphate content maintaining a more physiological pH. The higher pH favours the activity of DPG mutase which catalyses the conversion of 1,3 DPG to 2,3 DPG (Mollison, 1972). Platelets aggregate more readily in CPD than in ACD during the first few days of storage, possibly because the lower pH of ACD is less favourable for aggregation (Solis et al., 1974b). However because of better erythrocyte preservation, CPD is probably the anticoagulant of choice in most transfusion centres and so it was the anticoagulant used in this investigation.

Recipe:

Dextrose	25.5 g
Trisodium citrate (dihydrate)	26.3 g
Sodium dihydrogen phosphate (monohydrate)	2.22 g
Citric acid (Monohydrate)	3.27 g

Made up to 1L with distilled water.

2.5 METHODS

2.5.1 THE BLOOD COLLECTION PROCEDURE

This procedure was the same throughout the investigation. After resting for 10 minutes, in a supine position, the venous blood pressure in the arm of the volunteer was raised to 80 mmHg using a sphygmomanometer cuff. Topical application of Hibitaine was used to cleanse the needle entry site followed by the intradermal administration of 0.05 ml of Xylocaine as a local anaesthetic. 450 ml of blood was collected from the antecubital vein via a standard 15 gauge needle into a polyvinylchloride (PVC) blood pack containing 63.5 ml of CPD as the anticoagulant. The packs (McGaw Laboratories, USA) were specially prepared, and consisted of two packs connected by a Y junction, enabling simple division of the blood during or after collection (Figure 5). The packs were gently agitated by hand during the entire collection period, to ensure even mixing of the blood with the CPD.

2.5.2 BLOOD COLLECTION RESULTS (Table 1)

In total, there were 45 donations, of which 35 were undertaken in our laboratories. Of these, 15 donors volunteered twice, 38 had previously given blood and 33 were non-smokers. Donor age ranged between 19 and 55 years with a mean of 30 years. The bleeding time ranged between 4 and 21 minutes with a mean of 9 minutes. No difficulties were experienced during any donation in the laboratory, with the exception of one instance when we were unable to obtain
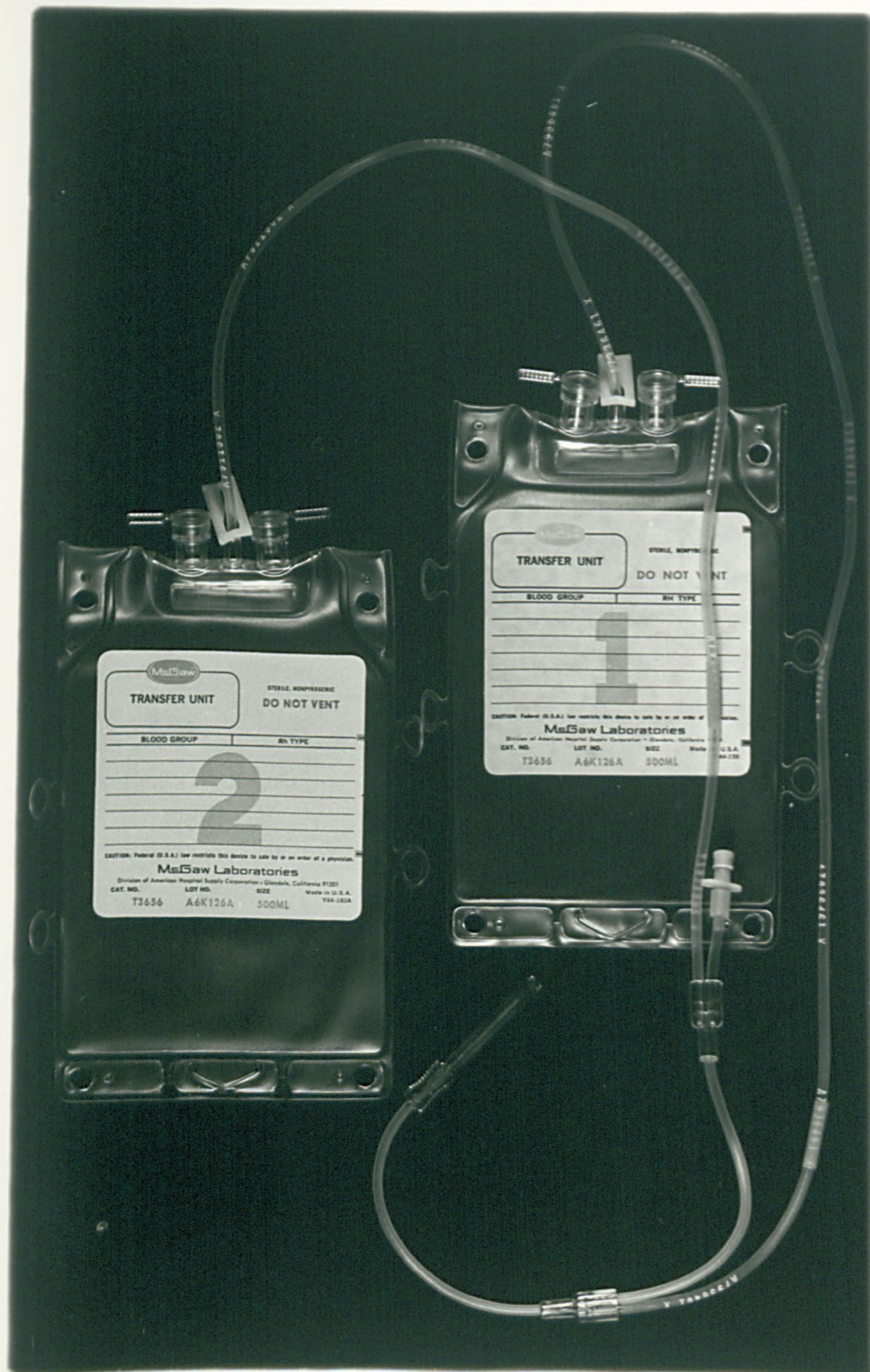
Figure 5

450 ml. This donation was excluded from the results. On 26 occasions the donor's blood pressure was taken before and after blood collection. Diastolic pressure showed little fluctuation with a mean of 78 mmHg, but systolic pressure fell by a small but statistically significant amount (students paired 't' test $p \leqslant 0.05$) from a mean of 131 to 121 mmHg.

2.5.3 SAMPLING FOR CATECHOLAMINE ESTIMATIONS

Samples of blood for catecholamine estimations at the start and finish of blood collection were taken from special sampling sites contained on the blood pack tubes (Figure 5). 15 ml samples were taken into sterile syringes containing 2.1 ml CPD.

Samples taken at the Transfusion Service Centres were taken from volunteers whose permission for sampling blood had been obtained. The objects of the research were briefly explained to the donors in order to alleviate any fears that might have developed, if they considered they had been singled out for undisclosed medical reasons. Sampling sites are not found on normal packs and so on these occasions, the first sample was taken immediately prior to collection directly from the vein, and the second sample from the blood pack tube after it had been clampled proximally to the sampling point at the end of collection. This meant a total of 480 ml of blood was taken from these donors, and so the same volume was taken from our own laboratory volunteers during the catecholamine experiments.

Subsequent blood collection, however, where separate sampling was not undertaken, involved the normal collection of 450 ml only.

2.5.4 PREPARATION OF SAMPLES FOR CATECHOLAMINE ESTIMATION

To minimize the loss of plasma catecholamines due to cellular uptake, blood samples were immediately cooled on ice and platelet free plasma (PFP) separated by centrifugation at the eariliest opportunity, usually within 15 minutes of collection. The addition of an antioxidant to fresh samples, such as sodium metabisulphite, which is recommended by some investigators (Anton and Syre, 1962; Shellenberger and Gordon, 1971; Diamont and Byers, 1975) was found to be unnecessary; a finding later endorsed (Callingham, 1977 personal communication; Sever et al., 1977). PFP plasma was separated by centrifugation at 2,500 g for 15 minutes at 4°C. PFP was carefully removed taking care not to disturb the 'buffy coat' layer on top of the erythrocytes. Subsequent microscopic examination of the PFP disclosed only trace contamination with residual platelets. It may be possible to freeze plasma samples (Callingham, 1975) without loss, though the effects of long storage are variable (Carruthers et al., 1970) and so all samples were assayed on the day of collection keeping the PFP at 4°C until required.

2.6 ASSAY FOR PLASMA CATECHOLAMINES

The concentrations of dopamine, adrenaline and noradrenaline in human plasma are normally very low, and therefore, require a highly sensitive and reliable procedure for their estimation. They can be estimated by biological or chemical assay, though, because of greater reliability and ease of execution, chemical methods are more desirable. For plasma samples, only methods based on spectrophotofluorimetry and radioisotope labelling have been claimed to

provide the required sensitivity. The isotope method is based on the o-methylation of the catecholamines using catechol-o-methyl transferase (COMT) and labelled S-adenosyl methionine (SAM) as the methyl donor, converting the catecholamines to labelled methoxy-tyranine, normetanephrine and metanephrine (Engleman and Portnoy, 1970; Siggers et al., 1970; Coyle and Henry, 1973; de Champlain et al., 1976; Ben-Jonathon and Porter, 1976; Callingham and Barrand, 1976). The fluorometric method is based on either oxidation of catecholamines to their respective trihydroxyindole derivatives, or the less specific ethylenediamine condensation. The trihydroxyindole procedure involves oxidation of adrenaline and noradrenaline to adrenochrome and noradrenochrome at a slightly acid pH, followed by the addition of a strong alkali and reducing agent resulting in rearrangement to fluorescent lutines. As the method operates near its limit of sensitivity with small plasma samples, many modifications have been proposed to improve sensitivity (Vendsalu, 1960; Haggendal, 1963; Anton and Sayre, 1962; McCullough, 1968; Jones and Robinson, 1975; Diamont and Byers, 1975).

Although greater sensitivity is claimed for the isotope method, the procedure is complex, costly, and for unknown reasons, low recoveries have sometimes been obtained (Siggers et al., 1970). As a consequence a spectrophotofluorimetric method was first used, and because of reported better specificity, the trihydroxyindole method was selected.

2.6.1 FLUORIMETRIC METHOD FOR THE DETERMINATION OF PLASMA CATECHOL-
        AMINES

The method used was a modification of the improved sensitivity

method described by Diamont and Byers (1975). However, catechol-
amines were extracted from plasma by ion exchange chromatography
(Dowex 50W-X8) rather than alumina, as it was easier to activate and
could be reactivated in the column (Jones and Robinson, 1975). Dif-
ferentiation between adrenaline and noradrenaline was catered for
by selective oxidation based on the differential influence of
tautomerization temperature as detailed by Diamont and Byers (1975).

The method, however, was never found to be suitably sensitive
to measure plasma samples, possibly due to the instability of the
fluorescent products making readings necessary in an impossibly short
time.

A more recent fluorometric method has been described using
fluorescamine (Roche) which is claimed to yield intense fluorescence
with many primary amines of biological importance including the cat-
echolamines (Udenfriend et al., 1972). Fluorescamine reacts directly
to give a stable fluorescent pyrrolinone product. The reaction
proceeds at room temperature and any unreacted fluorescamine is
rapidly hydrolysed naturally (Felix and Jiminez, 1974; Bohlen et al,
1973; Klein and Standaert, 1976). Its simplicity of reaction
made it an ideal candidate for estimation of plasma catecholamines.
However, preliminary studies showed the method to be insensitive
to concentrations below 1 ng/ml and therefore it was unsuitable for
estimating plasma levels.

2.6.2 DETERMINATION OF PLASMA CATECHOLAMINES BY THE RADIOLABELLED
       O-METHYLATION METHOD

As a consequence of the inadequacy of the fluorimetric methods

for assaying plasma catecholamines, it was decided to use the more
sensitive but more expensive and difficult radiometric method. The
procedure was a modification of the methods, described by Ben-
Jonathon and Porter (1976) and Callingham (1977 - personal commun-
ication). The enzyme, COMT is available commercially, but to reduce
costs, it was prepared in the laboratory.

2.6.2.1 PREPARATION OF COMT

The method of preparation was as described by Nikodejevic et al.,
(1970) but using ammonium sulphate fractionation of fresh pig's
liver rather than rats liver. The procedure followed that described
but with the following modifications. The paper refers to percentage
saturation steps maintaining a temperature around 0°C, when the
calculation was based on room temperature solubility. This was
corrected. Also, prior to the dialysis step, the dialysis tubing
was boiled for 5 minutes in 1mM EDTA followed by thorough rinsing
with deionised water. This helps to eliminate any heavy metal imp-
urities (Umbreit, 1964). The dialysate was divided into suitable
aliquots and stored at -20°C. Further purification through
Sephadex gel columns, via calcium phosphate gel adsorption and
ultracentrifugation, as suggested by Nikodejevic et al., (1970)
failed to provide a more sensitive assay (Callingham, 1977, personal
communication). The dialysate concentration represented about
50 mg protein per ml of 1mM phosphate buffer pH 7.

2.6.2.2 MEASUREMENT OF COMT ACTIVITY

The method used was as described by McCaman (1965) using dihdroxy-
benzoic acid as the substrate as this was more active than adrenaline

for testing COMT activity. Enzyme activity was calculated on the basis of the known specific activity of ^{3}H SAM (Radiochemical Centre) and expressed as μmoles of product formed per g of tissue per hour. Enzyme activity was linear for at least one hour. Stored COMT at -20°C remains active for at least a year and only one batch was required. This had a calculated activity of 68 nmol.mg^{-1} protein. hour^{-1}. Therefore, with a protein concentration of 50mg.ml^{-1}, 50 μl (2.5 mg) should, in theory, be sufficient to o-methylate 2.5 x 68 nmol of dihydroxybenzoic acid.

2.6.2.3 RADIOLABELLED SAM

The method described makes use of 3H labelled SAM as the methyl group donor. Unfortunately, ^{3}H SAM is extremely unstable unless stored at -140°C or below (over or in liquid nitrogen). ^{14}C labelled SAM is very much more stable and has been used as the methyl donor (de Champlain et al., 1976), but in the author's hands, it had a specific activity too low to allow the measurement of very low catecholamine concentrations. As a consequence, the use of ^{3}H SAM was unavoidable with resultant use of liquid nitrogen and its concomitant difficulties. Even at -140°C, decomposition is estimated to be in the region of 1% per month, but a stock was never maintained for more than two to three months and so it was not considered necessary to assess radiochemical purity during storage. (The sudden and inexplicable rapid decomposition notorious with ^{3}H compounds after prolonged storage, had never been observed in less than six months for ^{3}H SAM - Radiochemical Centre, personal communication, 1976).

2.6.2.4 ASSAY PROCEDURE

PFP samples were prepared as described. Samples were not deproteinised so that protein bound catecholamines were included. (Although binding, particularly of noradrenaline, takes place very quickly, mainly to albumin (Powis, 1975), and can account for up to 70% of the plasma level, it is uncertain to what extent released catecholamines are bound during blood collection). One ml of plasma was incubated in the presence of 85 mM TRIS buffer (pH 8.6), 20 μM magnesium chloride, 3.9 mM EGTA, 0.5 mM dithiothreitol, 2 μCi S-adenosyl (^{3}H methyl) methionine (specific activity 5 to 15 Ci/mmol) and 50 μl COMT (about 2.5 mg, protein), in a final volume of 1144 μl for 45 minutes at 37°C. 0.5 ng each of dopamine, adrenaline and noradrenaline (Sigma) added to an appropriate plasma sample served as internal standards. Blank values were obtained using plasma previously mixed with activated aluminium oxide (100 mg/ml PFP) at pH 8.4 for 10 minutes. This effectively removes catecholamines. Addition of 250 μl, 2M borate buffer (pH 11) terminated the incubation period, followed by the addition of 10 μg each of normetanephrine, metanephrine and 3 methoxy-tyramine (Sigma) as markers. The methylated catecholamines were extracted into 7ml toluene: isoamyl alcohol (3:2 v/v); the solvent was washed with 0.5 ml of 0.5 M borate buffer (pH 10), and back extracted into 250 μl of 0.1 N HCl. The HCl was dried off under a stream of nitrogen gas and the residue dissolved in acidified ethanol (40 μl). Each sample was spotted onto separate thin layer chromatography (TLC) plates (20 cm x 20 cm coated with 25 mm gypsum free, silica gel) and run in a two way TLC system to separate the catecholamines as detailed by

Ben-Jonathon and Porter (1976). After air drying, the spots were exposed with iodine vapour, scraped into scintillation vials, and eluted with 2 ml ethoxyethanol for 2 hours. Ten ml of toluene/PPO/POPOP were then added as the scintillation cocktail and the radio-activity counted on a liquid scintillation counter (Packard Tricarb). As with any technique for the measurement of radioactivity in biological samples, the efficiency of counting must be known. This is because efficiency may vary unpredictably from one sample to the next, due to chemical or colour quenching. Counting efficiency was calculated using automatic external standardisation (AES) which utilises an external gamma radiation source whose electrons are affected by quenching in the same way as the beta spectrum from the isotope being used. Efficiency was found to vary little from about 35%.

2.6.2.5 CALCULATION OF PLASMA CATECHOLAMINES

The assay has been reported to be linear for all three catechol-amines up to at least 5 ng. ml^{-1} (Ben-Jonathon and Porter, 1976), 10 ng.ml^{-1} (de Champlain et al., 1976) and even 30 ng.ml^{-1} (Coyle and Henry, 1973). This has been confirmed by the author up to 5 ng.ml^{-1}. As a result, the concentration of the unknowns was calculated from the standard. The plasma sample which contained the standard was also assayed without the standard, so that calculation of the counts per minute (cpm) due to the standard itself, was obtained by subtracting the cpm of the sample without the standard from the sample with the standard. The plasma blank cpm was subtracted from all samples. The assay was found to be particularly

sensitive to dopamine, but showed similar mean counts for the same concentration of adrenaline and noradrenaline. 500 pg/ml of adrenaline and noradrenaline resulted in counts of 235 $\pm$ (SEM) 36 cpm and 238 $\pm$ (SEM) 41 cpm respectively. Blanks were found to be 18 $\pm$ (SEM) 2 cpm for noradrenaline and 16 $\pm$ (SEM) 2 cpm for adrenaline. All cpm were above the ambient background. Calculation of the unknown samples corrected for blanks were compared with the standard. All results were then corrected for assay dilution and dilution due to the anticoagulant. i.e.

$$\text{catecholamine concentration (in pg.ml}^{-1}\text{ PFP)} = \frac{\text{cpm of unknown}}{\text{cpm of standard}} \times 500\text{pg} \times \frac{1000\mu l}{1144\mu l} \times \frac{513.5\text{ml}}{450\text{ml}}$$

The limit of sensitivity was taken as a sample to blank ratio of 2, giving an assay sensitivity of 25 to 35 pg.ml^{-1} for adrenaline, and 30 to 50 pg.ml^{-1} for noradrenaline. The assay was very specific for adrenaline and noradrenaline, and the small interference by DOPA reported by Ben-Jonathon and Porter (1976), affected only the dopamine assays. Intra assay variation between duplicates was less than 15%.

2.6.3 RESULTS OF CATECHOLAMINE ASSAYS DURING BLOOD COLLECTION (Table 2)

Dopamine is only a very weak potentiating agent for aggregation and probably does not aggregate platelets at all. As dopamine was found to have a plasma level only marginally higher than adrenaline,

- 75 -

TABLE 2

CATECHOLAMINE CONCENTRATIONS DURING BLOOD COLLECTION

Donor	Age	Duration of Donation (min)	Nervous (N) or Relaxed (R)	Plasma Catecholamine Concentrations (pg/ml.)			
				Ad Beginning	End	NAd Beginning	End
P.C.	33	14	N	0	55	23	108
C.H.	23	14	N	197	72	285	190
N.C.	34	9	R	44	57	224	236
S.J.	22	9	N	40	52	94	181
A.S.	28	7	N	49	53	86	102
D.B.	29	4	N	85	29	368	302
M.S.	21	6	N	17	26	87	44
A.B.	23	8	R	8	8	58	102
R.B.	32	7	R	14	97	114	323
J.D.	42	8	R	0	0	204	256
D.B.	24	17	R	77	175	174	335
J.H.	25	6	N	112	77	265	242
M.H.	23	15	N	0	23	52	247
F.G.	55	7	N	39	21	253	349
R.B.	23	5	N	14	29	210	233
K.G.	39	6	R	6	0	154	189
Mean	30	9		44	48	166	215
Range	21 - 55	4 - 17		0-197	0-175	23-368	44-349
S.E.M.				$\pm$12.8	$\pm$10.6	$\pm$23.8	$\pm$22.1

having a combined mean of 53 $pg.ml^{-1}$, the results have been excluded.

The plasma concentration of adrenaline and noradrenaline varied considerably between donors. The mean and standard errors at the beginning and end of blood collection were 44 $\pm$ 12.8 $pg.ml^{-1}$ and 48 $\pm$ 10.6 $pg.ml^{-1}$ for adrenaline, and 166 $\pm$ 23.8 $pg.ml^{-1}$ and 215 $\pm$ 22.1 $pg.ml^{-1}$ for noradrenaline. These figures were well within the "normal" ranges reported by some workers (Aronow et al., 1973; Hortnagl et al., 1977) but lower than others (Anton and Sayre, 1962; Carruthers et al., 1970; Fluck and Salter, 1973; Rawlinson et al., 1978). A paired Students 't' test revealed a significant difference (p $\leqslant$ 0.05) for noradrenaline only. In nine donors, the plasma noradrenaline concentration was relatively higher at the end of blood collection, and in three donors, it was relatively lower. There were no changes in the other four cases (the criteria for "no change" being within the minimum sensitivity limits). The adrenaline concentration rose in only four donations and remained the same in nine. Donor ages ranged between 21 and 55 years, and bleeding times between 4 and 17 minutes. There seemed to be no relationship between age or bleeding time, and catecholamine concentrations at the beginning or end of blood collection.

In six experiments, platelet counts were conducted. These always showed a small rise during blood collection, from a mean of 205 x 10^9 L^{-1} to 219 x $10^9 L^{-1}$ (Table 4i). However, the rise was variable and a paired Students 't' test showed it to be significant at the 6% level only.

In the six laboratory experiments, the main volume of blood was collected in two halves. The object was to examine the change in catecholamine concentration at different stages of blood donation. This

might demonstrate whether a smaller blood volume was associated with a lower catecholamine concentration increase and reduced platelet aggregation. Blood samples were taken from the packs immediately after collection. Generally, the pattern was similar for noradrenaline and adrenaline, the concentrations inceasing in two cases, falling in two, and remaining unchanged in the remaining two (Figure 6). Platelet aggregation occurred to the same extent in both packs, reaching a measurable maximum screen filtration pressure of 600 mmHg, within 24 hours.

2.6.4 DISCUSSION

Although errors involved in catecholamine assays, and variation between individuals make it difficult to give precise values (Callingham, 1975), the results obtained demonstrated only small changes in peripheral plasma catecholamine concentrations during blood collection. The increased platelet count, possibly due to splanchnic contraction (Magilligan and Schwartz, 1975), and the reduced systolic blood pressure suggest that the blood collection procedure stimulates adrenal medullary and neural synapse release of catecholamines. However, peripheral circulating, venous plasma catecholamine concentrations represent only that which remains after the interaction of many processes that tend to lower the concentrations. It has been estimated that catecholamine concentrations are reduced by 90% after only one passage of blood through the vascular beds of a hind limb (Critchley and West, 1977). A mild stimulus might be expected therefore, to produce little or no change in the peripheral venous blood concentration of adrenaline, and ante-cubital blood may contain only the noradrenaline washed out of tissues drained by this

Figure 6 HISTOGRAM SHOWING PLASMA CATECHOLAMINE CONCENTRATIONS AT VARIOUS
STAGES OF THE BLOOD COLLECTION

Sequence: 1st   15ml)
           Next   225ml)
           2nd   225ml)   Total of 480 ml (excluding anticoagulant)
           Last   15ml)

= Noradrenaline

= Adrenaline

pg.ml^{-1}

600
500
400
300
200
100

DONOR   P.C.   CH   N.C.   S.J.   A.S.   D.B.
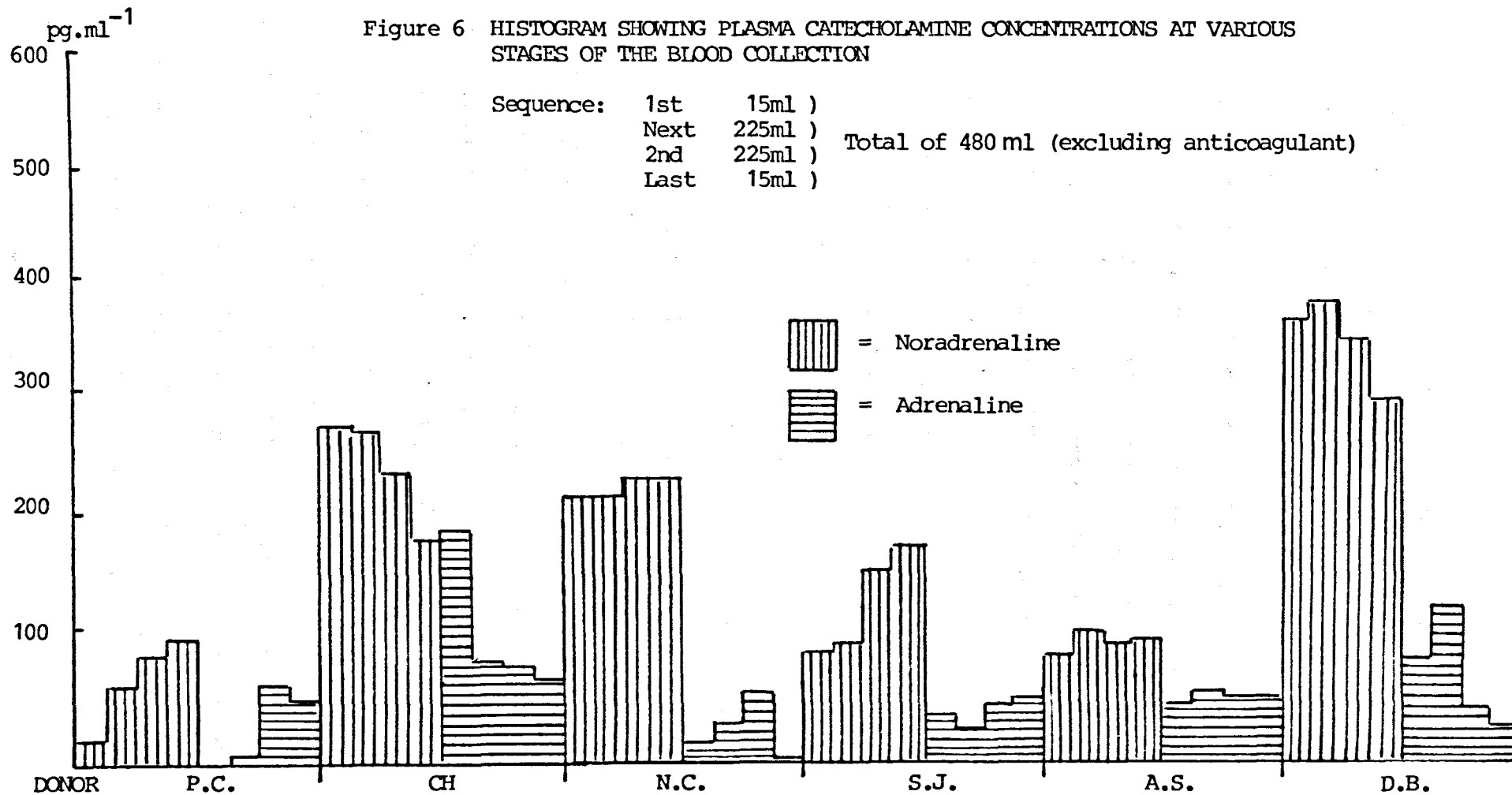
vein (Critchley and West, 1977). An increase in plasma noradrenaline concentrations during blood collection was frequently measured. However, changes in plasma catecholamine concentrations were probably affected by very variable individual responses to stress. The decrease in plasma noradrenaline concentrations measured in a few donors may have been produced as a result of a higher degree of emotional stress at the start of blood collection. This could have been due to fear or aggression, which is normally the predominant stimulus for noradrenaline release (Carruthers, 1975). These same donors produced a similar decrease, or no change, in plasma adrenaline concentrations. Of the sixteen volunteers, only six thought they had been subjected to no apparent stress. Five of the six showed an increased noradrenaline concentration from the beginning to the end of blood donation, and one, no change. In the four cases, in whom the plasma adrenaline concentration increased by the end of the collection, the noradrenaline concentration had also increased. It may be that these donors had a sufficiently high response to haemorrhage to be measurable in peripheral plasma.

It is possible that plasma catecholamines were liberated as a response to blood collection, but only in some donors. However, the plasma concentrations in ante-cubital blood never became high enough to potentiate ADP induced platelet aggregation according to in-vitro measurements (Born, 1967). This is particularly so, as protein bound catecholamines were included in the measurements, and these may not be physiologically active (Callingham and Barrand, 1976). There was no apparent relationship between the changes in catecholamine concentrations and the rate of platelet aggregation which occurred in

all the blood packs stored in the cold (see SFP results, chapter three).

It is possible that the measurement of peripheral blood plasma catecholamine concentrations are inadequate to test the hypothesis that catecholamines released during blood collection induce, or potentiate platelet aggregation. This is because the platelets may have been exposed to higher, local concentrations of adrenaline in the arterial blood (Callingham, 1975). Measurement of plasma free fatty acids might be a better index, as they would not be removed quickly, and increased catecholamines would cause a concomitant rise in plasma free fatty acids (Gordon et al., 1973).

However, as aggregation occurred to the same extent in the two halves of the collected blood, the indications are that the collection of 450 ml of blood (with the possible associated increase in catecholamine concentration) is not likely to induce platelet aggregation.

2.7 PLASMA CATECHOLAMINE CONCENTRATIONS IN STORED BLOOD

Blood platelets take up and carry all three catecholamines, releasing them in response to appropriate stimuli (Weil-Malherbe and Bone, 1954) to a level that may potentiate platelet aggregation (Thomas, 1967). Any catecholamines released from the adrenal medulla and nerve endings as a consequence of blood collection, may, as has been discussed, be taken up into platelets, and would add to any concentration that may already be there. During the platelet release reaction or degradation, this store of catecholamines would be released, and may potentiate secondary aggregation (see chapter one). Because

of this possibility, catecholamines were estimated at intervals
during blood storage, to monitor platelet release and/or degradation,
and to see if the concentration reached the level that has been
shown, in-vitro, to potentiate aggregation.

2.7.1 BLOOD STORAGE

Blood packs were stored in a refrigerator. Samples of blood for
catecholamine assay were taken after gentle agitation by hand to
ensure even mixing. Samples were taken by simple drainage through
a short length of the blood pack tubing left on the packs after col-
lection. These tubes were normally clamped during storage, and
residual blood in the tube was drained away before collecting the
samples.

Storage temperature was maintained at 4°C, the temperature used
by blood banks for long-term blood storage. During the early
experiments, the rate of increase in SFP was found to be higher
than it had been previously reported (Wright and Sanderson, 1974).
After several experiments, it was discovered that a fault in the
refrigerator thermostat was maintaining a temperature nearer 1°C
than 4°C. Although cold itself is thought to be a stimulus for
platelet aggregation (see chapter three), a temperature difference of
only 3°C has not previously been noted as an important difference
in the onset and rate of aggregation in blood. (Though a marked dif-
ference has been noticed between 0° to 1°C and 6°C in the extent
of cold induced aggregation in PRP (Kattlove and Alexander, 1971)).
It should be noted that at least one blood pack manufacturer recommends
that blood should be stored at any temperature between 1°C and 6°C.

Some preliminary comparative studies on SFP at 1°C, 4°C and ambient room temperature were subsequently undertaken. Since the rate of platelet aggregation differed markedly at the three storage temperatures, it was thought that it would be informative to study this further. Also, to include measurements of plasma catecholamine concentrations and other factors during blood storage to see what contribution, if any, they made to the overall extent of platelet aggregation.

The early studies were, therefore, inadvertently undertaken at 1°C. Comparative studies at 4°C and room temperature were carried out in later experiments. In experiments in which platelet aggregation and catecholamine release were compared at all three temperatures, blood was collected into one pack. This was then subdivided into the adjoining pack, and via the sampling site into a third pack. Weighing the packs allowed equal division of the total blood volume.

2.7.2 MEASUREMENT OF PLASMA CATECHOLAMINE CONCENTRATIONS DURING
        BLOOD STORAGE

Catecholamine measurements were performed at 2 and 4 day intervals primarily because of the logistic problems of shorter intervals. Sampling was undertaken at 0, 2, 4 and 8 days. No changes were measured after 8 days of blood storage because in early experiments at 1°C, the SFP had reached its measurable maximum of 600 mmHg after only 24 hours, so that subsequently measured plasma catecholamine concentrations could not be related to the SFP. In addition, the significance of such a relationship probably becomes less important as platelet viability decreases (see chapter three).

Subsequent measurement of catecholamines at 1°C, 4°C and room temperature were, therefore, also limited to 8 days.

2.7.3   RESULTS OF CATECHOLAMINE CONCENTRATIONS DURING BLOOD STORAGE

In three experiments at the three storage temperatures, the plasma catecholamine concentrations were measured as previously described. On all occasions, noradrenaline was the catecholamine present at the highest concentration by a factor of at least 2.5 at the time of blood collection, and by a factor of 4.5 after 4 days of storage. Only noradrenaline concentrations changed significantly during this storage period (Table 3i). The changes in adrenaline and dopamine concentration were often within the sensitivity limits of the assay method and so were not reliable. The concentrations of adrenaline and noradrenaline never reached the minimum (1800 pg.ml^{-1} for adrenaline, and 2 to 10 times 1800 pg.ml^{-1} for noradrenaline) that have been shown to be required to potentiate ADP induced platelet aggregation in-vitro (Born, 1967; Mills and Roberts, 1967).

However, it is not known to what extent, platelet aggregation may be potentiated by synergism between two or more aggregation inducers, or how cold affects catecholamine release. Nevertheless, noradrenaline was the predominant catecholamine measured in plasma, and was the only one whose concentration changed significantly during blood storage. Significant changes in total plasma catecholamines (the sum of the measured dopamine + adrenaline + noradrenaline concentrations) mainly represented changes in plasma noradrenaline. Measurement of total catecholamines in plasma is considerably more economical than measurement of individual catecholamines, and the separation procedure

- 82 -

TABLE 3

PLASMA CATECHOLAMINE CONCENTRATIONS DURING THE STORAGE OF WHOLE BLOOD

i) INDIVIDUAL CATECHOLAMINE MEAN CONCENTRATION ($\pm$ SEM) IN pg/ml PFP

Catecholamine	Temp	n	Sampling time = 0	2	4	8 days
	1°C	3	48 $\pm$ 6	58 $\pm$ 11	71 $\pm$ 7	66 $\pm$ 9
Adrenaline	4°C	4	49 $\pm$ 20	35 $\pm$ 13	52 $\pm$ 19	47 $\pm$ 12
	RT	3	31 $\pm$ 13	38 $\pm$ 17	17 $\pm$ 5	22 $\pm$ 7
	1°C	3	117 $\pm$ 9	190 $\pm$ 44	333 $\pm$ 35	295 $\pm$ 10
Noradrenaline	4°C	4	284 $\pm$ 100	240 $\pm$ 83	489 $\pm$ 89	353 $\pm$ 108
	RT	3	255 $\pm$ 136	248 $\pm$ 69	275 $\pm$ 101	187 $\pm$ 36
	1°C	3	41 $\pm$ 2	38 $\pm$ 6	42 $\pm$ 5	43 $\pm$ 4
Dopamine	4°C	4	42 $\pm$ 14	32 $\pm$ 7	38 $\pm$ 4	43 $\pm$ 10
	RT	3	47 $\pm$ 18	41 $\pm$ 21	27 $\pm$ 2	28 $\pm$ 7

TABLE 3 (cont)

ii) TOTAL CATECHOLAMINE CONCENTRATIONS IN pg/ml PFP

Temp.		0	2	4	8
1°C		212	324	478	371
		199	246	412	436
		165	723	904	996
		172	428	487	651
		135	242	731	984
		186	435	988	971
	mean $\pm$ SEM	177 $\pm$ 12	400 $\pm$ 73	666 $\pm$ 99	735 $\pm$ 118
4°C		480	239	669	239
		656	527	796	763
		118	109	350	262
		229	350	499	507
		165	474	672	1061
		172	234	315	492
		135	133	265	496
		186	455	210	185
	mean $\pm$ SEM	268 $\pm$ 69	315 $\pm$ 57	472 $\pm$ 77	501 $\pm$ 104
Room Temperature		656	398	522	292
		119	155	179	171
		226	426	253	248
		165	251	487	827
		172	219	301	558
		135	213	568	932
		186	158	163	484
	mean $\pm$ SEM	237 $\pm$ 71	260 $\pm$ 41	353 $\pm$ 64	502 $\pm$ 110
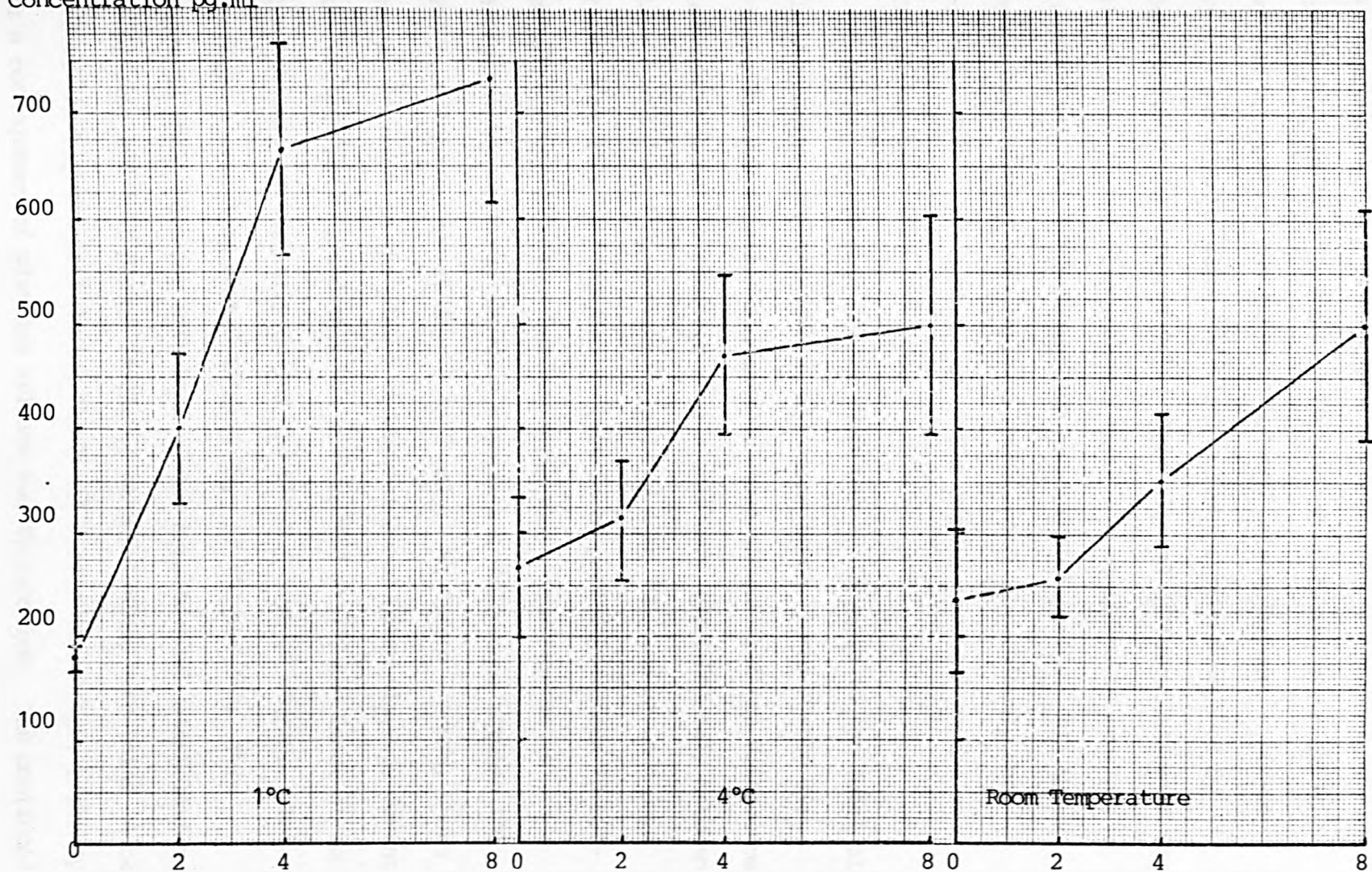
Figure 7.   PLASMA CATECHOLAMINE CONCENTRATIONS (± SEM) DURING BLOOD STORAGE

is simplified. In subsequent experiments, therefore, only total catecholamines were measured. Instead of utilising a two-way TLC system to separate the three methylated catecholamines, a one-way system was adopted using the first solvent system described by Ben-Jonathon and Porter (1976). This allowed differentiation of the three spots in one plane, though there was considerable overlap, particularly between metanephrine and normetanephrine. All three spots were scraped into one vial and counted as previously described. With this system, up to six samples were run on the same TLC plate.

During the storage period there was an increase in the total plasma catecholamine concentration at all three temperatures (Table 3ii, Figure 7), but a paired Students 't' test showed that the difference between consecutive readings were significant ($p \leqslant 0.05$) only between days 0 and 2 at 1°C, between days 2 and 4 at 1°C, and between days 2 and 4 at 4°C. A Students 't' test to compare differences in catecholamine concentration between consecutive measurements at different temperatures showed a significant difference between 1°C and room temperature at days 0 to 2 only. As figure 7 shows, there was an increase in the plasma concentration of catecholamines during the storage period, and this was most marked at 1°C and least at room temperature, particularly during the first 4 days of storage. The increase became less marked after 4 days at 1°C and 4°C. This suggests that there may have been a slow breakdown of catecholamines in plasma, and that plasma levels of catecholamines were being increased at the highest rate during the first 4 days of storage, probably as a consequence of platelet release and degradation. The continued upward trend in plasma catecholamine concentrations during blood

storage at room temperature may have been due to more prolonged preservation of platelet integrity.

As detailed earlier, catecholamines have been found to be quite stable in blood plasma, degrading only slowly, and the main source of their removal in fresh blood, in-vitro, is uptake into platelets, and to some extent, erythrocytes. Reuptake into remaining viable platelets is unlikely to be significant after several days storage and so the degradation of released catecholamines during blood storage probably occurs mainly in the plasma. The mechanism may be oxidation to adrenochrome and noradrenochrome catalysed by ceruloplasmin (Sharman, 1975)

2.7.4 DISCUSSION

The potential total catecholamine concentration in platelets has been estimated to be about 10% that of 5HT (Pletscher, 1968). This theoretical level is well above that required for the induction of platelet aggregation in-vitro (about 4 to 26 $ng.ml^{-1}$, assuming an average platelet count of 200 x $10^9 L^{-1}$ of blood). However, the measured total plasma catecholamine concentrations were well below those that might be expected to potentiate platelet aggregation. The slowness of plasma catecholamine degradation and the rapidity of platelet aggregation in the cold, might have been expected to result in a higher level of plasma catecholamines during storage. It is possible that this discrepancy was due to catecholamines remaining bound to the platelet membranes and since these membranes were removed during PFP preparation, the bound catecholamines were not assayed.

However, it seems unlikely that this possibility would account for the very large difference between the plasma catecholamine concentration measured during blood storage, and the concentration thought to be required to potentiate platelet aggregation. Therefore, it seems unlikely that catecholamines released during blood storage, significantly influence platelet aggregation. However, it is not known to what extent released catecholamines may act synergistically with other released factors in extending platelet aggregation.

2.8 PLATELET COUNTS DURING BLOOD STORAGE

Platelet counts were conducted at two day intervals during blood storage. However, after four days at 1°C and 4°C, the counts became unreliable, probably due to platelet aggregate formation, and were not continued.

The method for counting platelets was as described by Fiessly and Ludin (1949), in which erythrocytes were haemolysed using 3% cocaine in 0.25% sodium chloride and the platelets counted in a haemocytometer using dark phase microscopy.

On blood collection, the mean platelet count ($\pm$ SEM) was $191 \pm 11.2 \times 10^9 L^{-1}$ (n = 13). The platelet count fell during blood storage, but was most apparent at 1°C and least apparent at room temperature. Only the fall in count between days 0 and 2 at 1°C and 4°C were statistically significant (Students paired 't' test $p \leqslant 0.05$), the count falling from a mean of $210 \times 10^9 L^{-1}$ to $149 \times 10^9 L^{-1}$ at 1°C, and from $175 \times 10^9 L^{-1}$ to $153 \times 10^9 L^{-1}$ at 4°C. After 2 days the platelet count fell less dramatically. At room temperature, there was a gradual, but insignificant decline in the platelet count over 4 days of blood storage (Table 4ii).

TABLE 4 PLATELET COUNTS $(x\ 10^9.L^{-1})$

i) PLATELET COUNTS DURING BLOOD COLLECTION

	First 250 ml	Second 250 ml
	174	181
	175	184
	245	264
	228	230
	208	214
	197	239
mean ± SEM	205 ± 11.6	219 ± 13.2

TABLE 4 (cont)

ii) PLATELET COUNTS DURING BLOOD STORAGE

		Storage time in days		
		0	2	4
1°C		177	138	106
		179	147	162
		254	181	191
		229	129	109
		211	155	144
		218	142	–
	mean ± SEM	210 ± 11.8	149 ± 7.4	142 ± 16.1
4°C		142	121	120
		245	212	216
		218	193	164
		164	149	145
		191	164	153
		138	121	120
		129	108	98
	mean ± SEM	175 ± 16.7	153 ± 14.8	145 ± 14.6
Room Temperature		142	138	116
		245	246	230
		218	202	201
		164	164	149
		191	178	163
		138	130	104
		129	105	98
	mean ± SEM	175 ± 16.7	166 ± 18.0	152 ± 18.9

2.9 THE RISE IN PLASMA CATECHOLAMINES AND THE FALL IN THE PLATELET COUNT DURING BLOOD STORAGE

The decline in the platelet count during blood storage was probably due to platelet aggregation and platelet destruction. At 1°C, the rapid fall in the platelet count can be compared with the measured rapid rise in SFP during the first 2 days of blood storage (see chapter three). The rapid increase in plasma catecholamine concentrations at 1°C may also be compared with the fall in platelet counts over the first 2 days storage. At this temperature, aggregation occurred rapidly with a seemingly concomitant decline in the platelet count, and concomitant increase in plasma catecholamines released from platelets. Smaller, but similarly comparable changes, were observed at 4°C. At room temperature there was no measured increase in SFP, and it may be that the slow decline in the platelel count, combined with the gradual increase in plasma catecholamines, was mainly due to degrading platelets. The continued rise in catecholamine concentrations after 2 days storage, particularly at 1°C, was not comparable with the less pronounced fall in platelet counts. It is possible that the rise in plasma catecholamine concentrations in the first 2 days was mainly due to platelet aggregation, when the platelet count would decline rapidly, but that subsequent release was from degrading platelets, some of which may have been counted as normal platelets.

THE EFFECTS OF BLOOD STORAGE TEMPERATURE AND pH UPON

PLATELET AGGREGATION

3.1 COLD INDUCED PLATELET AGGREGATION

Cold induced platelet aggregation in PRP and platelet concen-

trates has been reported by Murphy and Gardner (1969), Shively et al.,

(1970), Kattlove and Alexander (1971), Hardeman and Heynens (1974a),

and Baldini et al., (1976). It also occurs in whole blood (Benner

and Brunner, 1973) where a greater volume of microaggregates were

found in blood stored at 4°C than in blood stored at room temperature

(Solis et al., 1974b). However, there is much debate as to whether

short term storage, particularly of platelet concentrates should be

at 4°C or room temperature. Long term storage of whole blood is still

carried out at 4°C, probably because of the unknown affects of

long term storage at room temperature, and to reduce any bacterial

proliferation. Whole blood and platelet concentrates are transfused

for different purposes. The majority of whole blood transfusions are

used to replace erythrocytes lost during haemorrhage (Becker and Asker,

1972), whereas platelet concentrates are often used to control severe

bleeding. The haemostatic mechanism depends upon platelet viability

(Baldini et al., 1960), so that the maintenance of platelet viability

during the storage of platelet concentrates is of paramount importance.

Viability is often defined as the platelets' ability to survive in the blood circulation after transfusion, and generally, viability is well maintained if normal discoid morphology is retained (Murphy and Gardner, 1975). Unfortunately, rapid platelet shape changes occur when platelet concentrates are stored either in the cold, or at 37°C (Hardeman and Heynens, 1974a). It seems that platelet viability is better maintained at 22°C, and measurement of platelet survival by ^{51}Cr labelling has shown that any temperature less than 20°C or above 24°C results in reduced survival (Murphy and Gardner, 1976). Following platelet storage for 72 to 96 hours, at 22°C, platelet survival was measured as 9 to 10 days after transfusion, whereas following storage at 4°C, the duration of platelet survival in the blood circulation was much shorter (Baldini et al., 1960; Murphy and Gardner, 1969; Lim et al., 1973). Baldini et al., (1960) suggested that the observed reduction in the number of circulating platelets after infusion of cold stored platelets, was due to surface damage making the platelets more susceptible to sequestration and phagocytosis by monocytes. Later evidence showed that this surface damage was due to a symmetrical loss of lipids, followed by the incorporation of unsaturated fatty acids which are primary targets for lipid peroxidation (Baldini et al., 1976).

The effects of cold are rapid. Changes in the intracellular levels of cAMP and cGMP have been detected after only 15 minutes storage at 4°C. cAMP showed a pronounced, but transient increase preceded by a rapid decrease in cGMP (Baldini et al., 1976). The association of platelet microtubule protein into tubular structures may be indicated by cyclic nucleotide dependent phosphorylation.

Baldini et al., (1976) found that microtubule protein is a hetero-dimer and that GTP is covalently bound to one of the subunits. He also found that chilling of platelets enhanced the incorporation of ^{32}P phosphate into microtubule protein. Because dissocation of micro-tubules occurs in the cold (Murphy and Gardner, 1969), it is possible that phosphorylation may be responsible for depolymerisation and resultant dissassociation of microtubule protein.

However, there is controversy as to the haemostatic effective-ness of platelets stored at room temperature. Though cooling destroys platelet function (Lim et al., 1973), immediate haemostatic function is alleged to be better preserved for 24 hours in platelets stored at 4°C than those stored at room temperature. However it was found that platelets stored at room temperature would shorten the bleeding time (a test of haemostatic effectiveness), 24 hours after transfusion (Aster et al., 1976). This delayed function is possibly due to a lesion developing in platelets stored at room temperature, prohibiting them from acting haemostatically for the first few hours after transfusion. This could be a consequence of a reduction in membrane sulphydryl groups involved in platelet adhesion, since this is particularly evident at room temperature. (Baldini et al., 1976). Therefore, it seems that immediate platelet function and long-term effectiveness are separate considerations, to the extent that, at least, American regulations allow platelet concentrate preparations at both 4°C and room temperature. 4°C storage for acute haemorrhage, because of its presumed rapid action, and room temperature for prophylaxis, because of the longer platelet survival at this temperature. However, a recent study presented new evidence. Studies on haemostatic effectiveness and survival have

usually been measured in thrombocytopenic patients who often have additional problems such as fever, sepsis, drug therapy, and disseminated intravascular coagulation, each of which may influence platelet behaviour. In Filip and Aster's (1978) study, patient selection was aimed at the minimisation of variables. It was found that platelets stored for 24 hours at 4°C or room temperature were equally effective in elevating circulating blood platelet levels, and in shortening bleeding times. Because at 72 hours only those platelets stored at room temperature were haemostatically effective, Filip and Aster (1978) suggest that there is no justification for routine platelet storage at 4°C.

Aggregation of platelets in the cold is thought to require no other stimulus (Hardeman and Heynens, 1974a), though the pattern of cold induced platelet aggregation, as seen in an aggregometer, resembles that of ADP induced platelet aggregation (Figure 2, chapter one). Also the exposure of platelets in PRP to a temperature of 4°C resulted in conformational changed in specific membrane proteins, similar to those changes postulated to occur during ADP induced platelet aggregation (Kattlove and Alexander, 1971). However, it does not appear that ADP is directly involved in cold induced platelet aggregation, because ADP was not released from chilled platelets and because the ADP inhibiting agent, apyrase, did not prevent cold induced platelet aggregation. On the other hand, the facts that calcium ions and fibrinogen are required, and that metabolic inhibitors prevent cold induced platelet aggregation, suggests that a specific energy requiring pathway exists. It is possible that, following the initial stimulus, whether cold or ADP, the biochemical pathways are identical (Kattlove and Alexander, 1971).

3.1.1 COLD AGGLUTININS

Cold agglutinins are naturally occurring, usually harmless, cold autoantibodies, which are most active at low temperatures (up to 15°C). They are usually IgM globulins, having specificity within the Ii antigen system found on erythrocytes and platelets (Lalezari and Murphy, 1967 - cited Kattlove and Alexander 1971). These cold agglutinins can cause haemagglutination of erythrocytes at low temperatures, and Kattlove and Alexander (1971) considered their possible role in cold induced platelet aggregation. However, a mediatory role for cold agglutinins and any other plasma proteins was excluded when Kattlove and Alexander (1971) demonstrated that cold induced platelet aggregation occurred in washed platelets resuspended in Tyrodes buffer.

3.2 PLATELET AGGREGATION DURING BLOOD STORAGE

The preliminary results for platelet aggregate formation in blood in the cold supported the findings of other workers, though the difference in the rate of aggregate formation between 1°C and 4°C was a new observation.   Because a difference of only 3°C appeared to have such a marked affect on the rate of platelet aggregation, it was important for comparison purposes to maintain the storage temperatures accurately at 1°C and 4°C. This was achieved by using cooled incubators (Gallenkamp) which maintained a temperature range of ± 0.5°C of the set temperature. It was not possible to maintain room temperature at 22°C, and so a maximum/minimum thermometer was used to monitor daily fluctuations in temperature. It was found that ambient room temperature fluctuated between 14°C and 21°C, temperatures close to the upper end of the range being more common.

3.2.1 THE METHOD FOR THE MEASUREMENT OF PLATELET AGGREGATION

The presence of platelet/granulocyte aggregates in blood was detected by the SFP technique described by Swank (1962). In this technique, the pressure generated proximal to a 20 μm screen filter is measured as a 2 ml blood sample is passed through a 6.25 mm^2 area of the screen at a constant flow rate of 0.33 ml.sec^{-1}. The more cellular aggregates that were present in the blood, the higher the SFP, to a measurable maximum of 600 mmHg. Fresh human, venous blood samples had a SFP of 30 $\pm$ (S.E.M.) 1.9 mmHg (n = 29).

3.2.2 SFP RESULTS (Tables 5a,b,c Figure 8)

SFP measurements were performed during blood storage on days 0, 1, 2, 3, 4, 8 and 14. The samples were obtained as described for catecholamines, except that samples were collected in polystyrene tubes, and SFP measurements were performed within a half hour of collection. Preliminary tests showed that SFP measurements were similar whether samples were maintained at the storage temperature, or allowed to warm to ambient temperature before the measurements were made.

The SFP results demonstrated a rapid rise in platelet aggregation at 1°C reaching the measurable maximum in 17 out of 19 blood packs within 24 hours (Table 5a). At 4°C, the results were variable, but showed a general increased rate of aggregation, usually reaching 600 mmHg between days 4 and 14 (Table 5b). At room temperature, minimal aggregation was observed, with no significant difference between results during the entire storage period (Table 5c). For the purpose of calculating statistical significance, the maximum measurable SFP of 600 mmHg was considered as 600 mmHg, even though

- 92 -

TABLE 5a

SFP CHANGES DURING BLOOD STORAGE (mm/Hg)

		0	1	2	
a) 1°C	Sampling Time				days
		38	600	600	
		32	600	600	
		27	600	600	
		27	600	600	
		22	600	600	
		28	600	600	
		20	600	600	
		18	600	600	
		20	600	600	
		30	600	600	
		26	600	600	
		23	600	600	
		20	600	600	
		32	580	600	
		42	600	600	
		50	485	600	
		48	600	600	
		42	600	600	
		52	600	600	
	mean	31	593	600	
	± SEM	2.3	6.1	0	
	n	19	19	19	

b) 4°C

Sampling Time	0	1	2	3	4	8	14	days
	38	83	380	–	600	600	600	
	26	33	47	–	48	60	167	
	37	43	52	–	76	85	108	
	26	–	40	–	82	152	600	
	43	–	31	–	61	240	340	
	20	57	165	–	600	600	600	
	20	75	340	–	600	600	600	
	20	36	60	–	65	145	231	
	20	28	60	–	83	190	600	
	26	92	185	330	600	600	600	
	20	54	178	330	540	600	600	
	18	43	75	105	215	430	600	
	20	30	42	132	370	600	600	
	30	34	45	95	130	240	460	
	26	120	190	240	420	600	600	
	23	30	25	–	160	600	600	
	20	36	28	45	55	460	600	
	32	130	255	325	350	–	600	
	42	58	50	58	86	233	600	
	50	53	65	112	170	290	600	
	48	140	155	180	180	260	–	
	42	133	600	600	600	600	600	
	52	67	345	600	600	600	600	
mean	30	65	148	242	290	399	523	
± SEM	2.3	8.1	30.9	52.1	47.2	44.0	33.6	
n	23	21	23	13	23	22	22	

TABLE 5c

SFP (cont)

c) RT

Sampling Time	0	1	2	3	4	8	14	days
	37	40	35	–	35	37	53	
	26	30	32	–	27	25	23	
	37	40	26	–	33	26	29	
	26	–	32	–	30	27	29	
	43	–	26	–	32	23	33	
	20	32	25	–	25	20	46	
	20	19	22	–	20	20	18	
	20	18	19	–	21	22	25	
	20	19	27	22	20	–	55	
	18	23	30	28	26	25	27	
	20	25	25	30	28	27	19	
	30	32	29	30	26	26	26	
	23	20	20	25	23	26	24	
	20	22	20	27	24	29	28	
	32	38	53	63	55	–	42	
	42	48	44	40	55	38	35	
	48	35	–	60	–	57	–	
	42	–	42	–	40	36	38	
mean	29	31	29	36	31	29	32	
$\pm$ SEM	2.4	3.0	2.3	5.1	2.6	2.3	2.7	
n	18	15	17	9	17	16	17	
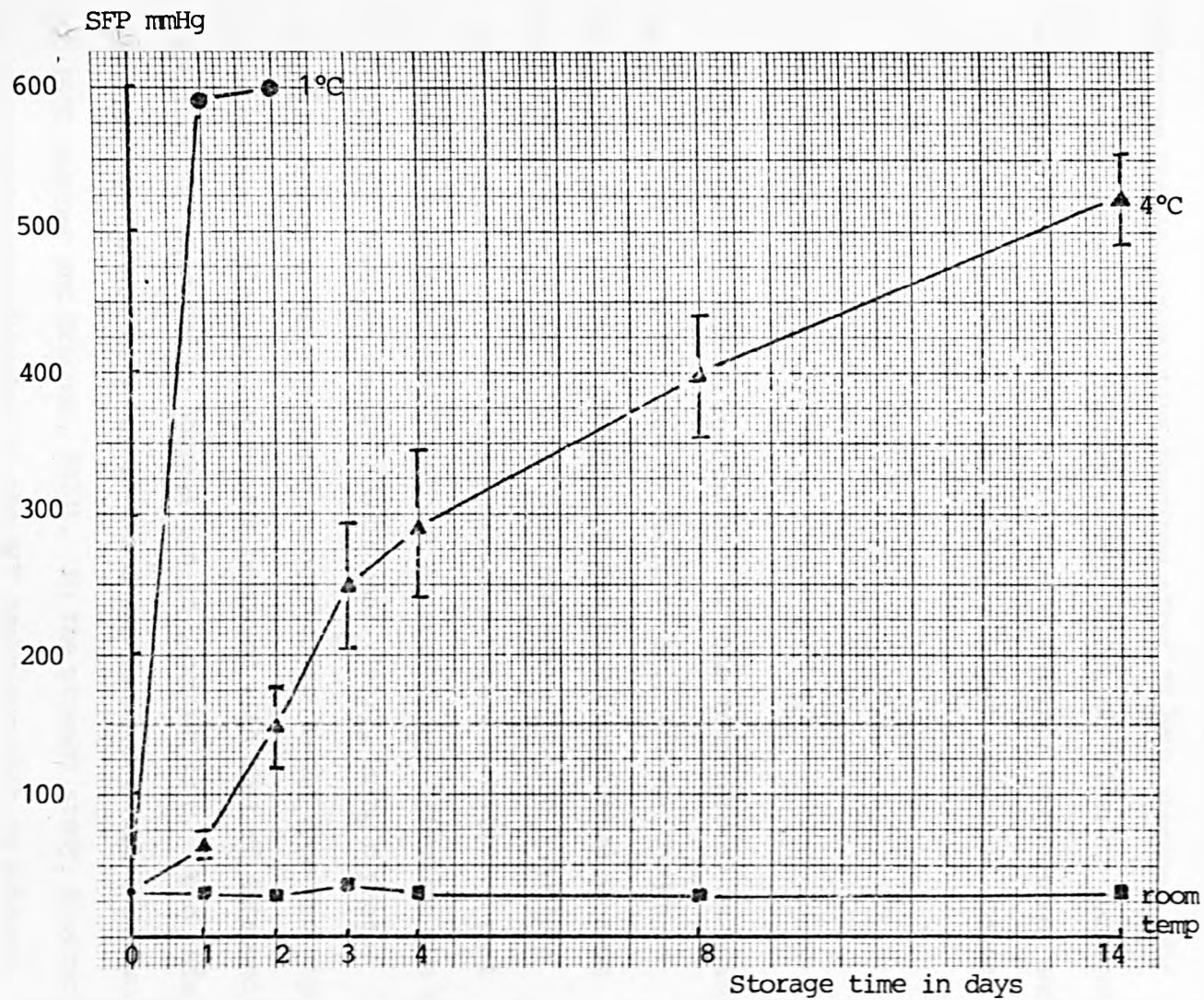
Figure 8   CHANGES IN SFP DURING BLOOD STORAGE (1 SEM)

the true SFP may have been higher than that. The difference between

consecutive SFP results at 4°C was significant throughout blood

storage (Students paired 't' test, p≤0.05). Because of the rapid

increase in SFP at 1°C, it was not possible to calculate dif-

ferences between consecutive readings after 24 hour storage. The

differences between the changes in SFP values at different temperatures

were significant between 1°C and 4°C, 4°C and room temperature, and

1°C and room temperature. The results have been published (Oakes

et al., 1978).

3.2.3 DISCUSSION

The apparent lack of aggregation at room temperature, as measured

by SFP was not found in blood by Solis et al., (1974b) though he

did report that platelet aggregation was much less marked at room

temperature than at 4°C. It is possible that by using a Coulter

counter, Solis et al., (1974b) achieved a higher sensitivity that

allowed him to detect small platelet aggregates not affecting the SFP

measurement. However, Benner and Brunner (1973), using a photo-

electric method to measure platelet aggregates (based upon the higher

transparency of platelet aggregates compared with that of surrounding

erythrocytes) observed no aggregation in blood at 22°C. Cold induced

platelet aggregation began at 20°C and was found to reach a maximum

at 15°C (Benner and Brunner, 1973). In the present study, however,

no aggregation was detected by the SFP technique with an ambient

temperature between 14°C and 21°C. The critical temperature for

platelet aggregation was not determined, but it appeared to be below

14°C. The rate and extent of platelet aggregation at 4°C (Table 5b)

was found to vary between individual blood packs, and so it is likely that the critical temperature for cold induced platelet aggregation is also variable.

3.3 BLOOD HYDROGEN ION CONCENTRATION DURING BLOOD STORAGE

Hydrogen ion concentration (pH) is an important factor for platelet aggregation. This occurs more readily in CPD than in ACD (Solis et al., 1974b; Wright and Sanderson, 1974) and may be due to the lower pH in ACD preserved blood (Solis et al., 1974b). Platelet aggregation and release are progressively inhibited as the pH is reduced (Murphy and Gardner, 1975) and at pH 6.7, platelet aggregation in PRP has been found to be totally inhibited (Kattlove and Alexander, 1971). Between pH 6.9 and 7.2, adrenaline induced platelet aggregation in PRP was very much reduced compared with aggregation at pH 7.4 (Rogers, 1972) and biphasic platelet aggregation, induced by ADP, has been shown to be gradually inhibited at pH values below 7.29 (Lamberth et al., 1974). On the other hand, platelet aggregation in PRP has been shown to be increased at pH values above 7.4 (Rogers, 1972; Rodman and Penick, 1972). Therefore, it appears that platelet aggregation is progressively inhibited at pH values below 7.4, but is enhanced at pH values above 7.4, with the rate of, at least, ADP and adrenaline induced platelet aggregation, reaching a maximum between pH 7.7 and 8 (Rodman and Penick, 1972; Coller et al., 1976).

During blood storage, both the plasma pH (Limbird and Silver, 1974) and whole blood pH (Bailey and Bove, 1975; Mishler et al., 1978) decreases. This decline was also observed during the storage of PRP,

when the decrease in pH at room temperature was greater than at lower temperatures, due partly to carbon dioxide retention arising from glucose and fatty acid oxidation, but mainly from lactate formation in glycolysis (Hardemen and Heynens, 1974a). (Platelet intermediate metabolism is mainly glycolytic, and resting platelets accumulate glucose and degrade it, almost exclusively, to lactate. Even on stimulation with rapidly increased glycogenolysis, lactate remains the predominant end product (Akkerman, 1978)).

Whole blood pH was monitored during stoage at 1°C, 4°C and room temperature.

3.3.1 THE MEASUREMENT AND THE RESULTS OF pH DURING BLOOD STORAGE

pH was measured in blood using a Radiometer pH meter with a combined electrode. Samples for pH measurement were taken as described for catecholamines, but into small polystyrene tubes and pH measurements were made immediately. The electrode was immersed in the blood for precisely 30 seconds before reading the pH. Measurements were taken on days 0, 1, 2, 3, 4, 8 and 14.

The results confirm that pH decreases during blood storage, particularly at room temperature. The pH decreased from a mean of 7.37 to 7.11 at 1°C, from 7.37 to 6.99 at 4°C and from 7.38 to 6.65 at room temperature during 14 days blood storage (Table 6, Figure 9). The decline in pH at 4°C was greater in this study than that observed by Bailey and Bove (1975) who measured a decrease from pH 7.1 at the beginning of storage to only 7 after 14 days blood storage. In contrast, Mishler et al., 1978, measured a mean decrease from pH 7.28 to 6.76 after 14 days blood storage at 4°C, though

TABLE 6

CHANGES IN BLOOD pH DURING BLOOD STORAGE AT 1°C, 4°C AND ROOM TEMP.

Sampling Time	0	1	2	3	4	8	14 days
i) 1°C	7.41	7.24	7.12	7.11	7.10	–	7.03
	7.40	7.38	7.20	7.20	7.13	7.11	7.09
	7.39	7.32	7.25	7.22	7.22	7.21	7.12
	7.37	7.33	7.25	7.23	7.21	7.20	7.10
	7.32	7.28	7.28	7.25	7.24	7.24	7.17
	7.33	7.31	7.28	7.21	7.18	7.16	7.16
mean	7.37	7.31	7.23	7.20	7.18	7.18	7.11
± SEM	0.02	0.02	0.02	0.02	0.02	0.02	0.02
ii) 4°C	7.40	7.35	7.31	7.26	7.21	7.10	7.00
	7.31	7.30	7.24	7.20	7.15	7.14	7.01
	7.41	7.34	7.28	7.25	7.21	7.11	6.88
	7.35	7.24	7.22	7.21	7.21	7.12	6.98
	7.32	7.32	7.21	7.19	7.17	7.10	7.01
	7.41	7.36	7.26	7.25	7.24	–	7.09
	7.40	7.31	7.28	7.25	7.19	7.13	7.00
mean	7.37	7.32	7.26	7.23	7.20	7.12	6.99
± SEM	0.02	0.02	0.01	0.01	0.01	0.01	0.02
iii) Room Temp.	7.40	7.32	7.21	7.11	7.03	6.81	6.71
	7.31	7.21	7.09	7.00	6.90	6.73	6.69
	7.41	7.26	7.11	7.05	6.95	6.79	6.61
	7.41	7.30	7.21	7.14	7.09	–	6.70
	7.40	7.25	7.21	7.08	7.00	6.71	6.57
	7.32	7.18	7.06	6.99	6.90	6.77	6.60
mean	7.38	7.25	7.15	7.06	6.98	6.76	6.65
± SEM	0.02	0.02	0.03	0.02	0.03	0.02	0.02
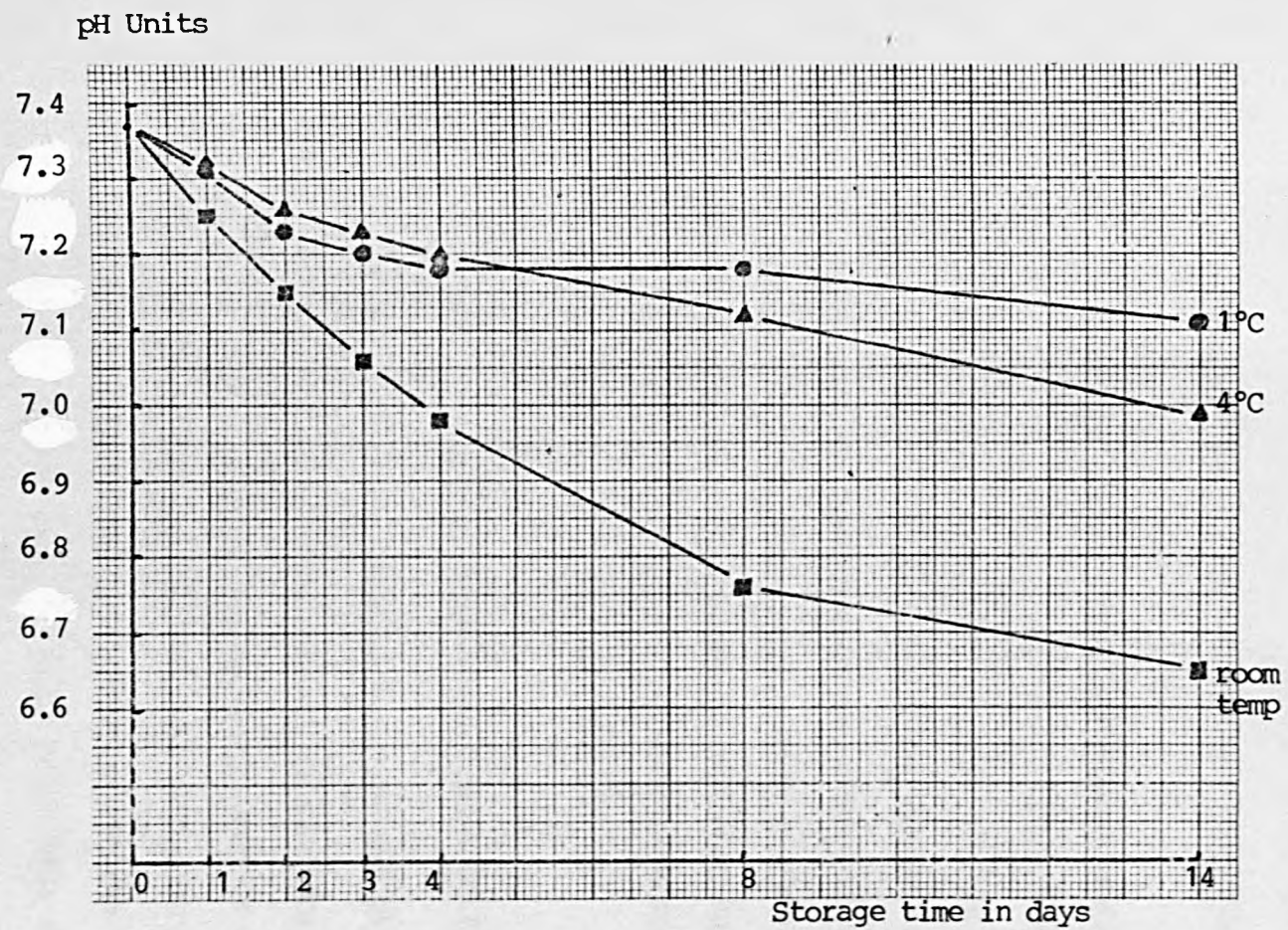
Figure 9   pH OF CPD ANTICOAGULATED BLOOD DURING STORAGE

measurements were made on samples warmed to 37°C before the pH was read.

On blood collection, the pH was in the range 7.31 to 7.41 with a mean of 7.36 ± (SEM) 0.02 (n = 11). These figures were within the "normal" range of 7.27 to 7.43 (mean 7.35) for adult male venous blood (Singer and Hastings, 1950 - cited Documenta Geigy Scientific Tables, 1962).

The decrease between consecutive daily pH values was significant (Students paired 't' test, $p \leqslant 0.05$) at all three storage temperatures for the first 3 days, and during the entire 14 days storage at 4°C and room temperature After 3 days, however, there was no significant decrease in pH at 1°C. The decline in blood pH was significantly different between 1°C and 4°C after 4 days of blood storage (Students unpaired 't' test, $p \leqslant 0.05$), though between 1°C and room temperature, and 4°C and room temperature, the decline in pH was significantly different during all of the first 8 days of storage.

3.3.2 A DISCUSSION OF THE CHANGES IN BLOOD pH DURING BLOOD STORAGE

The results suggest that the pH changes at 1°C and 4°C are indistinguishable during the first few days of blood storage, but that subsequently there is more metabolic activity in blood at 4°C. Blood stored at room temperature appears to have been very much more metabolically active, with the pH declining rapidly during the first 8 days of storage, reaching a mean pH of less than 7 after only 4 days. Presumably, the reduced pH also reduced the rate of glycolysis and may explain the slower decrease in pH after 4 days of blood storage.

Although it appears that the reduction in pH at 1°C and 4°C

would make the platelets less aggregable, it did not prevent platelet aggregation in the cold. The SFP results showed that platelet aggregation occurred rapidly in the cold, particularly at 1°C. Of course, the cold stimulus was not present at room temperature and the absence of measurable platelet aggregation at this temperature suggests that no other stimulus was present during the early part of storage. It is probable that aggregating agents released from degrading platelets may be of sufficient concentration to induce aggregation in remaining viable platelets. That this did not occur at room temperature may have been due to the slow rate of platelet breakdown (see platelet counts, chapter two) and the rapidly reduced pH, which may have prevented platelet aggregation.

CHAPTER FOUR

THE PLASMA CONCENTRATION OF 5-HYDROXYTRYPTAMINE

DURING BLOOD STORAGE

4.1 BIOCHEMISTRY AND PHYSIOLOGY

A secondary pathway (< 2%) for tryptophan metabolism is hydroxylation to 5-hydroxytryptophan in a process analogous to the conversion of phenylalamine to tyrosine. In fact, in the liver, the phenylalamine hydroxylating enzyme will also accomplish hydroxylation of tryptophan. Similarly to the catecholamines, 5-HT is formed by a pyridoxal phosphate dependent, amino acid decarboxylase reaction, in this case from 5-hydroxytryptophan (Figure 10). 5-HT is formed in many tissues, but mainly in the enterochromaffin cells of the gastrointestinal tract and in the brain (5-HT, like the catecholamines, does not cross the blood/brain barrier). The main rate limiting step in the synthesis of 5-HT is the hydroxylation step to 5-hydroxytryptophan (Lovenberg et al., 1968).

5-HT is produced in the organs where it is found, rather than being produced in one organ and carried by the blood to other organs. The presence of large amounts of 5-HT in the gastro-intestinal tract led to the suggestion that it acted there as a local hormone and is involved in the regulation of the motor activity of gastro-intestinal smooth muscle (Lewis, 1965). The distribution of 5-HT in the brain has led to the view that it has a physiological function

in the central nervous system. Its distribution in the brain is ass-
ociated with that of the enzymes 5-hydroxytryptophan decarboxylase and
monoamine oxidase so that it appears that the brain areas which
contain 5-HT can both synthesize and degrade it. There is a parallel
between the distribution of 5HT and noradrenaline in the brain, and
5HT may be present as a humoral transmitter which functions either to
stimulate parasympathetic centres or to inhibit sympathetic ones
(at which noradrenaline is the transmitter), (Lewis, 1965).

5-HT is only released into the blood circulation under certain
pathological conditions, for example, from malignant carcinoid tumours
(Vane, 1969). 5-HT is also released from the lungs and from platelets
during antigen/antibody reactions and anaphylaxis (Waalkes et al.,
1957 - cited Franzen and Eysell, 1969), and from platelets by
bacterial endotoxins (Rosenberg et al., 1959 - cited Garner, 1972).
Severe haemorrhagic and traumatic shock induced in dogs, have been
shown to cause an increase of 5-HT in the blood, probably released
from hypoxic enterochromaffin cells, and from unstable platelets
(Swank, 1968; Kusajima et al., 1974). Released 5-HT has a marked
effect on smooth muscle. It is a powerful broncho-constrictor, and
may be the cause of asthmatic attacks in patients suffering from
malignant carcinoids (Lewis, 1965). The actions of 5-HT on the heart
and circulation are complex and there are marked variations in
effects between species. In man, 5-HT usually causes the systemic
blood pressure to rise and cardiac output to increase, though the
effects are variable (Franzen and Eysell, 1969). 5-HT also causes
vasoconstriction of the pulmonary circulation, particularly the
pulmonary arterioles, and constriction of the coronary blood vessels.
5-HT is thought to activate the pressor reflex to the atrium and the

great blood vessels in the thorax through stimulation of the chemo-receptors of the carotid sinus (Kusajima et al., 1974). The per-ipheral blood vessels of the body are less sensitive to 5-HT than the pulmonary arterioles, but experimental findings of the effects of 5-HT are not consistent. In man, 5-HT introduced intra-arterially in the arm, seemed to be effective in causing constriction of the arteries and arterioles of the arm and hands, but dilated the cutaneous blood vessels (Roddie et al., 1955 - cited Franzen and Eysell, 1969). In contrast, Bock et al (1957 - cited Franzen and Eysell, 1969) found intra-arterial injections of 5-HT increased the blood flow in the forearm, and decreased the blood supply in the skin. Intra-venous injections of 5-HT were found to be similarly effective but only at much higher doses (above 100 μg).

Released 5-HT has a half life of only one to two minutes. It is removed from the plasma in the lungs where it is probably stored, and in the liver, where it is mainly oxidatively deaminated to 5-hydro-xyindole 3-acetic acid (Vane, 1969) (Figure 10). Newly formed plate-lets contain no 5-HT, and are probably not capable of synthesizing their own 5-HT. However, mature platelets contain high concentrations of 5-HT (Pletscher, 1968). Platelets do not remove 5-HT from the circulation very rapidly, but the liver and lungs reduce the plasma 5-HT concentration to less than 1% after only one circulation. 5-HT is taken up into platelets mainly from the lungs and from the gut mucosa. Platelets normally contain from about 0.2 to 1.3 μg 5-HT.10^9 platelets, but their capacity for 5-HT is much higher (Baumgartner, 1969).

Figure 10   THE MAIN PATHWAYS OF 5-HT METABOLISM

Tyyptophan   5-hydroxytryptophan
Hydroxylase   Decarboxylase

Tryptophan ⎯⎯⎯⎯⎯→ 5-hydroxytryptophan ⎯⎯⎯⎯⎯→ 5-HT
            < 2%

                                                    Monoamine   > 98%
                                                    Oxidase

5-methoxyindole 3-acetic acid ◄⎯⎯⎯ 5-hydroxyindole 3-acetic acid
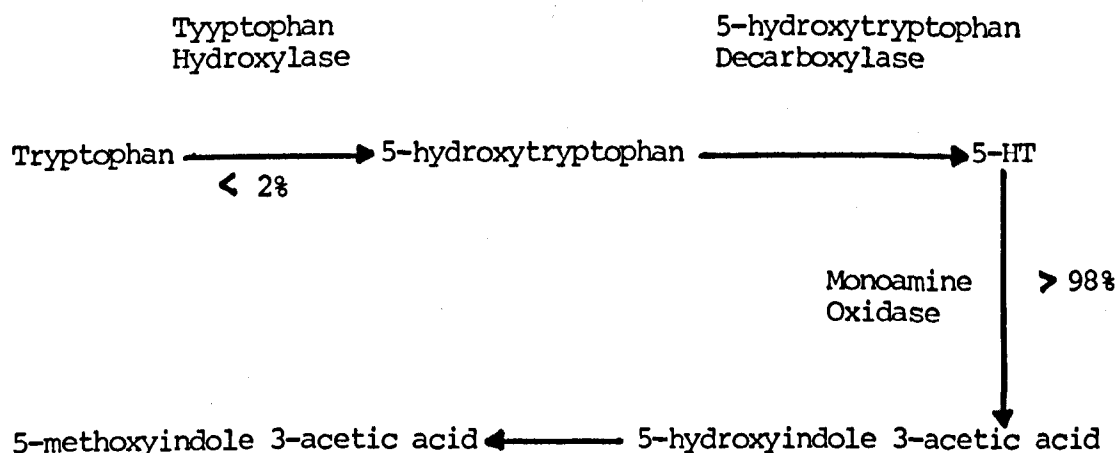
(Other products of 5-HT catabolism include, bufotenine (abnormal),

melatonin, 5-methoxytryptamine, and 5-hydroxytryptophol).

4.2   5-HT AND PLATELET AGGREGATION

Although human blood platelets are only weakly stimulated by

5-HT (Swank et al., 1963), a possible role for 5-HT in platelet

aggregation and potentiation has been discussed (chapter one). As

blood collection is only a mildly stressful procedure, it is unlikely

that plasma 5-HT concentrations will be markedly raised as a

consequence of blood collection alone. The release of 5-HT during

blood storage may be more important in the potentiation of platelet

aggregation. Although erythrocytes may contain up to 10% the plate-

let concentration of 5-HT (Franzen and Eysell, 1969), the integrity

of erythrocytes is thought to be maintained for at least several

days of storage, even at 4°C (Mollison, 1972). Any release of 5-HT

during the early stages of blood storage would, therefore, be from

platelets.

There is evidence that a low, though fairly stable, level of free 5-HT exists in plasma (Somerville and Hinterberger, 1975), although platelets contain the majority of circulating 5-HT. However, 5-HT concentrations were measured in plasma, rather than in platelets, since it has been found that an increase of 5-HT in plasma is complementary to a decrease in platelets in PRP (Hardeman and Heynens, 1974b).

4.2.1 METHODS OF ASSAYING PLASMA 5-HT CONCENTRATIONS

The assay procedures described in the literature for the estimation of 5-HT are varied, with specificity being the most common problem. Spectrophotofluorimetry is the basis for the more common techniques, though a more specific method is radioimmunoassay, which when compared with a fluorimetric method was found to be equally sensitive (O'Brien and Spector, 1975). However, this fluorimetric method, which depends on the natural fluorescence of 5-HT in strong acid, is thought to be eight times less sensitive than another fluorimetric method, that depends upon the formation of a fluorescent 5-HT derivative with ninhydrin (Snyder et al., 1965). A bioassay method is available and is claimed to be sensitive to 1ng of 5-HT in plasma (Vane, 1957). However, as with all bioassay procedures, it can be affected by the biological variability of the test system. Measurements that depend upon the interaction of chemical reagents are more reliable. The formation of natural fluorescence in strong acid with and without ultraviolet irradiation has been used, but both methods lacked sensitivity (Udenfriend et al., 1955; Anden and Magnusson, 1967). It is claimed that a method based upon

the formation of a fluorescent 5-HT/orthophthalaldehyde (OPT)
derivative, makes possible the measurement of minute quantities of
5-HT in body fluids (Maickel and Miller, 1966; Thompson et al.,
1969, 1970; Somerville and Hinterberger, 1975). According to Maickel
and Miller (1966), this method provides the greatest sensitivity, but
lacks specificity, since many related substituted indoles fluoresce
with OPT as strongly as 5-HT. These include melatonin, 5-methoxy-
tryptamine, 5-methoxyindole 3-acetic acid, and N-acetyl 5-HT. This
lack of specificity may be partly overcome by including a purification
process using a weak cation exchange resin (Thompson et al., 1970).
In fresh plasma samples, the interference due to substituted indoles
may not be important because their plasma concentrations are very low
compared with the concentration of 5-HT in platelets (Franzen and
Gross, 1965; Rao et al., 1976). However, during blood storage,
greater conversion of 5-HT to its metabolites may occur and so specif-
icity is important. The formation of a fluorescent derivative of
5-HT with ninhydrin has been described (Vanable, 1963; Snyder et
al., 1965; Shellenberger and Gordon, 1971) and has been shown to be
very specific for 5-HT (Snyder et al., 1965). Only bufotenin and
5-hydroxytryptophan caused interference, and this was only 3% and 2%
respectively. All other compounds that might interfere, including
related indoles, aromatic amino acids, and biogenic amines, gave no
measurable fluorescence.

The method probably depends upon the formation of a tetrahydro-
carbaline derivative formed by condensation of the ketone groups
of ninhydrin with the amino group of 5-HT.

4.2.2 THE METHOD USED FOR ASSAYING PLASMA 5-HT CONCENTRATIONS

The 5-HT concentration was measured in PFP prepared as described for catecholamines. It has been shown that small amounts of 5-HT can bind to plasma proteins in-vitro (Clarke et al., 1959). However, the small error in not measuring these amounts has to be accepted because it is necessary to precipitate the plasma proteins in order to reduce interference with fluorescence.

The method used was a modification of that described by Snyder et al., (1965), involving the solvent extraction of 5-HT from a salt saturated solution at pH 10. Blood for PFP preparation was taken from the blood packs immediately after collection and at intervals during storage as described for catecholamines. Plasma for 5HT estimation may be stored, though it is necessary to adjust the pH to 5.6 (Shellenberger and Gordon, 1971). However, all samples were assayed immediately after withdrawal from the blood packs.

PFP samples (2.5 ml) were deproteinised with the same volume of 1N perchloric acid. After thorough mixing, the protein was precipitated down by centrifugation at 2,500g for 10 minutes. The supernatants (3 ml) were carefully adjusted to pH 10±0.1 with sodium hydroxide, and 0.5M borate buffer pH 10 (0.5 ml) added. Each sample was then equally divided for duplicate assays, and sufficient salt was added to ensure saturation (about 0.5g; too much was found to result in gel formation on addition of the solvent). This was followed by the addition of n-heptanol (8 ml) as the solvent. n-heptanol was used in preference to the butanol/heptane solvent used by Snyder et al., (1965) as Shellenberger and Gordon (1971) found it gave better selectivity between 5-HT and 5-hydroxytryptophan,

lower solubility in water and therefore lower loss during the
washing stage, and a lower blank value. The mixture was vigorously
shaken by hand for two minutes and separated by brief centrifugation.
To the organic phase, salt saturated 0.1M borate buffer (2ml) was
added as the wash, followed by further vigorous mixing and centrif-
ugation. 0.05M phosphate buffer pH 7 (5ml) was then added to the
organic phase, mixed and centrifuged and followed by careful
removal of the aqueous phase (3ml). Fresh 0.1M ninhydrin (0.3 ml)
was added to the aqueous phase and incubated at 100°C for 25 minutes.
This was followed by cooling the tubes to room temperature for one
hour, during which time the condensation reaction with ninhydrin is
thought to occur. The fluorescence was read at an activation wavelen-
gth of 385 nm and emission, 490 nm in a spectrophotofluorimeter
(Perkin-Elmer). Fluorescence has been found to be relatively stable
for up to 6 hours (Snyder et al., 1965). After heating with ninhydrin
and reading at the above wavelengths, only 5-HT fluoresces with any
intensity compared with related indoles.

One of the problems found by several workers estimating 5-HT
has been to select a suitable blank. This problem was overcome by
using alkaline sodium sulphite which rapidly decays 5-HT fluorescence
(Shellenberger and Gordon, 1971). After measuring the fluorescence
in the samples, 2.5% alkaline sodium sulphite (0.1ml) was added. The
samples were then left for several minutes and measured again. In
this way, the samples acted as their own blanks.

5-HT creatinine sulphate standards were prepared fresh in buffer,
so that 10 μl added to one of the plasma samples gave a final 5-HT
concentration of 100 ng/ml. A linear relationship between fluores-
cence and concentration has been observed up to 500 ng.ml^{-1}

(Somerville and Hinterburger, 1975), and this has been confirmed by the author. The plasma sample in which the standard was included was also assayed without the standard, so that the subtraction of the fluorescence due to the plasma from the fluorescence of the plasma plus standard gave the fluorescence due to the standard itself. In this way, the standard was subjected to the same assay procedure as the plasma 5-HT. Therefore, by comparing the fluorescence of the unknown plasma samples with that of the standard, the concentration of 5-HT in the plasma samples was calculated. The spectrophotofluorimeter was set to measure a fluorescence of 100 (arbitrary units) with the standard in place. All of the unknown samples and blanks were then read off the scale without further adjustment. After correcting for blanks and allowing for the plasma dilution with CPD, the concentration of 5-HT was calculated:

$$5\text{-HT ng.ml}^{-1} = \frac{\text{fluorescence of the unknown}}{\text{fluorescence of the standard}} \times 100\text{ng} \times \frac{513.3 \text{ ml}}{450 \text{ ml}}$$

The resolving power of the spectrophotofluorimeter was not calculated, as the monochromator slit widths were not adjustable by the casual user. However, they were considered suitable for precise detection and optimum 5-HT sensitivity. Before each assay, the instrument was adjusted to a constant value for sensitivity by utilizing the Raman band for water (excitation wavelength of 365 nm and activation, 418 nm). Standards were set to 100 on the 8 x 10 scale, which was within the limits of sensitivity of the instrument. Because the samples were diluted, it was necessary to irradiate them only during the measurement period in order to reduce photo-decomposition (Rhys-Williams, 1976). The precision of the method was found

to be good, with the intra-assay variation between duplicates of
both standards and unknowns ranging between 0% and 10%.

4.2.3 THE RESULTS OF PLASMA 5-HT CONCENTRATIONS DURING BLOOD
       STORAGE (Table 7, Figure 11)

During the first two experiments at 4°C and room temperature,
measurements were made on days 0, 2, 4, 8 and 14. However, this
sequence was altered to days 0, 1, 2, 3, 4, 8 and 14 when it was
found that the 5-HT concentration decreased sharply on day 3.

The concentration of plasma 5-HT in the blood packs at the end
of blood collection was in the range 104 to 303 ng.ml^{-1}, with a
mean of 206 $\pm$ (SEM) 20.8 ng.ml^{-1} (n = 11). This was higher than
the normal free plasma 5-HT level reported by Somerville and
Hinterburger (1975) who found a concentration range between 13 and
31 ng.ml^{-1}. Though a review by Franzen and Eysell (1969) reported
that serum 5-HT concentrations were normally in the range between
20 and 150 ng.ml^{-1}, and an information handbook published by Lilly and
Co., (1971) gives a normal serum 5-HT concentration in the range bet-
ween 100 and 320 ng.ml^{-1} (Eli Lilly and Co., personal communication
1979).

It is not known why the concentration was in the order of ten
times that found by Somerville and the Hinterburger (1975), though
the different methods may have contributed to the contrast. A
problem associated with the minhydrin method is the instability
of the reaction, as it is sensitive to small changes in pH, and also
minor changes in the concentration of ninhydrin. Maximum efficiency
is dependent upon the final concentration of ninhydrin being close

TABLE 7

CHANGES IN PLASMA 5-HT CONCENTRATIONS DURING BLOOD STORAGE AT 1°C, 4°C AND ROOM TEMPERATURE ($ng.ml^{-1}$)

a) 1°C

	0	1	2	3	4	8	14 days
	301	203	202	146	256	241	163
	218	131	264	153	367	246	179
	197	147	233	174	368	297	218
	303	241	271	161	253	172	139
	241	127	251	202	206	196	153
	180	189	271	148	221	192	149
mean	240	173	249	164	279	224	167
± SEM	21.3	18.2	11.1	8.6	29.2	18.8	11.6

b) 4°C

	0	1	2	3	4	8	14 days
	104		98		191	142	162
	148		112		105	87	104
	128	112	100	90	119	101	124
	170	185	176	142	223	365	264
	279	143	146	113	171	377	302
	197	171	210	146	348	289	209
	303	300	254	163	210	166	126
	241	135	265	200	198	167	139
mean	196	174	170	142	195	212	179
± SEM	25.5	27.3	23.8	15.7	28.4	40.8	25.5

TABLE 7 (cont)

c) Room Temperature

	0	1	2	3	4	8	14 days
	104		149		203	138	152
	148		110		127	89	129
	128	104	107	90	130	112	112
	170	173	184	149	199	362	280
	218	144	268	126	342	269	217
	197	173	204	150	316	274	232
	303	269	217	165	225	166	129
	241	115	230	191	210	183	142
mean	188	163	184	145	219	199	174
$\pm$ SEM	23.0	24.2	20.4	14.1	27.3	33.2	21.5

5-HT Concentration ng.ml^{-1}

Storage time in days
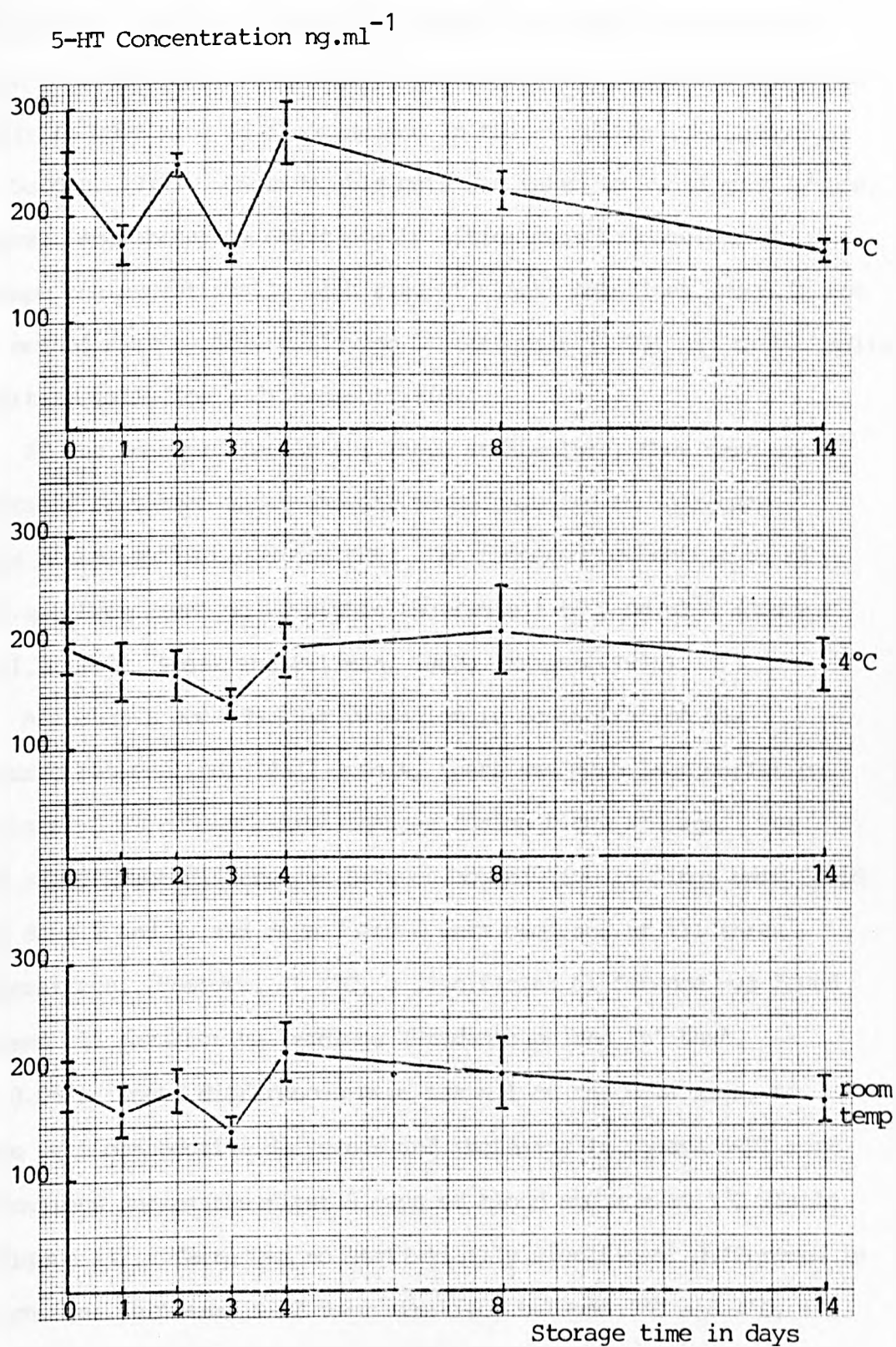
Figure 11   CHANGES IN PLASMA 5-HT CONCENTRATION ($\pm$ SEM) DURING
THE STORAGE OF BLOOD

to 0.01M. A reduction in concentration by only 25% markedly reduces fluorescence, and, as only small volumes are added, care must be taken to avoid loss. Conversely, increasing the concentration would result in quenching of fluorescence in the standards (Shellenberger and Gordon, 1971). Considerable care was taken to avoid such errors, however, and therefore other possibilities must be considered. Perhaps the exceptionally high reagent blanks sometimes shown in the OPT method used by Somerville and Hinterburger (1975) may have resulted in artificially low 5-HT concentrations.

If the measured levels are taken as absolute, they were considerably less than those thought to be required to aggregate human platelets in blood and PRP. The required concentration of 5-HT has been found to be within the range 1 to 5 μg.ml^{-1} (Swank et al., 1963; Swank and Fellman, 1967; Takano, 1975).

Although a considerable variation in concentration was measured between individual donors, there was much less variation in plasma 5-HT concentration changes during blood storage. The only significant differences between consecutive readings were found when days 2 and 3, and days 3 and 4 were compared at all three temperatures. However, at 1°C, a significant difference was found between all consecutive readings (Students paired 't' test, $p \geq 0.05$). These differences were not all of the same sign, as there were consecutive decreases and increases in plasma 5-HT concentrations during the first 5 days of blood storage at 1°C (Table 7, Figure 11). There was no statistically significant difference in the changes in plasma 5-HT concentrations between 1°C and 4°C, 1°C and room temperature, or 4°C and room temperature.

4.2.4 A DISCUSSION OF THE PLASMA 5-HT CONCENTRATIONS

The small changes in plasma 5-HT concentrations were not surprising as the mean concentration of normal 5-HT levels in human platelets has been estimated to be only 50 ng.10^8 platelets (Baumgartner, 1970). This is about one half the average platelet count in one ml of whole blood (2 x 10^8 platelets. ml^{-1}), and therefore, would only allow for a mean total difference of about 100 ng.ml^{-1} (although individual variation could be higher) assuming all the platelets 5-HT was released. Because erythrocytes have a capacity for 5-HT storage only about one tenth that of platelets (Franzen and Eysell, 1969), and remain viable for considerably longer during blood storage, they are unlikely to contribute to plasma 5-HT concentrations during the early stages of storage.

The 5-HT found in platelets, associated with platelet membranes is unlikely to be liberated as 5-HT, but rather as 5-hydroxytryptophol and 5-hydroxyindole acetic acid. This is due to the presence of monoamine oxidase and both alcohol and aldehyde dehydrogenases in platelets. However, the 5-HT in platelet storage granules is protected from these enzymes, though small quanitities may be converted on liberation during the release reaction (Pletscher, 1968).

Although there was no statistical difference in the changes in plasma 5-HT concentrations between the three storage temperatures, the pattern of change appeared to be most pronounced at 1°C during the first 4 days of blood storage (Figure 11). 5-HT degradation was measured in plasma and found to be less than 10% during the first 24 hours, at both 1°C and room temperature, though it was higher at room temperature. After 3 days storage, the degradation of 5-HT in

plasma was even less. This gradual degradation lends support to the results of Pletscher (1968) who found that 60% to 80% of endogenous 5-HT in plasma remained unchanged. It was, therefore, difficult to account for the observed declines in the plasma 5-HT concentrations by plasma degradation alone, particularly as the decline during the first 24 hours was most apparent at 1°C. It is possible that this decline was due to the uptake of 5-HT into platelets, and that the ADP produced as a result of the ATP consumed in this uptake, was responsible for platelet aggregation, as suggested by Baumgartner (1970). However, there is no reported evidence that cold as an aggregating stimulus requires 5-HT, and there was no statistically significant difference in the changes in plasma 5-HT concentrations between any of the three storage temperatures. Indeed, the changes in 5-HT concentration at 4°C were less than those at room temperature, at which no measurable platelet aggregation occurred. Also, the rate of 5-HT uptake into platelets has been found to decrease when the temperature is decreased (Maynert and Isaac, 1968) possibly due to reduced platelet viability (Hardeman and Heynens, 1974b). Therefore, if any importance is to be attached to the greater decrease in 5-HT concentration during the first 24 hours, at 1°C, it is likely to be mainly due to another factor, perhaps increased binding to plasma proteins, which would have been removed in the assay.

An increase in the plasma 5-HT concentration after 2 days storage may have been due to release from platelets, and this was also particularly apparent at 1°C. However, the SFP had reached its measurable maximum after only 24 hours at 1°C. Therefore, if 5-HT release is a marker for platelet release, then cold induced during the first 24 hours did not appear to be associated with platelet

release. After 3 days blood storage, a marked decrease in plasma 5-HT concentrations was measured in all samples at all three temperatures, but particularly at 1°C. This again suggested the binding of released 5-HT to plasma proteins or perhaps to erythrocytes. The increased plasma 5-HT concentrations measured at 4 days blood storage, and again most apparent at 1°C, may have been due to general platelet degradation.

A gradual decrease in the plasma 5-HT concentration after 4 days or 8 days storage may have been due to slow degradation or binding of the released 5-HT. Daily samples were not taken after 4 days blood storage, because platelets were not likely to contribute to the plasma 5-HT pool after this time, particularly at the lower temperatures.

Although the changes in 5-HT concentrations during blood storage were more apparent at 1°C, they also occurred at 4°C and room temperature, indeed seemingly least so at 4°C. Because platelet aggregation did not occur at room temperature, there did not appear to be a direct relationship between the plasma concentration of 5-HT and platelet aggregation measured by the SFP technique.

4.4.3.1 ELECTRON MICROGRAPHS OF PLATELET AGGREGATES FORMED IN
STORED BLOOD AT 1°C

Electron micrographs were taken of aggregates collected on the discs during the measurement of SFP. The discs were prepared for microscopy as follows. The discs were fixed in 3% glutaraldehyde (in 0.16M phosphate buffer, pH 7.1) for about 1 hour and the aggregate mass lifted off the disc and fixed for a further 2 to 3 hours. The aggregate mass was then rinsed in the same phosphate buffer

and post fixed in 1% osmium tetraoxide (in phosphate buffer) for 45 minutes. After rinsing in distilled water for 5 minutes, the aggregate mass was then dehydrated in 30% acetone for 10 minutes. This was followed by block staining in 2% uranyl acetate (in 30% acetone) for 15 minutes, and then dehydration continued in 60% acetone for 10 minutes, 90% acetone for 10 minutes, and 100% acetone (three times) for 10 minutes. The aggregate mass was then impregnated in three changes of Spurr's resin and polymerised at 60% for 24 hours. Sections were cut on an ultramicrotome (silver sections) and viewed in a Phillips electron microscope (E.M. 200) at 60 KV.

The final form of the platelet aggregates may not have been the original form because of deformation of the aggregates in the SFP apparatus. Nevertheless, marked morphological differences can be seen between platelets stored for 1 day and those stored for 4 days at 1°C. After 24 hours of blood storage, most of the platelets appeared to be morphologically intact but they were deformed (cf. a normal unactivated platelet in chapter one). The aggregates seemed to be only loosely packed, and the dense granule content of individual platelets appeared to be centralised (Figures 12 and 13). After 4 days blood storage at 1°C, the aggregates were more densely packed and the grossly deformed platelets were surrounded by a mass of amorphous material. This was probably platelet debris from degraded platelets. Although dense bodies were still apparent, they were much fewer in number (figures 14 and 15). The electron micrographs demonstrate that platelet degradation seemed to have occurred more extensively after 4 days blood storage at 1.°C than after 24 hours. Nevertheless, the granular content of several platelets after 24 hours do appear to have been discharged and might have been expected
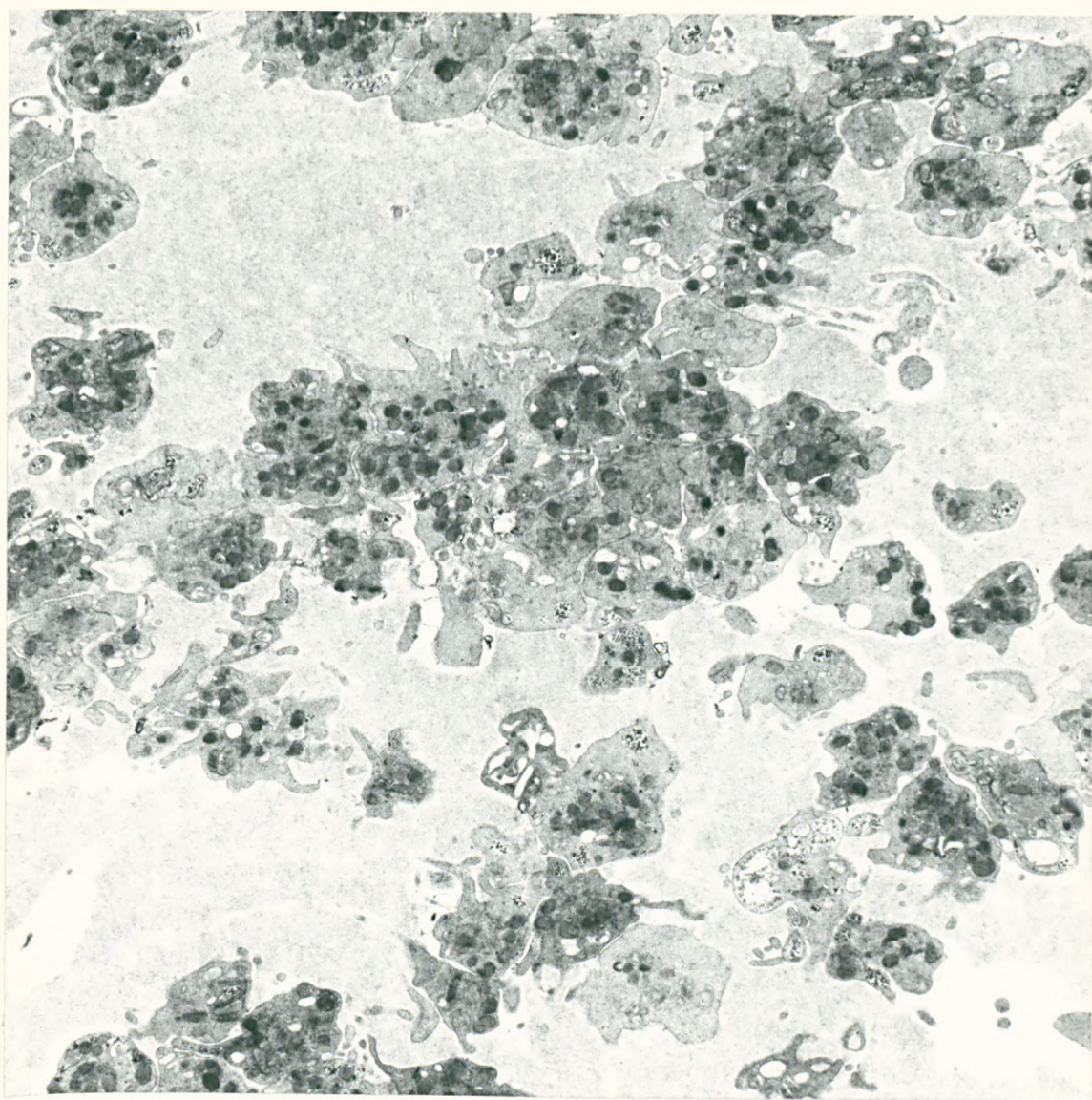
FIG. 12 Platelet aggregates from blood stored for 24 hours at 1^{O}C
(x 8,900)
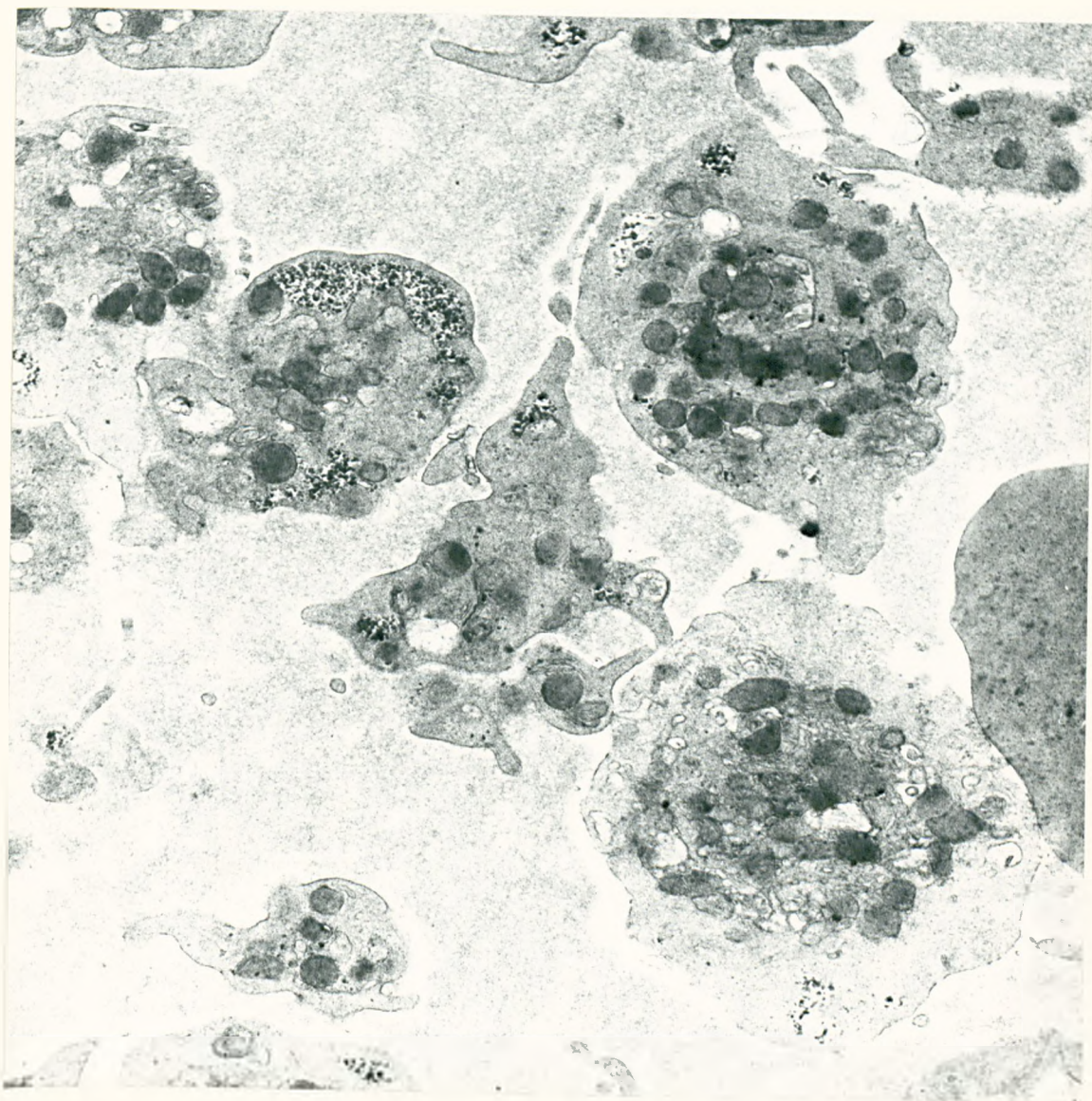
FIG. 13   Platelet aggregates from blood stored for 24 hours at 1°C
(x 19,700)
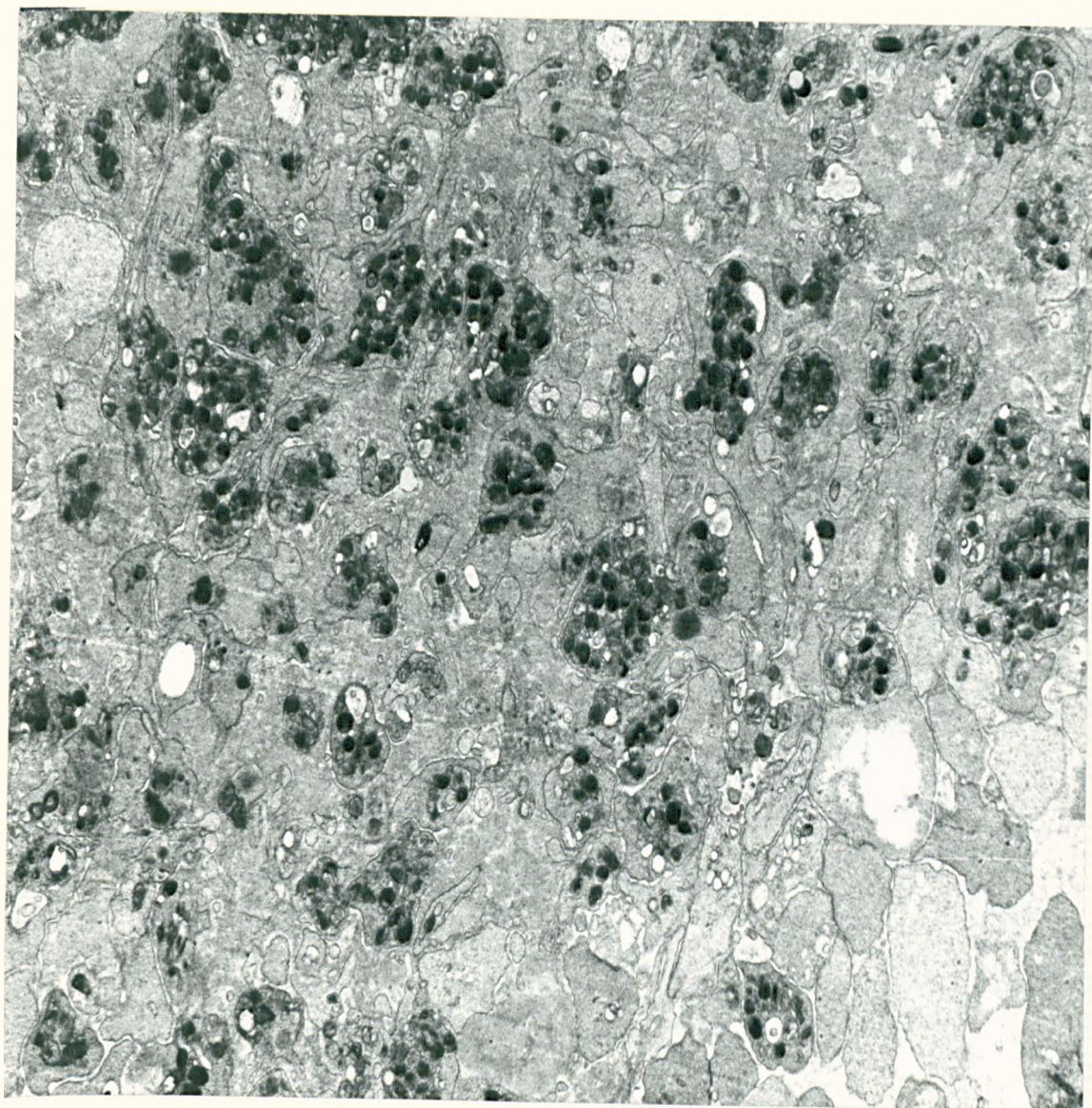
FIG.14 Platelet aggregates from blood stored for 96 hours at 1ºC
(x 8,900)
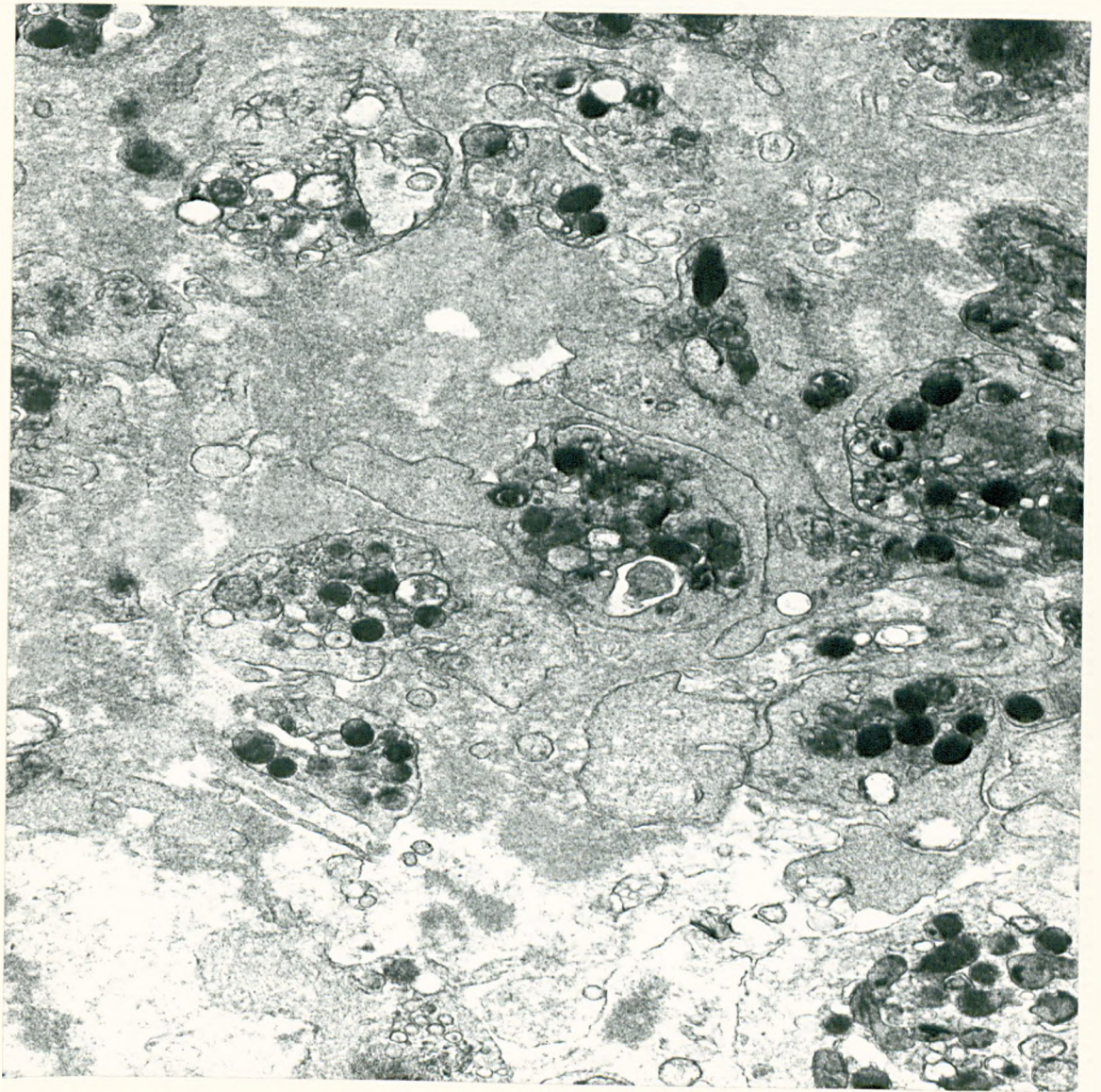
FIG. 15 Platelet aggregates from blood stored for 96 hours at 1ºC
        (x 19,700)

to cause an increase in the plasma concentration of 5-HT. This did not usually occur (Table 7) and suggest that any released 5-HT from platelets is rapdily removed. If this is so, then the measurement of plama 5-HT is probably not a suitable marker for platelet release during the early stages of blood storage.

CHAPTER FIVE

CHANGES IN ADP, ATP AND PLASMA HAEMOGLOBIN CONCENTRATIONS

DURING BLOOD STORAGE AT 1°C, 4°C AND ROOM TEMPERATURE

5.1 ADP CONCENTRATIONS DURING BLOOD STORAGE

ADP is a nucleotide of primary biological importance, as an immediate precursor of polynucleotides, and, with ATP, as a participant in oxidative phosphorylation. ADP is the molecule that captures, in the form of high energy phosphate bonds, some of the energe resulting from catabolic processes. The resultant ATP passes on this evergy to drive processes requiring energy, resulting in ADP and AMP formation.

ADP is a potent platelet aggregating agent and a mediator of secondary aggregation by several other aggregating agents in-vitro (see chapter one). Its role in platelet aggregation during the storage of whole blood, therefore, may be important as a consequence of platelet release. However, platelet aggregation in anticoagulated whole blood is associated with a loss of potassium ions from erythrocytes (Swank and Porter, 1963), a phenomenon which also occurs with ADP release from erythrocytes (Harrison and Mitchell, 1966). This ADP is thought to play an important role in platelet aggregation in haemostasis (Gaarder et al., 1961). Also drug inhibition of ADP release from erythrocytes has been shown to increase bleeding times from a small hole in a plastic tube circuit containing circulating blood (Born et al., 1976). The increase bleeding times were due

to slower platelet activation, and, therefore, an important contribution to platelet aggregation in blood may be ADP released from erythrocytes.

5.1.1 ASSAY PROCEDURE FOR ADP

It is difficult to measure absolute values of ADP in blood and plasma, because it is rapidly degraded in plasma at a rate calculated to be about 0.1 nmol min^{-1} ml^{-1} (Haslam and Mills, 1967). Also, the best available method, enzyme assay, is not specific for ADP as it is affected by all nucleoside diphosphates (Haslam, 1964). However, guanosine diphosphate (GDP), inosine diphosphate (IDP) and uridine diphosphate (UDP) react at considerably lower rates, and since their normal blood concentrations are very low compared with ADP (D'Souza and Glueck, 1977), they are unlikely to significantly interfere.

The method used was as described by Adam (1970), based on the enzymic conversion by pyruvate kinase of ADP (in the presence of phosphoenolpyruvate) to ATP and pyruvic acid, the latter in quantities equivalent to the available ADP. In the presence of lactate dehydrogenase, this pyruvate is reduced to lactic acid with NADH, the amount used of which is equivalent to the pyruvate and hence the ADP concentration. NADH was determined in a spectrophotometer by the absorption change at 366 nm against an air blank. The procedure depended upon deproteinisation of the blood or plasma sample using 0.5 M, perchloric acid (final concentration). Excess perchlorate was removed using potassium carbonate, which precipitates out as potassium perchlorate in the cold, and separated by centrifugation.

Care was taken to maintain a temperature around 2°C during separation since potassium perchlorate becomes increasingly soluble as the temperature rises and would interfere with the assay. Whole blood and PFP were assayed identically.

5.1.1.1 CALCULATION

The concentration of ADP was calculated using the general formula

$$\text{concentration} = \frac{\text{change in absorption} \times \begin{array}{c}\text{molecular}\\\text{weight of}\\\text{ADP}\end{array} \times \text{final volume}}{\begin{array}{c}\text{extinction coefficient}\\\text{of NADH at 366 nm}\end{array} \times \begin{array}{c}\text{cuvette light}\\\text{path length}\end{array} \times \begin{array}{c}\text{sample}\\\text{volume}\end{array}} \quad mg.L^{-1}$$

This was corrected for dilution taking into account the specific gravity and water content of blood and plasma, though these made only minimal differences to the overall concentrations (specific gravity of blood is about 1.06, and of plasma, about 1.03; the water content of blood is about 80% and of plasma, about 90%).

5.1.1.2 ACCURACY OF THE ADP ASSAY

The assay was tested using a known concentration of ADP added to buffer and run through the procedure. Calculation of the change in absorption gave a mean result greater than 95% of the original concentration. This demonstrated that the use of the formula was suitable, and that the use of standards for comparison were unnecessary.

5.1.2 PLASMA AND BLOOD ADP CONCENTRATION RESULTS (Tables 8, 9, 10, Figures 16, 17, 18)

ADP concentrations were measured daily during the first week

TABLE 8

CHANGES IN ADP CONCENTRATION DURING BLOOD STORAGE AT 1°C, 4°C AND ROOM TEMPERATURE

1°C

a) Whole Blood (ug.ml^{-1})

	0	1	2	3	4	8	14	days
	21.8	–	24.0	–	15.0	24.5	–	
	25.5	–	41.0	–	24.5	–	–	
	30.0	–	35.0	–	27.0	–	–	
	17.1	18.9	20.3	16.5	12.7	–	3.3	
	22.1	–	28.2	25.3	20.6	29.1	17.5	
	24.8	32.3	35.4	26.9	20.7	24.5	33.7	
	22.8	32.0	30.6	25.5	20.1	29.2	33.3	
	34.0	50.0	39.4	38.8	39.8	47.6	21.1	
	28.2	33.7	31.6	25.5	17.0	29.9	33.3	
mean	25.1	33.4	31.7	26.4	21.9	30.8	23.7	
± SEM	1.7	4.5	2.3	2.9	2.7	3.5	5.0	

b) PFP (ug.ml^{-1})

	10.5	0	13.6	14.8	2.4	0	11.6
	0	0	19.0	0	3.1	0	3.4
	2.4	0	0	7.5	0	1.4	2.9
	0	7.5	6.8	0	0	3.4	5.1
	3.1	5.4	0	9.5	18.7	0	2.1
	5.9	2.4	6.8	0	0.3	5.1	1.0
mean	3.7	2.6	7.7	5.3	4.1	1.7	4.3
± SEM	1.6	1.3	3.1	2.6	3.0	0.9	1.6

c) Intracellular level (calculated from blood – PFP means)

	21.4	30.8	24.0	21.1	17.8	29.1	19.4

TABLE 9

ADP concentrations (cont)

4°C

a) Whole Blood

	0	1	2	3	4	8	14 days
	19.9	–	20.8	–	34.1	24.2	41.2
	18.1	–	17.0	–	27.0	22.9	21.5
	14.4	–	25.7	–	29.5	23.7	24.2
	16.3	–	20.0	–	17.0	18.5	26.1
	20.0	–	53.7	–	27.9	25.7	34.4
	26.0	17.0	20.3	–	18.9	28.6	–
	10.2	5.7	12.3	19.4	28.8	19.4	–
	17.1	14.0	14.9	15.1	13.0	–	11.6
	22.1	19.4	21.0	22.9	22.9	26.6	10.9
	24.8	22.8	23.1	23.1	19.7	30.3	38.5
	22.8	22.4	21.4	23.8	17.7	30.9	34.0
	34.0	36.4	42.8	37.4	39.1	36.0	42.8
mean	20.5	19.7	24.4	23.6	24.6	26.1	28.6
± SEM	1.8	3.6	3.4	3.1	2.3	1.6	3.7

b) PFP

	0	1	2	3	4	8	14 days
	7.6	0.3	0	–	9.3	2.6	0.8
	3.8	0	0	–	0.9	3.8	0
	1.5	0	6.2	7.8	9.6	7.6	–
	0	0	16.7	2.4	0.7	0	1.7
	2.4	0	0	10.9	2.7	2.4	3.4
	0	1.7	7.1	1.0	0.7	6.8	7.3
mean	2.6	0.3	5.0	5.5	4.0	3.9	2.6
± SEM	1.2	0.3	2.7	2.3	1.8	1.2	1.3

c) Intracellular Level (calculated)

0	1	2	3	4	8	14 days
17.9	19.4	19.4	18.1	20.6	22.2	23.9

TABLE 10

ADP Concentrations (cont)

Room Temperature

a) Whole Blood

	0	1	2	3	4	8	14 days
	18.1	–	12.7	–	25.6	34.8	20.8
	14.4	–	25.3	–	34.4	26.9	24.9
	16.3	–	15.9	–	24.6	28.0	11.3
	20.0	–	49.5	–	37.8	44.6	29.1
	26.0	14.0	28.6	–	26.9	44.4	0.4
	17.1	8.7	24.1	25.0	12.7	–	11.8
	22.1	13.1	18.3	25.8	24.1	36.3	19.5
	24.8	16.7	20.4	25.8	23.8	53.0	42.2
	22.8	15.3	20.1	22.1	22.8	47.9	29.6
	34.0	28.6	44.9	46.9	53.0	64.6	28.9
mean	21.6	16.1	26.0	29.1	28.6	42.3	21.9
$\pm$ SEM	1.8	2.7	3.8	4.5	3.4	4.1	3.7

b) PFP

	0	1	2	3	4	8	14 days
	7.6	0	0	–	5.3	0.9	3.4
	3.8	0	0	–	0	2.3	2.8
	10.5	0	3.7	15.2	0	1.0	14.6
	0	0	16.7	1.0	3.1	0	2.0
	2.4	0	0	8.5	8.2	0.7	3.0
	0	0	4.4	0	0	3.4	5.1
mean	4.1	0	4.1	6.2	2.8	1.4	5.2
$\pm$ SEM	1.7	0	2.6	3.6	1.4	0.5	1.9

c) Intracellular Level (Calculated)

	0	1	2	3	4	8	14 days
	17.5	16.1	21.9	22.9	25.8	40.9	16.7

ADP Concentration μg.ml^{-1}
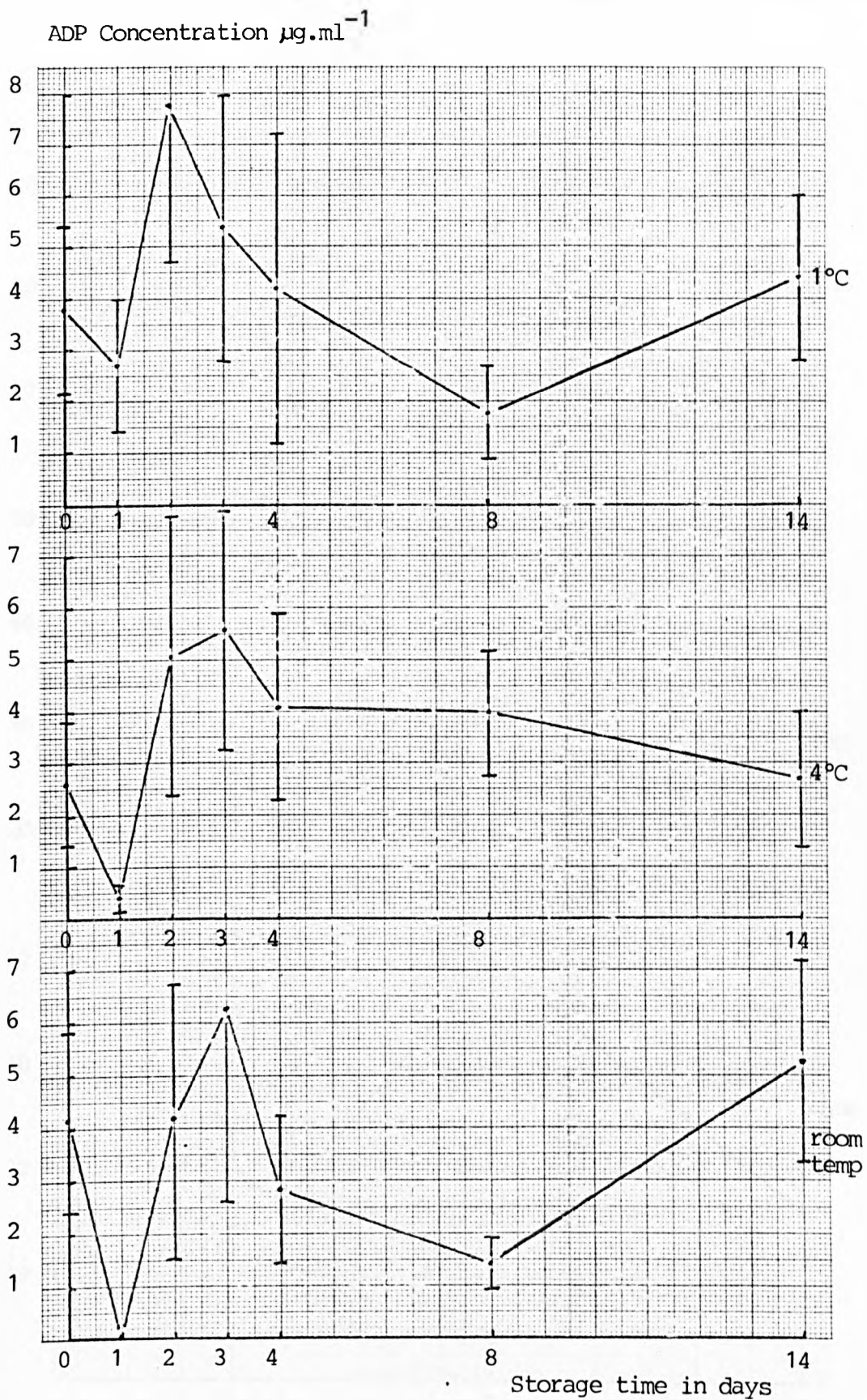
Figure 16   CHANGES IN PLASMA CONCENTRATIONS OF ADP (+ SEM) DURING
             BLOOD STORAGE

ADP Concentration µg.ml^{-1}

Storage time in days
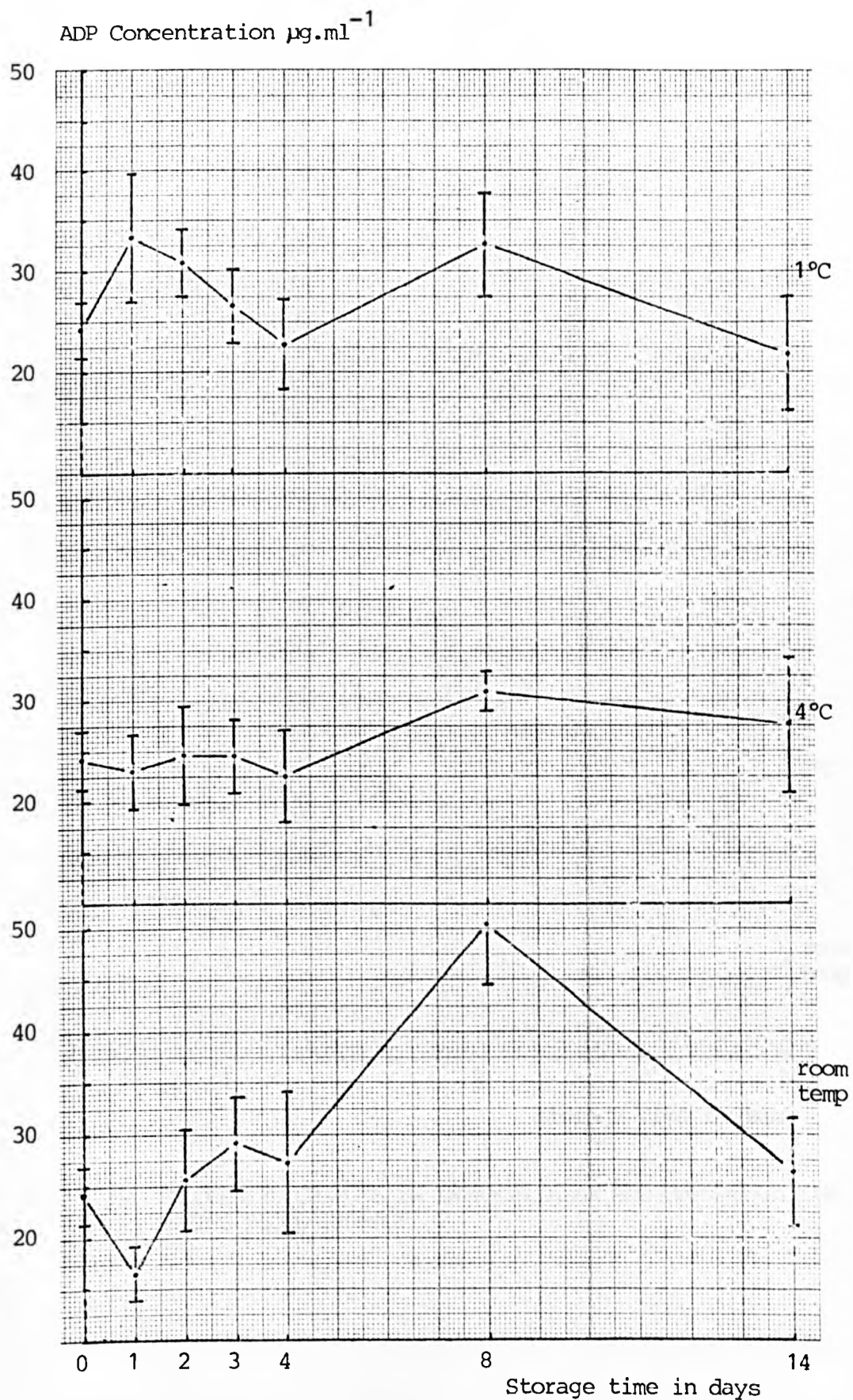
Figure 17   CHANGES IN WHOLE BLOOD CONCENTRATIONS OF ADP ($\pm$ SEM)
DURING BLOOD STORAGE

ADP Concentration μg/ml^{-1}

Storage time in days
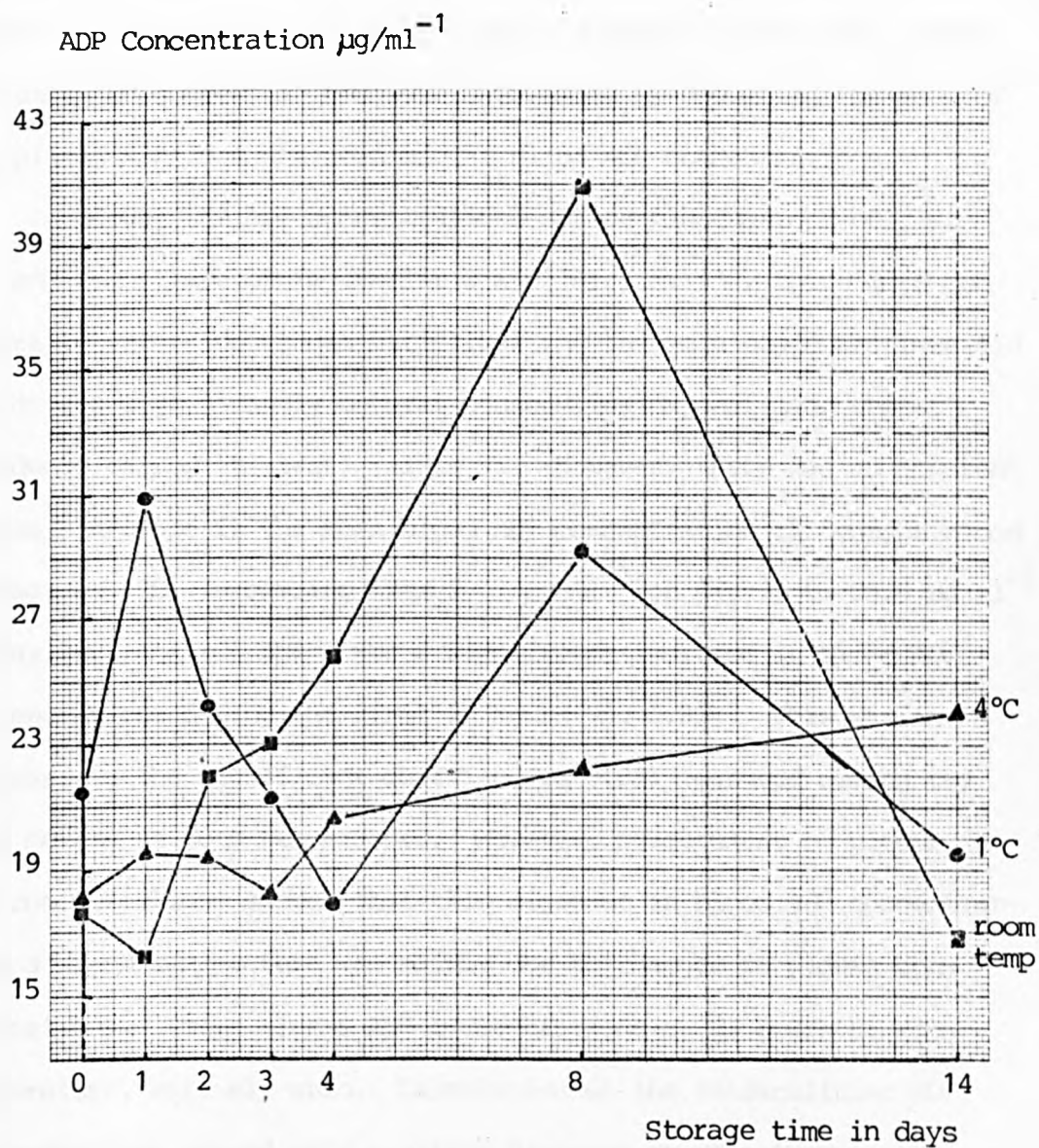
Figure 18 CALCULATED CHANGES IN INTRACELLULAR ADP CONCENTRATIONS
DURING BLOOD STORAGE

of storage and then after 8 and 14 days.

Increases in blood ADP concentrations are most likely to be a consequence of ATP consumption. ADP leaking from erythrocytes or platelets would have been measured as plasma ADP concentrations. To give an impression of events occurring within blood cells, intra-cellular ADP concentrations were calculated by deducting the mean of the plasma ADP from the mean of the blood ADP concentrations.

In blood, the ADP concentrations showed fluctuations only at 1°C and room temperature (tables 8 and 10). At 4°C, there were con-siderable inter-individual variations and the mean concentrations did not differ significantly between consecutive days of measurement (Students paired 't' test) (table 9). However, there was an apparent gradual increase in the mean blood ADP concentration throughout blood storage at 4°C, increasing from 20.5 μg ml^{-1} on day 1, to 28.6 μg ml^{-1} on day 14. At 1°C, there was a significant increase in blood ADP concentrations during the first 24 hours ($p \leqslant 0.05$). This was in contrast to the significant decrease ($p \leqslant 0.05$) measured during the same period at room temperature. However, measurement of plasma ADP concentrations showed that this decrease in blood ADP concentrat-ions at room temperature was mainly due to changes in plasma con-centrations. These plasma ADP concentrations at 24 hours at room temperature, were all zero. Calculation of the intracellular ADP concentrations showed only a slight decrease at room temperature, a slight increase at 4°C, but still a large increase at 1°C during the first 24 hours of blood storage (Figure 18).

There was considerable variation in the plasma ADP concentrations between individuals during blood storage. With the exception of the decrease in plasma ADP concentration during the first 24 hours at

room temperature, there was no statistically significant difference between consecutive readings at any temperature. There was also no significant difference in the rate or extent of ADP release into plasma between the storage temperatures (Students unpaired 't' test), all showing increased concentrations mainly between 48 hours and 96 hours. This increase reached an apparent peak after 48 hours at 1°C, but only after 72 hours at both 4°C and room temperature.

The mean values for blood ADP concentrations gradually declined after 2 days at 1°C, a significant decrease being measured between days 2 and 3, and between days 3 and 4. However, a significant increase in blood ADP concentrations then occurred between days 4 and 8 (p $\leqslant$ 0.05) followed by another decrease between days 8 and 14. This increase was also measured between days 4 and 8 at room temperature and again was statistically significant. However, in this group, the increase was a continuation of a significant increase measured between days 1 and 2, and days 3 and 4. Hence, the two patterns of blood ADP concentration changes at 1°C and room temperature differed. With the exception of the first 24 hours, the pattern of change in blood ADP concentrations at all storage temperatures was the same for the calculated intracellular ADP concentrations. The difference in blood ADP concentration changes was significant (Students unpaired 't' test p $\leqslant$ 0.05) between all the storage temperatures at 24 hours, and also at 48 hours between 1°C and room temperature, and between 4°C and room temperature. Between 48 and 72 hours, the change in blood ADP concentration was significantly different between 1°C and 4°C, and between 1°C and room temperature, but not between 4°C and room temperature. However, only between 4°C and room temperature were

any further significant differences seen in blood ADP concentration changes, and these were from days 4 to 8, and from days 8 to 14.

During the first 4 days of blood storage, therefore, a contrasting pattern can be seen between blood ADP concentration changes at 1°C and at room temperature with the changes at 4°C being somewhere inbetween.

The mean ADP concentration in whole blood immediately after collection was $24.1 \pm$ (SEM) $1.6 \mu g \ ml^{-1}$, and the range was 10.2 to $36.4 \mu g \ ml^{-1}$. This was lower than the "normal" range reported to be from 21 to 48 $\mu g \ ml^{-1}$ by Laudahn (1959) (though of the 19 samples in the range, 14 had a concentration over 20 $\mu g \ ml^{-1}$).

The possibility that the increases in plasma ADP concentrations during the first few days of blood storage were due to activities in platelets, was examined by performing ADP assays during the storage of platelet rich plasma (PRP) at 4°C (Table 11a). PRP was prepared by centrifugation of blood at 100 g for 10 minutes at 4°C, followed by careful removal of the PRP, under aseptic conditions, into sterile polystyrene jars for storage. There was considerable variation in the results with an apparent, though statistically insignificant, mean increase in the plasma ADP concentration after 24 hours storage. There was a significant decrease in the plasma ADP concentration between days 1 and 2 (p $\leqslant$ 0.05) followed by no significant change between days 2 and 3, and between days 3 and 4, and then (in those samples that were measured) a decline to zero after 8 days of PRP storage. Results with PRP must be treated with caution, as the PRP preparation procedure may be a stimulus for platelet release (Barrett et al., 1976), and may have accounted for the apparent increase in ADP concentration during the first 24 hours. However,

TABLE 11

a) Changes in Plasma ADP Concentrations During Storage of PRP at 4°C

| | | Storage time in days | | | | | Platelet Count |
	0	1	2	3	4	8	$(x\ 10^9.L^{-1})$
	3.2	8.1	4.3	5.5	0.4	0	293
	3.0	11.3	2.1	–	1.2	–	247
	5.4	3.7	0	10.2	17.0	–	261
	7.1	16.0	5.1	5.1	0	0	324
	5.8	–	5.8	1.0	15.3	0	340
	2.1	0.3	5.1	13.2	0	0	291
	3.1	16.3	3.7	3.7	0	0	308
mean	4.2	9.3	3.7	6.5	4.8	0	295
± SEM	0.7	2.7	0.8	1.8	2.9	0	12.4

b) SFP Changes in PRP Stored at 1°C

| | | Storage Time in Days | | | | Platelet Count |
	0	2	4	8	14	$(x\ 10^9.L^{-1})$
	7	11	55	600	600	329
	14	23	26	110	600	193
	11	600	600	600	600	244
	10	110	285	600	600	261
mean	11	186	241	478	600	257
± SEM	1.4	140	133	123	0	28

the overall pattern of plasma ADP concentrations during PRP

storage, suggests that platelets may have contributed to plasma

ADP concentrations during the first few days of blood storage at

4°C. The higher mean plasma ADP concentrations in stored PRP compared

with stored blood may have been due to the higher platelet count of

PRP.

5.1.3 DISCUSSION OF THE ADP RESULTS

During the first 24 hours of blood storage, the concentration of

ADP in plasma decreased particularly at room temperature. This may

be explained as being due to the more rapid ADP degradation at higher

temperatures. The different rates of ADP degradation were confirmed

by comparing the breakdown rates of ADP added to plasma at 1°C with

those at room temperature. ADP, dissolved in 0.05M phosphate buffer,

pH 7, was added to fresh blood plasma under aseptic conditions to

give a final concentration of 100 μg ml^{-1}. The concentration of ADP

was measured after 24 hours and was found to be reduced by a mean of

91% at room temperature and 58% at 1°C. This rate of degradation was

still apparent in plasma prepared after 48 hours and stored with added

ADP, for 24 hours.

If ADP were being released from erythrocytes during this period,

then it must have been at a slower rate than the rate of ADP degrad-

ation. However, whether it was due to a reduced rate of degradation

or to an increased rate of ADP leakage from erythrocytes, the concen-

tration of plasma ADP was generally higher at 1°C than at 4°C or

room temperature after 24 hours blood storage.

The subsequent increase in plasma ADP concentrations occurred

at all three temperatures, and, with the exception of room temperature between 24 and 48 hours, seemed to generally follow increases in intracellular ADP. The plasma concentration then decreased as ADP was degraded. Perhaps the sharp increase in plasma ADP concentrations at room temperature, and to an extent at 1°C and 4°C, during the 24 to 48 hour period was due to the dephosphorylation of ATP during the first 24 hours (see Figure 23).

The plasma ADP concentration in some samples at 24 hours at 1°C, and on occasions throughout blood storage at all temperatures, reached a value of about 10^{-5}M (4.3 μg.ml^{-1}). Platelet aggregation has been shown to occur following the exposure of platelets in PRP to ADP concentrations as low as 3×10^{-8}M (Takano, 1975). However, higher ADP concentrations are usually required. Born (1962) found that 10^{-6}M was the lowest concentration that would induce irreversible platelet aggregation in PRP, and Swank et al. (1963) observed that ADP concentrations in the range 1 to 5 μg.ml^{-1} were necessary to aggregate platelets in whole blood in-vitro. This concentration, however, caused only a loose reversible aggregation, that required the presence of other factors such as 5-HT or adrenaline before the aggregates became tightly bound. Indeed, increases in the concentration of ADP alone up to 15 μg.ml^{-1} had no further affect on the extent of platelet aggregation in blood (Swank and Fellman, 1967).

Using the absolute values measured in plasma it appears that the ADP concentrations were not high enough to initiate platelet aggregation. It should be born in mind, though, that the high rate of ADP degradation was a problem in measuring absolute values. Very many more measurements, perhaps hourly, would be required to reduce this problem. However, this was impractical. It is possible that

the measurement of plasma ADP was not a suitable indicator of the effect of ADP as it did not account for any locally high concentrations. This might have occurred in stagnant blood in which sedimentation results in the concentration of platelet populations. This hypothesis may also be forwarded for other released factors such as 5-HT and catecholamines and could be prevented by constant, gentle agitation of stored blood, thus reducing the close proximity of platelets. The value of agitation in reducing platelet aggregation during CPD anticoagulated blood storage was suggested by Arrington and McNamara (1975). However, agitation also destroys platelets and breaks down aggregates, so that the extent of this reduction is now in doubt (Gervin et al., 1978). The effect of many aggregating agents is thought to be the relase of ADP following the consumption of platelet ATP (Haslam, 1964) possibly due to the stimulation of ATPase (O'Brien, 1964b). The importance of ADP has been shown particularly by the use of enzymes to remove released ADP, such as creatine phosphate/creatine phosphokinase (Izrael et al., 1974; Tschopp and Baumgartner, 1976), apyrase (Wang et al., 1975) and pyruvate kinase/phosphoenolpyruvate (Haslam, 1964). A possible exception however, is cold, which has been shown to aggregate platelets without the mediation of released ADP (Kattlove and Alexander, 1971). Indeed, ADP is thought not to be able to induce platelet aggregation in the cold (O'Brien, 1962). However, it has also been observed that platelets stored as PRP for 10 days at 5°C showed a response to high ADP concentrations that was above that induced by cold (Shively et al., 1970), though the authors considered that platelets exposed to temperatures between 0°C and 4°C aggregate "spontaneously".

To complicate the problem further, any ADP involved in aggregation

may be bound to platelets and not assayed as plasma ADP, but would be included in the calculation of intracellular concentrations. It was shown that intracellular ADP concentrations were higher after 24 hours at 1°C than at 4°C or room temperature (at which a decline occurred) (Figure 18). It was assumed that intracellular ADP concentration increases were due to ATP consumption, and indeed at 1°C this appeared to be so (Figure 24). However, ADP consumption was also observed equally at 4°C and room temperature, without the expected resultant increases in ADP. This suggested more rapid breakdown which would occur more readily in plasma. Therefore, the "intracellular" ADP levels at 24 hours may be a measure of bound ADP which was highest at 1°C, and lowest at room temperature as would be expected with the different rates of degradation. In an attempt to further elucidate the role for ADP, specific inhibitors were employed.

5.1.4 INHIBITION OF ADP AND ADP RELEASE

Chlorpromazine, used clinically as a tranquilliser, is known to inhibit platelet function in-vitro, particularly the release of ADP (Warlow et al., 1976), in the platelet release reaction (Born et al., 1976). It is not thought to interfere with the platelet aggregation mechanism (Born, 1967) and therefore, was a suitable candidate for judging the importance of released ADP in platelet aggregation during blood storage in the cold. Apyrase is an enzyme that breaks down ADP, thus preventing its activating effect on platelets (Wang et al., 1975; Born et al., 1976) and was used to complement the studies with chlorpromazine.

5.1.4.1 ADDITION OF CHLORPROMAZINE

Chlorpromazine was prepared fresh in 0.05M phosphate buffer
pH7, and suitably diluted so that the addition of 1 ml to 20 ml
of blood gave the required concentration. A concentration of 2.8
x 10^{-4}M was used which would inhibit the release reaction in-vitro
(Born et al., 1976). 20 ml blood samples were taken from blood
packs by drainage into sterile polystyrene "Universal" jars
under aseptic conditions. The drug solution was sterilised
by microfiltration through 0.02 μm pore diameter "Millipore" filters.
The containers were stored in the same conditions as the blood packs,
and samples taken aseptically when required. It was not known
to what extent the drug would deteriorate during storage, and so
no further addition of chlorpromazine could be made, lest the
final concentration be higher than required. The drug was tested
on blood stored at 1°C.

5.1.4.2 THE EFFECTS OF CHLORPROMAZINE AT 2.8 x 10^{-4}M ON PLATELET
AGGREGATION IN BLOOD STORED AT 1°C

Using the SFP technique, chlorpromazine was shown to inhibit
platelet aggregation for at least 8 days of blood storage (Table 12a,
Figure 19). The difference between consecutive readings was
significantly reduced by chlorpromazine, (Students paired 't' test,
$p \leqslant 0.05$), though statistical significance could not be calculated
after 24 hours, as the SFP measurable maximum had been reached
for all of the untreated samples. The results seem to demonstrate
that, either platelet aggregation at 1°C depends upon the platelet
release of ADP, or that chlorpromazine at 2.8 x 10^{-4}M interferes

TABLE 12

EFFECTS OF CHLORPROMAZINE AND APYRASE ON PLATELET AGGREGATION IN BLOOD STORED AT 1°C (SFP – mm/Hg)

	0	1	2	3	4	8	14	days
a) Chlorpromazine								
2.8×10^{-4}M	18	20	25	40	65	185	–	
	20	37	80	130	168	220	260	
	30	43	55	60	75	120	190	
	20	53	65	25	25	75	530	
	32	47	48	50	40	64	435	
	42	43	85	80	82	78	198	
mean	27	41	60	64	76	124	323	
± SEM	3.8	4.6	9.0	15.2	20.4	26.5	68.3	
b) Chlorpromazine								
2.8×10^{-5}M	26	275	600	600	600	600	600	
	22	230	600	600	600	600	600	
	42	45	75	145	162	600	600	
	50	62	84	266	275	330	600	
	48	340	600	600	600	600	600	
	42	600	600	600	600	600	600	
mean	38	259	427	469	473	555	600	
± SEM	4.7	83.3	109.7	84.6	81.7	44.9	0	
c) Chlorpromazine								
2.8×10^{-6}M	50	58	112	190	270	360	600	
	48	460	600	600	600	600	600	
	42	600	600	600	600	600	600	
	52	70	177	190	290	600	600	
mean	48	297	372	395	440	540	600	
± SEM	2.2	137.5	132.1	118.4	92.5	60.0	0	

TABLE 12 (cont)

	0	1	2	3	4	8	14 days
d) Apyrase							
50 ug.ml^{-1}	42	60	98	100	110	190	385
	50	45	66	112	162	450	600
	48	270	385	600	600	600	600
	42	600	600	600	600	600	600
	52	75	95	180	220	360	600
mean	47	210	249	318	338	440	557
$\pm$ SEM	2.1	106.0	105.6	116.1	108.5	77.7	43.1

Extent of Aggregation without inhibitor (for all comparisons)

	31	600	600	600	600	600	600

SFP mm/Hg
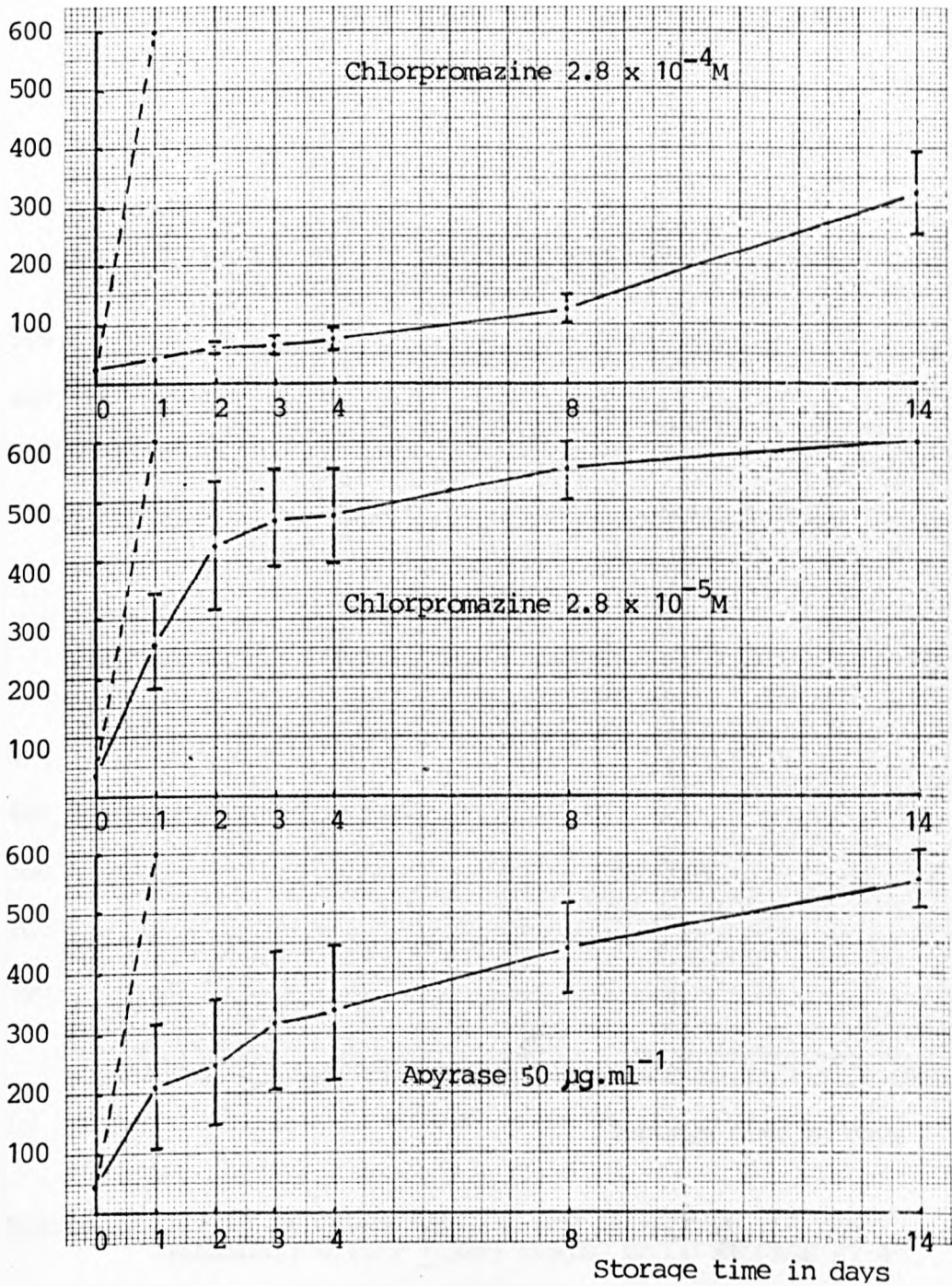
Figure 19   EFFECT OF CHLORPROMAZINE AND APYRASE ON PLATELET AGGRE-
            GATION (SFP + SEM) DURING BLOOD STORAGE AT 1°C

            •—–.   SFP Without Inhibitor
            •—–.   SFP With Inhibitor
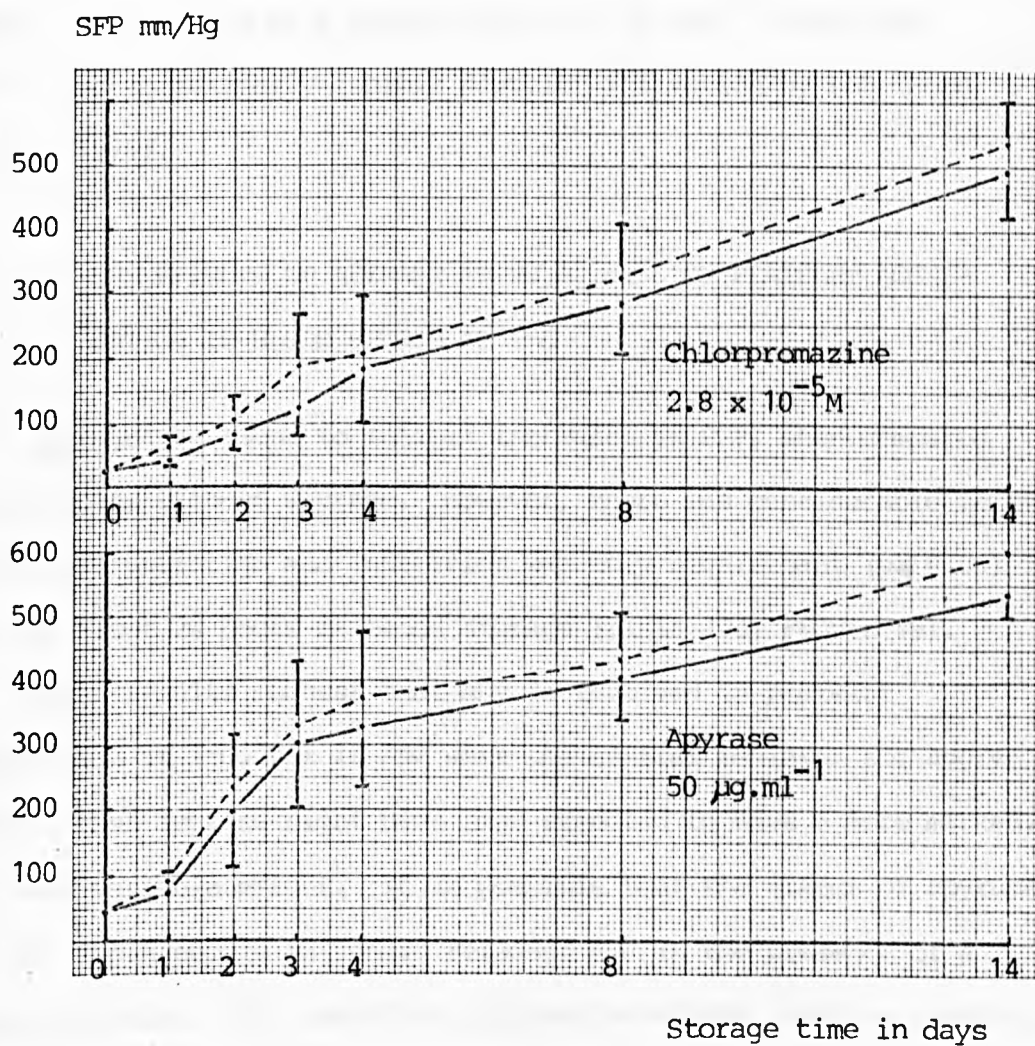
Figure 20   EFFECT OF CHLORPROMAZINE AND APYRASE ON PLATELET
AGGREGATION (SFP ± SEM) DURING BLOOD STORAGE AT 4°C

.---. SFP Without Inhibitor

.___. SFP With Inhibitor

with the aggregation mechanism.

5.1.4.3 ADDITION OF APYRASE

The possibility that platelet aggregation in blood stored at 1°C, depends upon released ADP, was tested using apyrase. Lyophilised potato apyrase was prepared as described for chlorpromazine. It was used at a concentration of 50 μgml^{-1} which has been found to maximally inhibit platelet activation by ADP (Born et al., 1976).

5.1.4.4 THE EFFECTS OF APYRASE AT 50 μg.ml^{-1} ON PLATELET AGGRE-
GATION IN BLOOD STORED AT 1°C

Apyrase was found to inhibit increases in SFP, when compared with samples without apyrase. However, there was considerable inter-individual variation and inhibition was only significant ($p < 0.05$) for the first 24 hours of blood storage (Table 12d, Figure 19).

These results suggest that ADP is involved in platelet aggregation at 1°C, but if ADP were the central mediator for aggregation, then apyrase might have been expected to have a more marked, and less variable effect. It is possible that the effect of apyrase was not rapid enough to remove released ADP in the presence of a strong stimulus. The use of an alternative enzyme, such as creatine phosphate/creatine phosphokinase, which is thought to act more rapidly than apyrase (Izrael et al., 1974) was not tried. However, ADP from erythrocytes may also contribute to platelet aggregation and it may have been this ADP which was inhibited by apyrase.

5.1.5   THE ROLE OF ERYTHROCYTES IN PLATELET AGGREGATION DURING BLOOD
        STORAGE

Platelet aggregation has been shown to occur in PRP in the
cold (Kattlove and Alexander, 1971). This was confirmed by the
author in PRP stored at 1°C in "Universal" jars (Table 11b).
However, the rate of aggregation was not as rapid as in whole blood
at 1°C, and this suggests that factor(s) other than plasma and
platelets contribute to platelet aggregation in blood at 1°C. This
may, in part, be the presence of granulocytes (see chapter one), and
the volume of cells in blood compared with PRP. However, it may
also have been ADP from erythrocytes which accelerated platelet
aggregation in blood at 1°C. This possibility was tested using
chlorpromazine and apyrase.

At low concentrations, chlorpromazine will increase the res-
istance of erythrocytes to haemolysis, and may alter their membranes
so as to diminish or prevent ADP leakage (Born et al., 1976).
Chlorpromazine, prepared as previously described, was used at a
concentration of 2.8×10^{-5}M, which was about half the concentration
required to reduce the platelet release reaction by 50% in-vitro.

5.1.5.1   THE EFFECTS OF CHLORPROMAZINE AT 2.8×10^{-5}M ON PLATELET
          AGGREGATION IN BLOOD STORED AT 1°C AND 4°C

Chlorpromazine inhibited platelet aggregation, as measured by
SFP, at 1°C (Table 12b, Figure 19). However, the difference bet-
ween the SFP's of treated and untreated blood samples were signif-
icant only after the first 24 hours storage (Students paired 't'
test, $p \leqslant 0.05$). After 24 hours, the SFP in the treated samples

increased rapidly. These results suggest that ADP released from erythrocytes may contribute to platelet aggregation in blood stored at 1°C. It was possible that the reduced platelet aggregation in chlorpromazine treated blood might have been due to partial inhibition of the release reaction. To test this possibility, chlorpromazine was used at 2.8 x 10^{-6}M, at which concentration, platelet release was unlikely to be inhibited. Only 4 experiments were conducted using this concentration, but the results (Table 12c) showed an apparently similar effect on platelet aggregation as chlorpromazine at 2.8 x 10^{-5}M. It was therefore unlikely that chlorpromazine at 2.8 x 10^{-5}M inhibited the platelet release reaction, and so reduced platelet aggregation in stored blood at 1°C was probably due to inhibition of ADP release from erythrocytes.

The observations with chlorpromazine at 4°C showed no significant inhibition of platelet aggregation (Table 13a, Figure 20). This suggests that at 4°C, erythrocytes make no significant contribution of ADP.

5.1.5.2   THE EFFECTS OF APYRASE AT 50 μg.ml^{-1} ON PLATELET AGGREGATION
IN BLOOD STORED AT 1°C AND 4°C

It has been shown that in blood stored at 1°C, apyrase reduced the rate of platelet aggregation (section 5.1.4.4). This therefore supports the observations with chlorpromazine at 2.8 x 10^{-5}M and 2.8 x 10^{-6}M,   that platelet aggregation during blood storage at 1°C was partly activated by ADP from erythrocytes.

Like chlorpromazine, apyrase had no significant effect on platelet aggregation in blood stored at 4°C (table 13b, Figure 20)

TABLE 13

EFFECTS OF CHLORPROMAZINE AND APYRASE ON PLATELET AGGREGATION IN BLOOD STORED AT 4°C (SFP - mm/Hg)

a) Chlorpromazine, 2.8×10^{-5}M – SFP with (D) and without (ND) drug

	0		1		2		3		4		8		14		days
	D	ND	D	ND	D	ND	D	ND	D	ND	D	ND	D	ND	
	18	20	23	36	35	60	–	–	36	65	165	145	280	231	
	20	20	25	28	36	60	–	–	130	83	340	190	600	600	
	25	26	45	92	123	185	235	330	600	600	600	600	600	600	
	20	20	38	36	35	28	31	45	86	55	235	460	600	600	
	32	32	58	130	175	255	122	325	100	340	165	–	600	600	
	42	42	55	58	80	50	104	58	141	86	195	233	265	600	
mean	26	27	41	63	81	106	123	189	182	205	283	326	491	537	
+ SEM	3.8	3.6	6.0	16.4	23.7	37.3	42.2	79.7	84.9	90.4	68.7	87.7	69.1	63.1	

TABLE 13 (cont)

EFFECTS OF CHLORPROMAZINE AND APYRASE ON PLATELET AGGREGATION IN BLOOD STORED AT 4°C (SFP - mm/Hg)

b) Apyrase, $50\mu g.ml^{-1}$

	0		1		2		3		4		8		14		days
	D	ND	D	ND	D	ND	D	ND	D	ND	D	ND	D	ND	
	24	26	60	92	320	185	600	430	600	600	600	600	600	600	
	42	42	55	58	50	50	60	58	80	86	255	233	385	600	
	50	50	55	53	60	65	120	112	155	170	265	290	600	600	
	48	48	75	140	110	155	133	180	186	180	265	260	445	-	
	42	42	125	133	560	600	600	600	600	600	600	600	600	600	
	52	52	67	67	75	345	290	600	350	600	450	600	600	600	
mean	43	43	73	91	196	233	301	330	329	373	406	431	538	600	
± SEM	4.2	3.8	10.9	15.5	83.6	85.1	99.6	99.9	93.1	102.4	68.2	76.2	39.8	0	

and suggests that these platelets are not, in part, stimulated by ADP released from erythrocytes. Although only speculative, it is possible that the difference in the rates of platelet aggregation between blood stored at 1°C and at 4°C may have been due to this ADP contribution from erythrocytes.

The apparent role of erythrocytes in platelet aggregation during blood storage at 1°C, was further tested by measuring plasma haemoglobin concentrations as an index of haemolysis.

5.2 PLASMA HAEMOGLOBIN CONCENTRATIONS DURING BLOOD STORAGE

ADP release is not necessarily associated with erythrocyte haemolysis, as it may leak out from erythrocytes that are structurally and metabolically normal. For example, ADP leaks out during reversible erythrocyte deformation, and experimentally, the adhesiveness of platelets to glass was increased in the presence of erythrocytes without evidence of haemolysis (Born, et al., 1976). However, the plasma haemoglobin concentration is increased during blood storage at 4°C in CPD (Limbird and Silver, 1974; Bailey and Bove, 1975; Mishler et al., 1978) which would be associated with the release of erythrocyte constituents including ADP.

5.2.1 HAEMOGLOBIN ASSAY

The method used to estimate haemoglobin in plasma separated from whole blood was as described by Crosby and Furth (1956). This method is based on the catalytic action of heme containing proteins bringing bout the oxidation of benzidine by hydrogen peroxide, to

give a green colour which changes through blue to violet. The colour intensity can be compared with a known standard in a spectrophotometer. In the experiments, a cyanmethaemoglobin standard solution was used containing 57.2 mg.dl^{-1}. As a control, water was substituted for the plasma.

Methods using the peroxidase activity of heme as the basis of colorimetric procedures are subject to variable interferences, and they do not distinguish between different heme containing compounds, which may show different degrees of peroxidase activity. A spectrophotometric scanning technique is available, and it is claimed that this measures plasma haemoglobin accurately, even in the presence of the usual interferences by bilirubin, turbidity and methaem-albumin (Blakney and Dinwoodie, 1975). However, without scale expansion facilities on the scanning spectrophotometer, the method was found to be unsuitable for measuring low concentrations, and, therefore, despite its advantages, was not used.

5.2.2 THE RESULTS OF PLASMA HAEMOGLOBIN CONCENTRATION MEASUREMENTS

Haemoglobin measurements were made after 0, 2, 4, 8 and 14 days storage. Concentrations varied between donors but showed a gradual increase throughout the storage period at 1°C, 4°C and room temperature (Table 14, Figure 21). On collection, plasma haemoglobin concentrations had a mean of 1.7$\pm$ (SEM) 0.3 mg.dl^{-1} (n = 19), a concentration also found in another study (Bailey and Bove, 1975). The extent of haemolysis was most apparent at 1°C, increasing to a mean of 81.1 mg.dl^{-1} after 14 days blood storage.

TABLE 14

PLASMA HAEMOGLOBIN CONCENTRATION DURING BLOOD STORAGE AT 1°C,

4°C, ROOM TEMPERATURE (in mg.dl^{-1} of PFP)

a) 1°C	0	2	4	8	14 days
	4.3	10.6	–	20.7	39.4
	2.1	9.0	11.2	18.9	38.7
	1.9	19.9	27.0	39.6	67.5
	5.2	13.9	40.2	82.9	168.7
	1.6	–	18.8	30.8	58.8
	1.6	12.9	11.1	18.8	61.5
	1.4	11.8	8.2	18.2	–
	0.3	4.6	14.1	46.8	132.9
Mean	2.3	11.8	18.7	34.6	81.1
± SEM	0.6	1.8	4.3	7.9	18.8

TABLE 14 (cont)

b) 4°C	0	2	4	8	14 days
	0.6	9.1	13.5	22.5	31.9
	0.1	8.4	19.2	34.0	105.0
	1.2	3.6	3.8	9.8	18.8
	0.3	2.8	5.7	6.2	21.3
	1.1	3.4	4.3	8.7	13.3
	1.7	3.7	7.3	10.2	14.4
	1.2	4.6	8.2	14.7	33.8
	3.9	8.7	12.7	21.7	47.1
	2.1	13.8	19.8	21.8	32.5
	1.9	13.8	21.9	31.4	50.6
	1.4	8.9	13.1	18.9	-
	0.3	16.3	45.0	58.6	91.5
	1.1	7.7	12.0	29.5	36.1
	0.9	11.8	15.3	30.4	45.8
	1.7	10.0	18.1	34.0	51.2
mean	1.3	8.4	14.7	23.5	42.4
± SEM	0.2	1.1	2.6	3.5	7.2

TABLE 14 (cont)

c) Room Temp.	0	2	4	8	14 days
	0.6	4.2	6.1	8.6	23.2
	0.1	4.1	6.7	18.1	61.0
	1.2	3.8	4.1	11.9	21.7
	0.3	2.6	7.6	8.0	21.8
	1.1	2.9	3.4	7.3	20.7
	1.7	3.5	5.7	9.9	14.3
	1.2	4.7	7.9	12.1	27.3
	2.1	6.9	7.7	17.6	30.2
	1.9	7.5	18.3	20.4	49.9
	1.6	—	3.7	8.4	18.1
	1.4	13.4	9.7	18.2	—
	0.3	6.7	9.4	13.5	57.6
mean	1.1	5.5	7.5	12.8	31.4
± SEM	0.2	0.9	1.1	1.3	5.0

Haemoglobin Conc. mg.dl^{-1}

Storage time in days
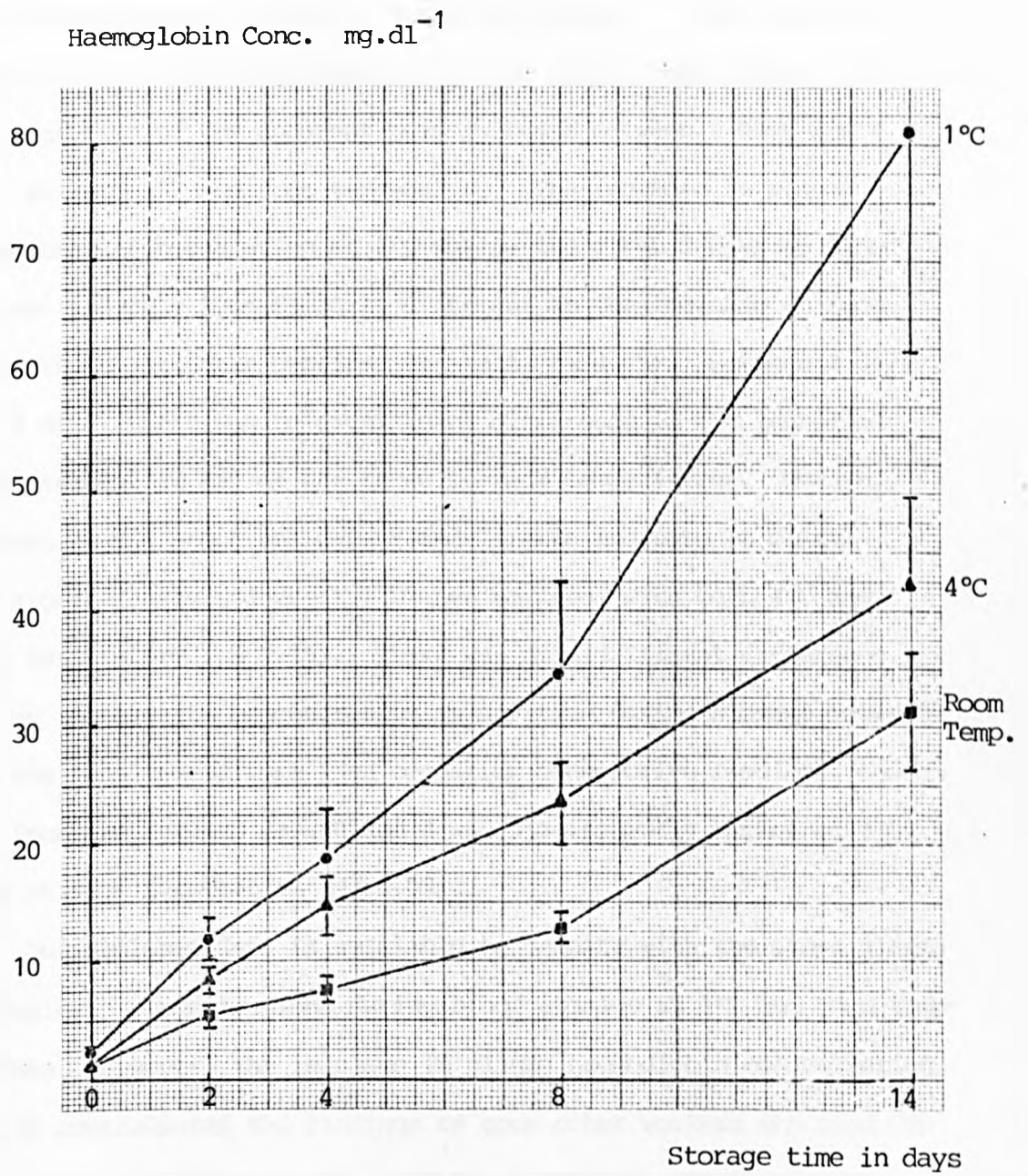
Figure 21 CHANGES IN PLASMA HAEMOGLOBIN CONCENTRATION ($\pm$ SEM)
DURING BLOOD STORAGE

At 4°C, the plasma haemoglobin concentration increased to 42.4 mg.dl^{-1}, and at room temperature, to 31.4 mg.dl^{-1}. The increase in concentration was significant (Students paired 't' test, p$\leqslant$0.05) between most consecutive readings at all three temperatures, with the exception of the apparent mean increase between 2 days and 4 days at both 1°C and room temperature. The increase in plasma haemoglobin concentration at 1°C during the first 2 days of blood storage was only significantly different to the increase at room temperature (Students unpaired 't' test, p$\leqslant$0.05). Between 2 days and 4 days, there was no significant difference in the increased concentration at any of the three storage temperatures. However, between days 4 and 8 and days 8 and 14, the increase in plasma was significantly higher at 1°C when compared with both 4°C and room temperature (p$\leqslant$0.05). There was no significant difference in the increase in concentration at any time during storage between 4°C and room temperature when comparing consecutive readings, though the increase between days 0 and 4 was significantly higher at 4°C, than at room temperature (p$\leqslant$0.05).

No published data is available to compare with the above plasma haemoglobin concentrations during blood storage at 1°C and room temperature. However, the increase in plasma haemoglobin concentration at 4°C contradicted the findings of some other workers who used CPD anticoagulated blood. Bailey and Bove (1975) observed a gradual increase to a mean of only 12.5 mg.dl^{-1} after 14 days blood storage. Limbird and Silver (1974) found little change during the first 14 days blood storage but recorded a plasma haemoglobin mean concentration of about 42 mg.dl^{-1} after only 1 day of storage. The increase in

plasma haemoglobin concentrations reported here are, however, very close to the results of Mishler et al., (1978) who measured a mean concentration of 43.5 mg.dl^{-1} after 14 days blood storage.

The extent of haemolysis was only very small, even at 1°C. Considering a maximum haemoglobin concentration of about 16 g.dl^{-1} of venous blood in the average adult male, then the mean extent of haemolysis after 14 days blood storage was only about 0.5% at 1°C, about 0.25% at 4°C and about 0.2% at room temperature. Thus haemolysis in stored blood was most evident at 1°C and least evident at room temperature with the plasma haemoglobin concentrations at 4°C being closer to those at room temperature than at 1°C. Although ADP release from erythrocytes alone was not measured, because haemolysis was most evident during blood storage at 1°C, it is probable that ADP was released more readily from erythrocytes stored at 1°C, than from erythrocytes stored at 4°C or room temperature.

5.3 ATP CONCENTRATIONS DURING BLOOD STORAGE

ATP probably plays two essential roles in platelet aggregation. Firstly it provides the energy required, and the consumption of metabolic ATP is the earliest biochemical effect of aggregating agents so far detected (MacFarlane and Mills, 1975). Secondly, it is the precursor of the majority of ADP released from platelets (Haslam, 1964), with resultant second stage aggregation.

ATP is a nucleotide of primary biological importance not only providing energy for the various kinds of work performed by living cells, but also as a precursor of the coenzymes NAD and NADP.

- 131 -

These operate as hydrogen and electron transfer agents by virtue of reversible oxidation and reduction essential in dehydrogenase reactions.

ATP itself is released from platelets aggregated by thrombin (Detwiler and Feinman, 1973) by as much as 40% of its platelet content (D'Souza and Glueck, 1977) Evidence is available that ATP will inhibit ADP induced aggregation, possibly, like AMP, by competing for specific aggregating sites on the platelet membrane (Born, 1962). However, a high concentration is required (about 200 times the concentration of ADP, in-vitro) and only involves first stage aggregation (MacFarlane and Mills, 1975).

ATP was measured to see to what extent it was utilised during blood storage at 1°C, 4°C and room temperature, and to try and relate changes in ADP concentrations to energy utilisation.

5.3.1 ASSAY FOR ATP IN PLASMA AND WHOLE BLOOD

Enzymic estimation of ATP presents the same problems as it does for ADP. Inosine triphosphate, uridine triphosphate and guanosine triphosphate are also determined, but like their diphosphate relatives, they are only present in very small quantities. ADP may be estimated by recording luminescence resulting from the reaction of ATP with firefly luciferin/luciferase (Detwiler and Feinman, 1973), though this has no advantage over the enzymic method of Adam (1962). This method is based on ATP phosphorylation of glycerate-3-phosphate to glycerate 1,3 diphosphate in a reaction catalysed by 3-phosphoglycerate kinase (Figure 22). The glycerate 1,3 diphosphate is reduced with NADH, and glyceraldehyde 3 phosphate dehydrogenase to

form glyceraldehyde 3 phosphate which is converted to dihydroxy-
acetone phosphate by triosephosphate isomerase. Finally, dihydroxy-
acetone phosphate is reduced to glycerol 1.1 phosphate by glycero-
phosphate dehydrogenase and NADH. The method is in effect, a
reversal of part of glycolysis and gluconeogenesis and normally the
equilibrium of the first reaction lies to the left. However, by the
use of an excess of glycerate 3 phosphate and removal of the glycer-
ate 1,3 diphosphate in the second reaction, the equilibrium of which
lies to the right, ATP can be completely converted. The NADH used
in this reaction is equivalent to the glycerate 1,3 diphosphate
and hence to the ATP concentration. The further reactions double
the rate of conversion. NADH is the reaction indicator.

Spectrophotometric measurements and application of the cal-
culation are as detailed for ADP.

Glycerate 3 phosphate ⇌ glycerate 1,3 diphosphate

ATP → ADP

Pi → NADH + H^+ / NAD

dihydroxyacetone phosphate ⇌ glyceraldehyde 3 phosphate

NADH + H^+ / NAD

glycerol 1 phosphate
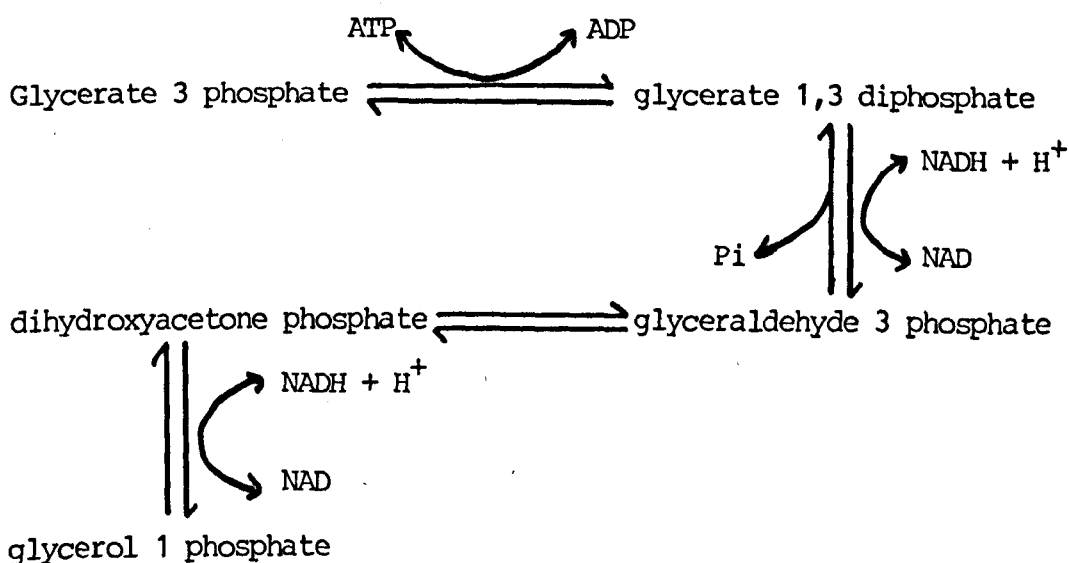
Figure 22

(Tables 15, 16 and 17, Figures 23 and 24). ATP concentrations were measured at the same intervals as for ADP, and similarly, 'intracellular' levels were calculated from the measured plasma and whole blood concentrations. These showed large individual variations, though the mean whole blood concentrations at all three storage temperatures showed a similar trend throughout blood storage. There was no statistically significant difference between any consecutive reading of ATP in PFP (Students paired 't' test) at any temperature, nor was there any significant difference in the change in concentration, at any time during storage, between any temperature. Therefore, because there was little to distinguish between the ATP concentrations in PFP, no graph has been included.

Whole blood concentrations of ATP immediately after collection were in the range 26.7 to 37.7 mg.dl^{-1} with a mean of 31.0 $\pm$ SEM 1.7 mg.dl^{-1}. (n = 6). This was marginally higher than the 'normal' reported range of 19 to 32 mg.dl^{-1} (Boehringer), though lower than the range of 38 to 54 mg.dl^{-1} reported for adult males (Buell, 1935 – cited Documenta Geigy Scientific Tables, 1962). In whole blood, a significant decrease in concentration (Students paired 't' test, $p < 0.05$) was observed during the first 24 hours of storage at all three temperatures, but with a significantly larger decrease at 1°C (mean 31.0 to 17.8 mg.dl^{-1}) ($p < 0.05$). The decreases in concentration at 4°C and room temperature were similar (mean, 31.0 to 20.6 mg.dl^{-1} at 4°C, and to 20.5 mg.dl^{-1} at room temperature). Because of the lack of difference between plasma ATP concentrations at different temperatures, the calculated intracellular concentrations showed similar changes to whole blood levels. All consecutive

TABLE 15

ATP CONCENTRATIONS DURING BLOOD STORAGE AT 1°C, 4°C AND ROOM TEMPERATURE (x 10^4ug.L^{-1})

1°C

a) Whole Blood	0	1	2	3	4	8	14 days
	26.7	7.4	17.5	24.1	38.1	–	15.8
	27.6	–	23.2	20.6	9.6	26.3	27.2
	33.3	17.1	27.6	27.6	25.8	19.7	3.1
	32.0	16.2	25.8	24.1	15.3	14.5	4.8
	37.7	25.4	13.6	25.8	24.1	17.9	14.0
	28.4	22.8	15.3	21.9	13.6	29.3	13.6
mean	31.0	17.8	20.5	24.0	21.1	21.5	13.1
± SEM	1.7	3.1	2.4	1.0	4.2	2.7	3.5

b) PFP							
	6.6	3.9	4.4	3.0	2.2	2.6	0.9
	5.7	3.1	5.3	2.0	0	3.1	1.1
	0	0.9	0	0	0	12.7	0
	0	3.1	11.8	0	0	4.4	0
	0	0	0.4	4.4	0	0	0
	0.9	0	0	1.8	0	0	0
mean	2.2	1.8	3.7	1.9	0.4	3.8	0.2
± SEM	1.3	0.7	1.9	0.7	0.4	1.9	0.2

Intracellular Level (calculated from whole blood minus PFP)

	28.8	16.0	16.8	22.1	20.7	17.7	12.9

TABLE 16

ATP CONCENTRATIONS (cont)

4°C

a) Whole Blood	0	1	2	3	4	8	14	days
	26.7	13.1	19.3	27.2	35.9	–	17.1	
	27.6	21.0	25.0	25.0	9.6	25.4	24.1	
	33.3	21.9	31.1	28.0	27.6	24.1	12.7	
	32.0	17.6	24.5	21.9	14.0	19.7	11.8	
	37.7	27.2	21.0	26.7	28.5	25.4	14.0	
	28.4	22.8	17.5	21.9	17.5	26.3	20.6	
mean	31.0	20.6	23.1	25.1	22.2	24.2	16.7	
± SEM	1.7	2.0	2.0	1.1	4.1	1.1	2.0	

b) PFP	0	1	2	3	4	8	14	
	6.6	2.6	4.8	2.0	1.3	2.2	1.2	
	5.7	2.6	4.4	1.9	1.3	3.9	1.8	
	0	0.9	0	0	0	7.9	0	
	0	0	9.6	0	0	0.9	0	
	0	0	3.9	4.4	2.6	0.4	0	
	0.9	0	0	1.8	0	0	0	
mean	2.2	0.6	3.8	1.7	0.9	2.6	0.5	
± SEM	1.3	1.2	1.5	0.7	0.4	1.2	0.3	

c) Intracellular Level (calculated)

	28.8	20.0	19.3	23.4	21.3	21.6	16.2

TABLE 17

ATP CONCENTRATIONS (cont)

Room Temperature

a) Whole Blood

	0	1	2	3	4	8	14	days
	26.7	13.1	17.5	26.3	51.2	–	3.9	
	27.6	17.5	26.7	24.1	17.1	17.5	9.6	
	33.3	21.5	29.3	28.0	23.6	17.5	0	
	32.0	17.1	25.8	21.9	9.2	15.3	1.3	
	37.7	30.7	20.1	25.4	27.6	16.2	0	
	28.4	22.8	19.2	23.2	14.0	23.2	4.4	
mean	31.0	20.5	23.1	24.8	23.8	17.9	3.2	
± SEM	1.7	2.5	2.0	0.9	6.1	1.3	1.5	

b) PFP

	0	1	2	3	4	8	14
	6.6	1.8	4.4	1.9	1.8	2.6	0.8
	5.7	2.2	3.5	1.8	1.3	3.1	1.1
	0	0.9	0	0	0	7.9	0
	0	0	4.8	0	0	0	0
	0	0	4.8	2.6	1.3	0	0
	0.9	0	0	2.2	·0	0	0
mean	2.2	0.8	2.9	1.4	0.7	2.3	0.3
± SEM	1.3	0.4	0.9	0.5	0.3	1.3	0.2

c) Intracellular Level (calculated)

0	1	2	3	4	8	14
28.8	19.7	20.2	23.4	23.1	15.6	2.9

ATP Concentration x 10^4 µg.L^{-1}

Storage time in days
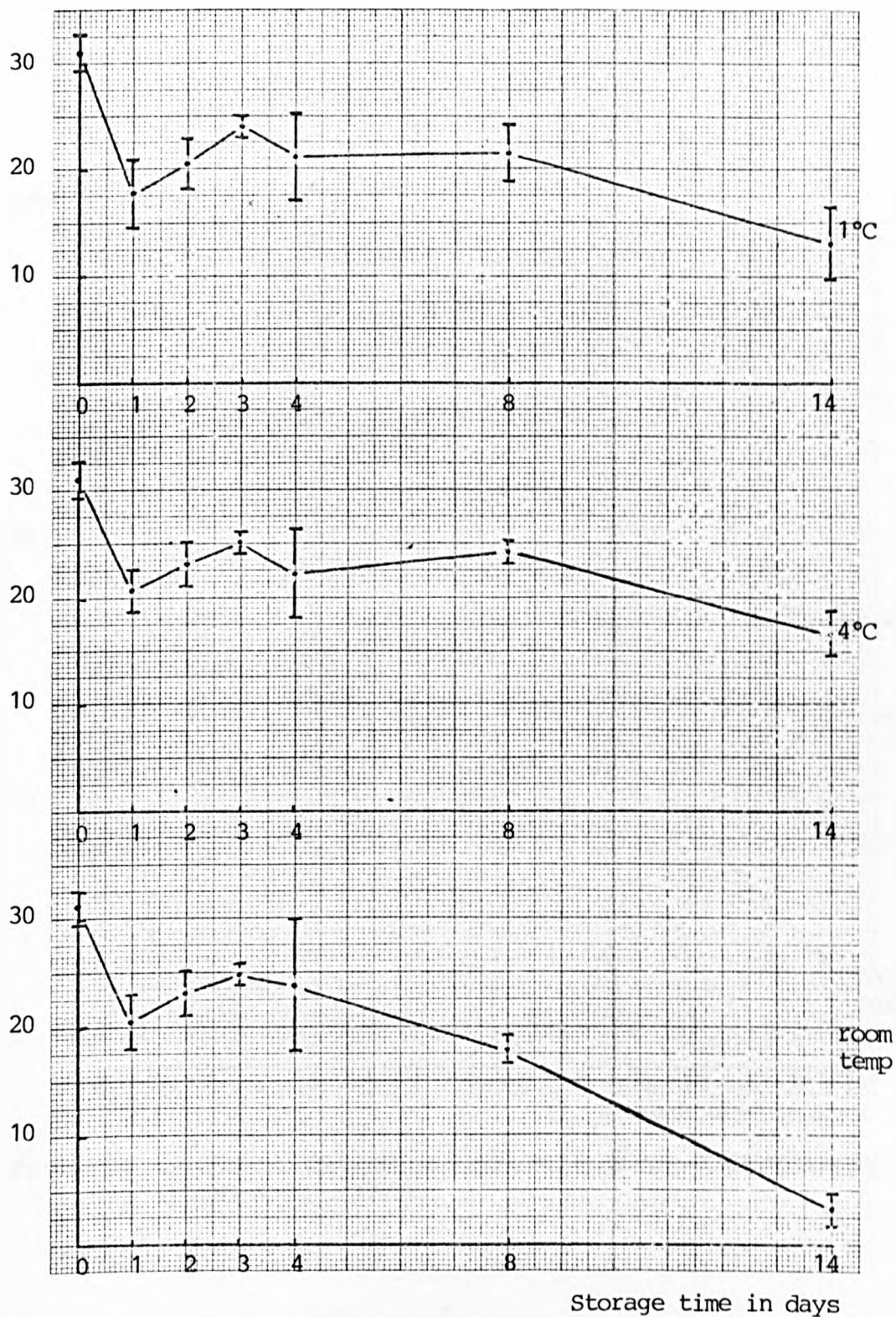
Figure 23   CHANGES IN WHOLE BLOOD CONCENTRATIONS OF ATP ($\pm$ SEM)
DURING BLOOD STORAGE.

ATP concentration x 10^4 µg.L^{-1}
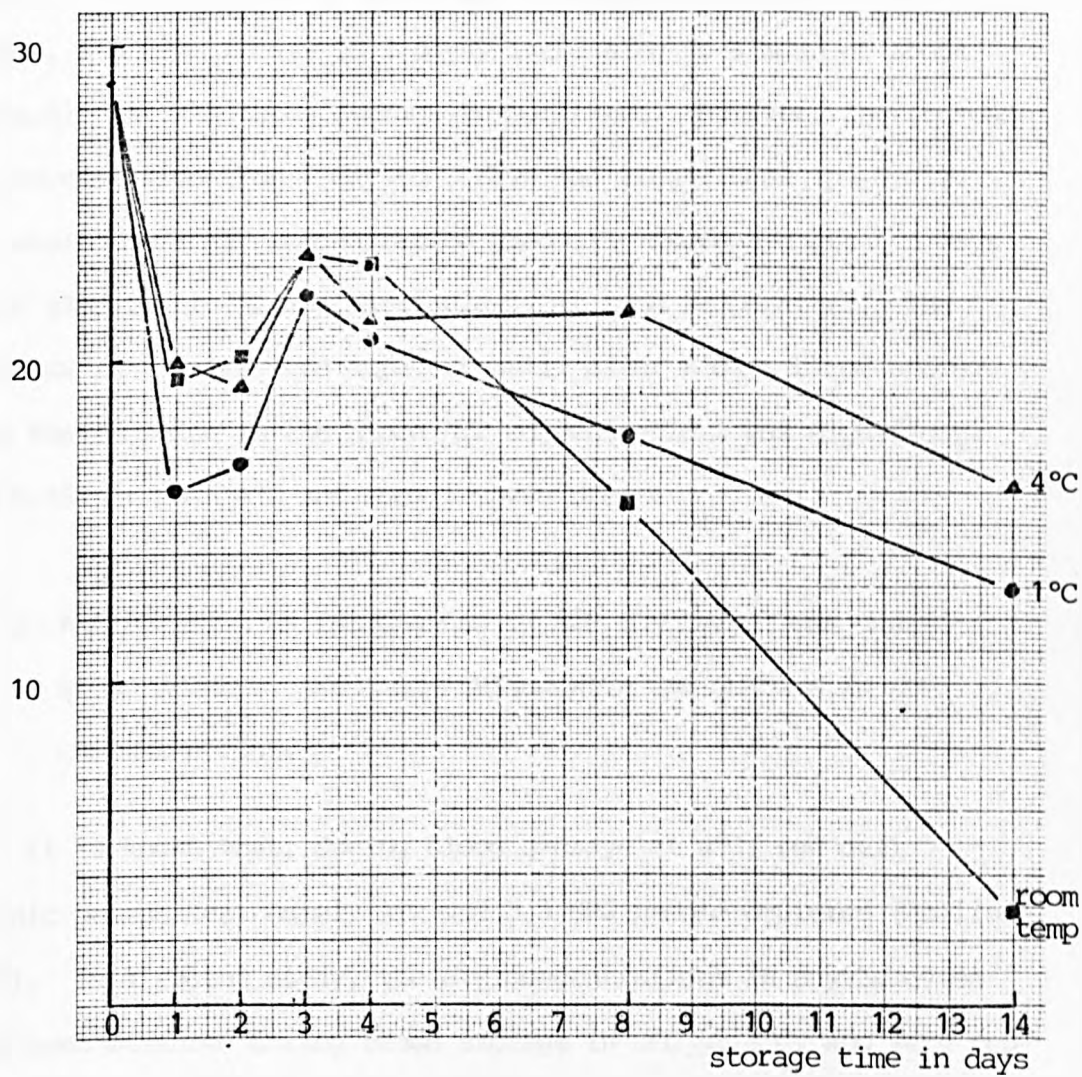
Figure 24   CALCULATED CHANGES IN INTRACELLULAR ATP CONCENTRATIONS
DURING BLOOD STORAGE

readings from 1 to 8 days at 1°C, 4°C and room temperature showed a similar pattern of change for ATP concentrations in whole blood and calculated intracellular levels. There was no significant difference in the changes in ATP concentration between any of the storage temperatures. There was an apparent, though statistically insignificant increase in blood ATP concentrations from 1 day to 2 days, and from 2 days to 3 days, increasing to a mean of about 24 mg.dl^{-1} at all three storage temperatures. However, the increase in concentration from 1 day to 3 days was significant ($p \leqslant 0.05$). The whole blood ATP concentration gradually decreased after 3 days blood storage at 1°C and particularly at room temperature. The decrease at 4°C was less apparent until after 8 days blood storage when the decrease in the blood ATP concentrations was significant ($p \leqslant 0.05$) at both 4°C and room temperature.

5.3.3 A DISCUSSION OF THE CHANGES IN ATP CONCENTRATIONS DURING BLOOD STORAGE, AND A COMPARISON WITH THE CHANGES IN ADP CONCENTRATIONS.

It is known that, during blood storage at 4°C, red cell organic phosphates, namely ATP and 2,3 DPG become depleted (Mollison, 1972). In a recent study, the ATP concentrations in erythrocytes have been measured during blood storage in CPD at 4°C, and were reported to show a "gradual decline during storage" when measured after 1 day, 3 days, 7 days and 14 days (Mishler et al., 1978). In fact, a closer examination of the reported mean ATP concentrations shows a marked decrease between the "pre-donation" and the 1 day concentration, and a small increase in erythrocyte ATP concentration

between 1 day and 3 days blood storage. The results reported here show a very marked decrease in whole blood and intracellular ATP concentrations during the first 24 hours of storage and a significant increase in concentration between 1 day and 3 days blood storage at all three storage temperatures. Does this suggest measurable activity in blood cells other than erythrocytes? Erythrocytes require energy to maintain structure, shape and membrane functions, and this energy is usually furnished by ATP produced from glycolysis (Sasakawa and Tokunaga, 1976). Although erythrocytes are directly permeable to the dextrose in the CPD anticoagulant, glycolysis would be very much reduced in the cold and hence existing ATP might well be rapidly depleted. Platelets are also metabolically very active, even in their resting state. One process that constantly consumes ATP in the resting platelet is the rapid utilisation of ATP by actin to keep this molecule in the G-form (Holmsen, 1977a). Platelet aggregation has been shown to occur very rapidly in blood stored in the cold (chapter three) and although this aggregation has been described by Kattlove and Alexander (1971) as "spontaneous", the use of metabolic inhibitors such as potassium cyanide, considerably reduced cold induced platelet aggregation in PRP. Not only aggregation, but even the very early phases in platelet function - shape change and pseudopod formation - involve the activation and/or relaxation of a contractile mechanism. It is well known that in platelet function, energy metabolism and the content of metabolically active ATP play an important role (Schneider, 1974). It is, therefore, likely that the ATP content of platelets would also be rapidly depleted. During blood storage in ACD at 4°C, it has been shown that polymorphonuclear neutrophils remain functionally active for

between 1 day and 4 days, and lymphocytes for much longer (McCullough et al., 1969). It is therefore likely that energy would be required by these cells to maintain functional activity.

Nevertheless, the significantly larger decrease in the concentration of intracellular ATP at 24 hours blood storage at 1°C (Figure 24) where platelet aggregation was very rapid suggests that at least a part of the decrease was due to platelet activity. However, White (1974) has suggested that pseudopod extension and platelet shape change (i.e. primary aggregation) in cold activated platelets are passive phenomena resembling relaxation, while the internal transformations caused by other aggregating agents are probably active processes similar to contraction. Thus ATP may not be involved in this stage of aggregation which precedes the platelet release reaction. The results in this study have shown that platelet release probably does not occur concomitantly with the rapidly increased SFP after 24 hours blood storage at 1°C. Plasma ADP, (Table 8b) plasma ATP (Table 15b) and plasma 5-HT (Table 7) all showed a mean decrease in concentration after 24 hours, and Kattlove and Alexander (1971) demonstrated that at low temperatures, platelets in PRP become sticky and aggregate spontaneously, without releasing endogenous stores of ADP. But if this is the case, then why did the concentration of intracellular ATP decrease significantly more in blood stored at 1°C than in blood at 4°C?

There is no evidence that erythrocyte or leucocyte ATP consumption should be greater in blood stored at 1°C. Also, the increase in intracellular ATP concentration mainly between 2 days and 3 days blood storage is very much greater in the results reported here than the erythrocyte ATP concentrations in Mishler et al.'s (1978) study. Because of the uncertainty it is worth speculating that some of the changes in ATP concentration measured during the early stages of blood storage were due to platelet activity. The reason for the consumption of energy during the first 24 hours of blood storage probably depended upon the storage temperature. In the cold, platelet aggregation may have been a major energy consumer, and this would have been most apparent at 1°C where platelet aggregation was very rapid when compared with aggregation at 4°C. At room temperature, where no platelet aggregation occurred, the maintenance of cell function might have accounted for the decrease in ATP. The decrease in the intracellular ATP concentration was similar in blood stored for 24 hours at 4°C and room temperature, and an alternative explanation for the decrease in ATP concentration could be as follows. Platelets in stored blood were exposed to the same aggregating stimulus regardless of temperature, but that aggregation at room temperature was inhibited. The main degradation products of ADP are

AMP, adenosine, inosine, inosine monophosphate and hypoxanthine.
Both AMP and adenosine are powerful inhibitors of ADP induced
platelet aggregation (Born and Cross, 1963; Born, 1967), possibly
by activation of platelet adenyl cyclase with resultant c.AMP
mediated inhibition (Haslam and Lynham, 1973). If a stimulus other
than cold were involved in the initiation of platelet aggregation in
stored blood, then, as a consequence of ATP consumption, the above
products would be formed very quickly at room temperature and might
inhibit aggregation. However, it seems unlikely that ATP consumption
at room temperature was due to platelet stimulation, as energy
consumption for platelet aggregation would have occurred during the
aggregation process itself, and not just as a result of the presence
of an aggregating agent. There was no evidence for platelet aggreg-
ation, as measured by the SFP technique, in blood stored at room
temperature (Table 5c, chapter three), though it is possible that
any aggregation which took place within the first 24 hours was
reversed by the increasing presence of aggregation inhibitors before
the 24 hour SFP measurement was taken. This possibility was not
investigated, but would not explain why ATP consumption during the
first 24 hours in blood stored at 1°C was significantly greater than
ATP consumption at 4°C and room temperature (Figure 23).

The decrease in the concentration of intracellular ATP in
blood stored at 1°C for 24 hours appeared to be associated with an
increased intracellular ADP concentration. However, the increase
in intracellular ADP concentrations apparent at 1°C was very much
less obvious at 4°C and was decreased at room temperature. This
may, in part, have been due to different ADP degradation rates,
dependent upon temperature, and, particularly at room temperature,

rephosphorylation of ADP to ATP during glycolysis.

When the comparative concentrations are considered, it is evident how much degradation of ADP took place. The concentrations of ATP were measured in units ten times those of ADP, and hence a relatively small fall in ATP concentrations might have been expected to produce a relatively large increase in intracellular ADP. This did not occur at any of the three storage temperatures. It may be explained at 1°C and 4°C by the observation that so called "release energy" ATP may have both of its energy rich phosphoryl groups used during the release reaction, resulting in the eventual formation of ribose-1-phosphate and hypoxanthine which leave the cell (Holmsen et al., 1969a). However, this is thought to account for all the ATP consumed in the release reaction (Holmsen et al., 1969b) and yet the intracellular ADP concentration was highest at 1°C (Figure 18) where platelet aggregation had occurred most rapidly. This suggests that the platelet release reaction did not take place during the first 24 hours of blood storage in the cold. As mentioned earlier, this possibility is endorsed by the other parameters that were measured, as the release reaction might have been expected to cause a concomitant increase in the plasma concentration of ADP, ATP and 5-HT after 24 hours blood storage in the cold. (Plasma catecholamines were not measured at 24 hours). The plasma concentration of these three factors decreased during the first 24 hours, and only showed an increase in concentration at 48 hours storage. It is not likely that these increases were due to the release reaction because they occurred to a greater or lesser extent at all three storage temperatures and were not paralleled by a further decrease in the intracellular ATP concentration. This might have

been expected since the release reaction is an energy dependent

process (Holmsen et al., 1969a, b). In fact, the intracellular

ATP concentration showed an apparent increase between 24 and 72 hours

blood storage at all three temperatures. It is possible that the

platelet release reaction, per se, took place gradually and was not

recognised as a distinct event by 24 hour monitoring.

The increase in plasma ADP concentrations after 48 hours blood

storage seemed to follow the utilisation of ATP during the first

24 hours. Although the increase in the concentration of plasma

ADP was similar at all three storage temperatures, it is likely that

this was apparent and not real. There was considerable variation

in the results between individuals and ADP degrades more quickly

in plasma at room temperature than in the cold. It was possible

that the source of plasma ADP varied at different storage temperatures.

At room temperature possibly the main source was from metabolic

energy demands in blood cells. At 1°C, ADP released from erythro-

cytes may have contributed, and plasma ADP concentrations at 1°C and

4°C would probably have been contributed to by platelet destruction,

the platelet release reaction, and ADP from ATP utilisation in

primary aggregation. Some ADP released from platelets and erythro-

cytes in blood stored in the cold may also have been bound to plate-

lets and measured as intracellular ADP.

The increase in intracellular ATP concentrations observed

particularly between 2 and 3 days blood storage were similar at

all three storage temperatures. Platelets are known to be very

sensitive to changes in adenine nucleotide levels so that reduced

ADP or ATP concentrations results in increased phosphorylase activity

and hence glycogenolysis (Mustard and Packham, 1970). This may

have caused the increase in intracellular ATP concentrations.
Although the electron micrographs seemed to show that the majority
of platelets were morphologically intact during the early stages
of blood storage in the cold (Figures 12 to 15, chapter four),
platelet viability is thought to be much reduced after 24 to 48
hours storage at 4°C (Lim et al., 1973). Therefore, increased ATP
concentrations after 48 hours blood storage in the cold were unexp-
ected, particularly as they appeared to be similar to the increases
observed at room temperture. However, Estes et al., (1962) recorded
continued glycolysis in platelets stored for considerably longer
than 48 hours at 4°C even though viability (as measured by the
platelets life in the circulation, after reinfusion) was much
reduced. The differences in the intracellular ADP concentrations
between 24 and 96 hours in blood stored at the different temperatures
might have been due to the use of this increased ATP. At 1°C,
with a much reduced normal cellular metabolism, and with already
extensive platelet aggregation, there would have been little demand
for energy. Hence, the decrease in the intracellular ADP concen-
tration (Figure 18). Whereas at room temperature with nearer normal
blood cell functions to maintain, there would have been a greater
demand for energy with a resultant increase in the intracellular ADP
concentration. At 4°C, the cellular metabolic requirements would
have been much reduced but platelet aggregation was still taking
place and hence the comparatively small change in the ADP concen-
tration. Therefore, the similarity in intracellular ATP concentration
increases at 3 days blood storage at the three storage temperatures
might only have been apparent. Had no ATP been consumed, then ATP
production might well have been seen as a considerably higher

concentration at room temperature than in the cold.

The intracellular ATP concentration decreased after 3 days blood storage at 1°C and at 4°C, and after 4 days at room temperature. This decrease is in accord with other recent reported studies, (Sasakawa and Tokunaga, 1976; Mishler et al., 1978). The decline in ATP is associated with parallel alterations of red cell deformability in blood stored at 4°C with disc to sphere transformation as the cells become more rigid (Haradin et al., 1969). The rate of decrease was particularly rapid at room temperature and probably accounted for the rapid increase in the intracellular ADP concentration between 3 and 8 days blood storage followed by the rise in the plasma ADP concentration between 8 and 14 days storage. However, the decrease in the intracellular ATP concentration at room temperature may have been largely contributed to by the rapidly decreasing pH observed in blood stored at room temperature (Figure 9, chapter three). This decreased pH would have interfered with the function of glycolytic enzymes and quickly halt glycolysis. At all three storage temperatures, the decrease in intracellular ATP concentrations after 3 or 4 days storage was associated with an increase in the plasma ATP concentration at 8 days storage. This was most apparent at 1°C and probably paralleled reduced cell viability. However, the increased intracellular ADP concentration at 8 days blood storage at 1°C suggested either continued ATP consumption or degradation. It was not clear why the changes in ADP and ATP concentrations in blood stored at 4°C were less pronounced than those at 1°C or room temperature. At 4°C there was an apparent very slow decrease in the concentration of intracellular ATP up to 8 days blood storage with a concomitant small increase in the intracellular ADP concentration.

After 8 days blood storage, the intracellular ATP concentration began to decrease more rapidly. It seems that the changes seen at 1°C and room temperature were delayed in blood stored at 4°C.

Only an extensive investigation probably involving the separation and storage of individual blood cell types would make clear what contribution blood platelets make to overall ADP and ATP concentration changes. It is not possible from the investigations undertaken in this study to state whether platelet aggregation in cold stored blood is an energy dependent process, though the evidence seems to favour this possibility. However, it does seem that platelet aggregation in blood stored at 1°C and 4°C did not depend on the platelet release reaction.

CHAPTER SIX

THE MEASUREMENT OF THE OVERALL EFFICIENCY OF THE

INTRINSIC AND EXTRINSIC BLOOD CLOTTING SYSTEMS

6.1 THE BLOOD COAGULATION PATHWAYS

The following introduction is not intended as a comprehensive
review of the blood coagulation sequence as it is understood today,
but as a brief revision of the relationships of the various coagu-
lation factors as described by Bennett and Ratnoff (1972). The
extrinsic clotting system is basically the mechanism responsible for
the clotting of plasma when it is mixed with tissue extract (throm-
boplastin) and calcium. It is likely that the extrinsic system
provides the first traces of thrombin necessary for the initiation
of vascular repair, for unlike the intrinsic system, the reaction is
complete in a few seconds. The nature of the tissue factor is unknown,
perhaps being some lipoprotein complex. The triggering mechanism
responsible for intrinsic prothrombin activation probably involves
the activation of factor XII by negatively charged surfaces with a
particular spatial arrangement of charges. The intrinsic pathway
is so named to contrast with the extrinsic as no material extrinsic to
the blood is known to be involved. The two systems are only different
up to the formation of activated factor X (Xa)

The sequence of reactions culminating in the production of fibrin
has interested many workers though two hypotheses are favoured. One
is the "autocatalytic theory" (Seegers et al., 1967 - cited Garner,
1973) and the other is known as the "sequential theory" (Macfarlane

1964 - cited Garner, 1973). The essential difference between the theories concerns the interpretation of the nature of prothrombin (factor II). In the autocatalytic theory, prothrombin is regarded as a single complex molecule composed of several sub-units which are liberated by traces of thrombin (produced as the result of a chemical change of the parent molecule occurring after contact with a foreign surface). Other specialised proteins and phospholipids present in the plasma, or derived from platelets, accelerate the reaction. The sequential theory regards prothrombin as a single protein, a pro-enzyme, with only one product, thrombin. The other clotting factors are distinct entities present as inactive precursors. Each factor is activated by the preceding one in a chain of events that ultimately leads to the production of thrombin.

Neither theory is thought to provide a completely satisfactory explanation and a modification was suggested, which regarded the different stages of the coagulation pathway as active complexes. (Mammen, 1971 - cited Garner, 1973). There have been other modific-ations, such as the possible activation of factor IX by factor VII (Di Scipio et al., 1978). However, the general concept, as detailed, is still acceptable (Figure 25).

6.2 PLATELET AGGREGATION AND BLOOD COAGULATION

Platelet activation and aggregation by ADP and collagen have been shown to result in activation of factor XII in the intrinsic coagulation pathway (Walsh, 1973). The interaction of platelets with ADP also leads to activation of PF3 which functions in the interaction of coagulation factors Xa and V. Thus by activating
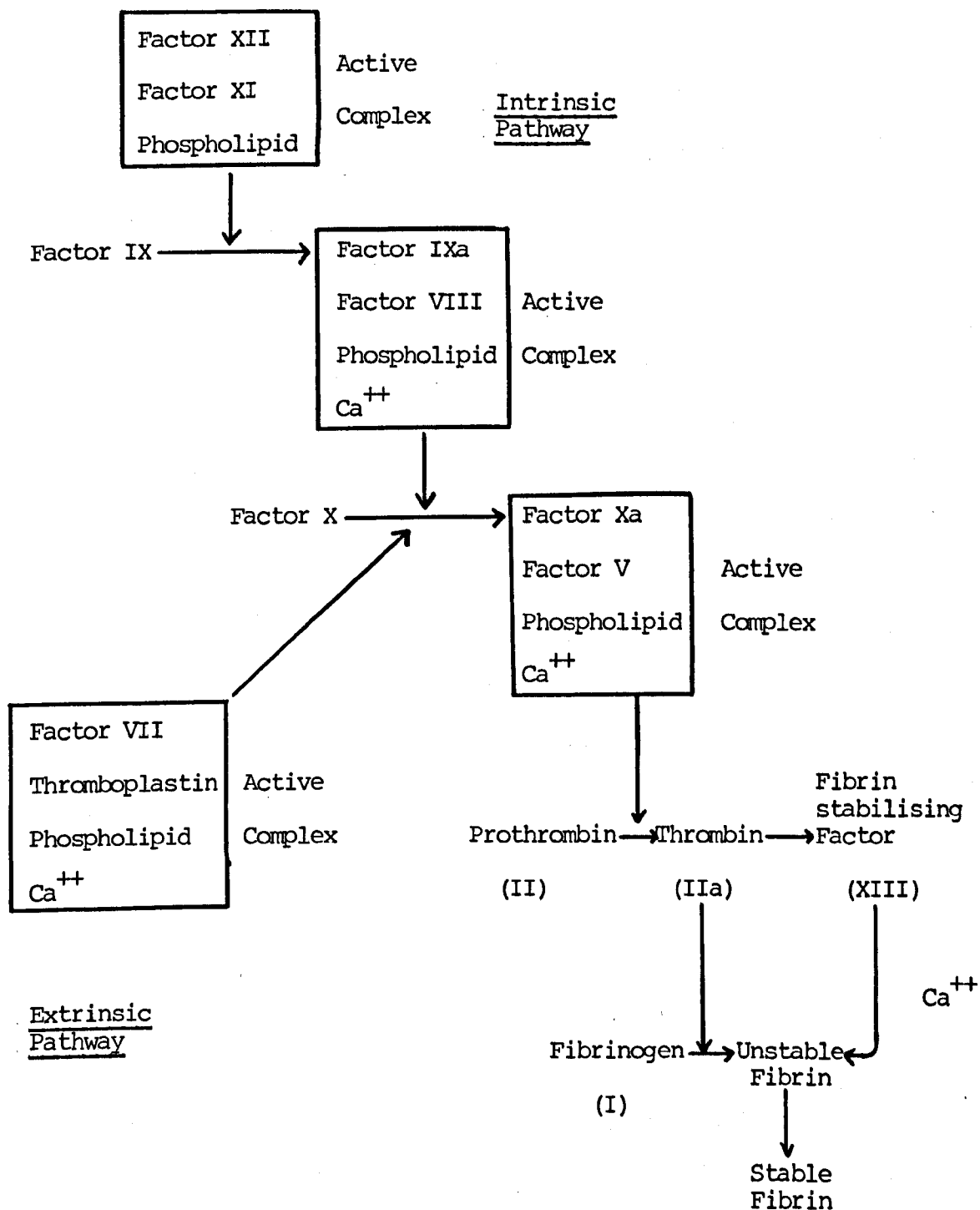
Figure 25   THE PATHWAYS OF BLOOD COAGULATION

factor XII, ADP initiates coagulation and this reaction can proceed
to completion through interaction between PF3 and the intermediate
clotting factors which are adsorbed onto the platelet membrane (Ardlie
and Han, 1974). Thrombin may therefore, be generated in association
with platelet aggregates and can cause release of platelet constitu-
ents (Holmsen et al., 1969a). It has been suggested that blood
coagulation and platelet aggregation are inseparable phenomena (Ardlie
and Han, 1974) and therefore, tests of coagulation have been performed
to see if there were an increased tendency of anticoagulated blood to
clot in association with platelet aggregate formation. In the pres-
ence of CPD anticoagulant, it seemed unlikely that sufficient calcium
ions would be available for blood coagulation and this was shown by
Born and Cross (1963), who found that ADP added to citrated PRP did
not lead to blood clotting. However, during blood storage, a reduced
pH may alter binding affinities and allow the release of small amounts
of ionised calcium (Limbird and Silver, 1974) and there is a rapid
release of calcium ions during the platelet release reaction (see
Chapter 1). Therefore, sufficient calcium for coagulation may become
available during blood storage and blood clots have been observed in
dogs' venous blood stored in ACD for 7 days at 4°C (Wright, 1975).
Fibrin, the end product of blood coagulation, is considered important
for stabilising platelet aggregates (Mustard et al., 1966) and has been
observed in stored blood after about 10 days storage (Arrington and
McNamara, 1974).

The two basic methods that measure the overall efficiency of the
intrinsic and extrinsic blood coagulation pathways are the kaolin
partial thromboplastin time and the prothrombin time, respectively.
Both of these test times are prolonged during the storage of CPD

anticoagulated blood at 4°C (Limbird and Silver, 1974; Mishler et al., 1978) which may be interpreted as either activation of the coagulation cascades or as reduced efficiency of the coagulation factors. The tests were repeated in this investigation so as to compare any changes in the coagulation test times with platelet aggregation at 1°C, 4°C and room temperature.

6.3 METHODS

The efficiency of the extrinsic clotting system was measured using the one stage prothrombin time test described by Hardisty and Ingram (1965). In this test, the time taken to clot is measured after addition of 0.025M calcium chloride to a mixture of the test anticoagulated plasma with rabbit brain thromboplastin (Diagen diagnostics) at 37°C. The normal range for fresh plasma is 12 - 17 seconds.

The efficiency of the intrinsic clotting system was measured using a development of the partial thromboplastin time, recalcification time and whole blood clotting time tests, known as the kaolin cephalin clotting time. In this test, platelet activity is standardised by the use of a platelet substitute, and contact activation of factors XI and XII is standardised by preincubation of the plasma and platelet substitute mixture with a suspension of kaolin for a standard time before recalcification. The method used was as described by Dacie and Lewis (1975) in which the clotting time was measured on the addition of calcium chloride, two minutes after mixing kaolin/platelet substitute (Diagen diagnostics) with the test plasma and incubated at 37°C. The normal time for fresh plasma is within the range of 40 to 55 seconds.

In both tests, plasma samples were assayed in duplicate after
testing the procedures with "normal" plasmas (Diagen diagnostics)
standardised to give control times of 12 to 14 seconds for the pro-
thrombin time, and 40 to 45 seconds for the kaolin cephalin clotting
time. The duplicates never varied by more than 2 seconds for the
prothrombin time and 5 seconds for the kaolin cephalin clotting time.

6.4 RESULTS

Both of the blood coagulation tests were undertaken at 0, 2, 4,
8 and 14 days of storage. Prothrombin times on plasma samples pre-
pared immediately after blood collection were sometimes higher than the
extreme of the normal range. The reason for this is not known, though
longer times were observed in plasma from blood samples with the lower
platelet counts. Prothrombin times after blood collection had a mean
of 15.6 $\pm$ (SEM) 0.76 seconds, and a range of 12 seconds to 19.5 seconds
(n = 10). Plasma prothrombin times generally increased during blood
storage at all three temperatures (Table 18). This was only statis-
tically significant between the times recorded at blood collection and
at 2 days storage (Students paired 't' test, $p \leqslant 0.05$), the times
increasing from a mean of 16 seconds to 19 seconds at 1°C and at 4°C,
and to 18 seconds at room temperature. Prothrombin times continued
to increase slowly reaching a mean of about 22 seconds at all three
temperatures. There was no significant difference in the prothrombin
times between 1°C, 4°C or room temperature throughout the 14 days of
blood storage and so no graph has been presented.

Kaolin cephalin clotting times in plasma samples after blood
collection all lay in the normal range with a mean of 42.5 $\pm$ (SEM) 0.77
seconds, and a range of 40 seconds to 46 seconds (n = 10). The kaolin

TABLE 18

ONE STAGE PROTHROMBIN TIME (seconds)

		Storage Time in Days				Platelet Count On Blood Collection $(\times 10^9.L^{-1})$
a) 1°C	0	2	4	8	14	
	17.5	19.5	19.5	21	21	177
	18.5	20.5	20	21.5	20	179
	15	17	18	20	21.5	254
	14	16	16.5	19	19	229
	12	16	16.5	18	–	211
	19.5	25	25.5	28	29	
mean	16	19	19.5	21.5	22	
± SEM	1.2	1.4	1.3	1.4	1.7	
b) 4°C	15.5	19.5	20	20	22	
	14.5	17	16.5	17.5	17.5	
	17	19	21	19	21	142
	15	17	17	19	19	245
	13	17	17	17	21	218
	19.5	26	27.5	29	29	
mean	16	19	20	20	21.5	
± SEM	1.1	1.6	1.9	2.0	1.8	
c) Room Temperature	14	16	18	18.5	20.5	
	16	18	20	19	23	142
	18	20	22	22.5	24.5	
	15	16	17	18	20	245
	13	15	16	17	18	218
	19.5	23.5	27	28	29	
mean	16	18	20	20.5	22.5	
± SEM	1.4	1.9	2.5	2.5	2.4	

cephalin clotting times also increased during blood storage at all three temperatures (Table 19, Figure 26) though the increase during the first 2 days storage was only significant at 1°C and 4°C (p≤0.05), increasing from a mean of 43 seconds to 54 seconds at 1°C and to 50 seconds at 4°C. There was no mean change at room temperature. There were no further significant increases between consecutive readings, the kaolin cephalin clotting times remaining at about the same level at 1°C and 4°C, but slowly rising to a mean of 51 seconds after 14 days storage at room temperature. The increased time during the first 2 days storage was significantly higher at both 1°C and 4°C compared with room temperature (Students unpaired 't' test p≤0.05). There was no significant difference between the apparently longer increased kaolin cephalin clotting time at 1°C with the increased time at 4°C. No significant differences were observed between changes at any temperature after 2 days storage.

6.5 DISCUSSION OF THE INCREASED CLOTTING TIMES DURING BLOOD STORAGE

The change in the blood coagulation times at 4°C compared favourably with the results published by Mishler et al., (1978) for both the prothrombin time and kaolin cephalin clotting time. The results published by Limbird and Silver (1974) were produced as percentages of the control times, but demonstrated a sharp increase during the first 2 days storage for kaolin cephalin clotting time. Their results for prothrombin time showed little variation during the first 14 days blood storage.

The results showed that the decline in the efficiency of the extrinsic blood clotting system is particularly apparent during the first 2 days of blood storage, but with no statistical difference

TABLE 19

KAOLIN/CEPHALIN CLOTTING TIME (seconds)

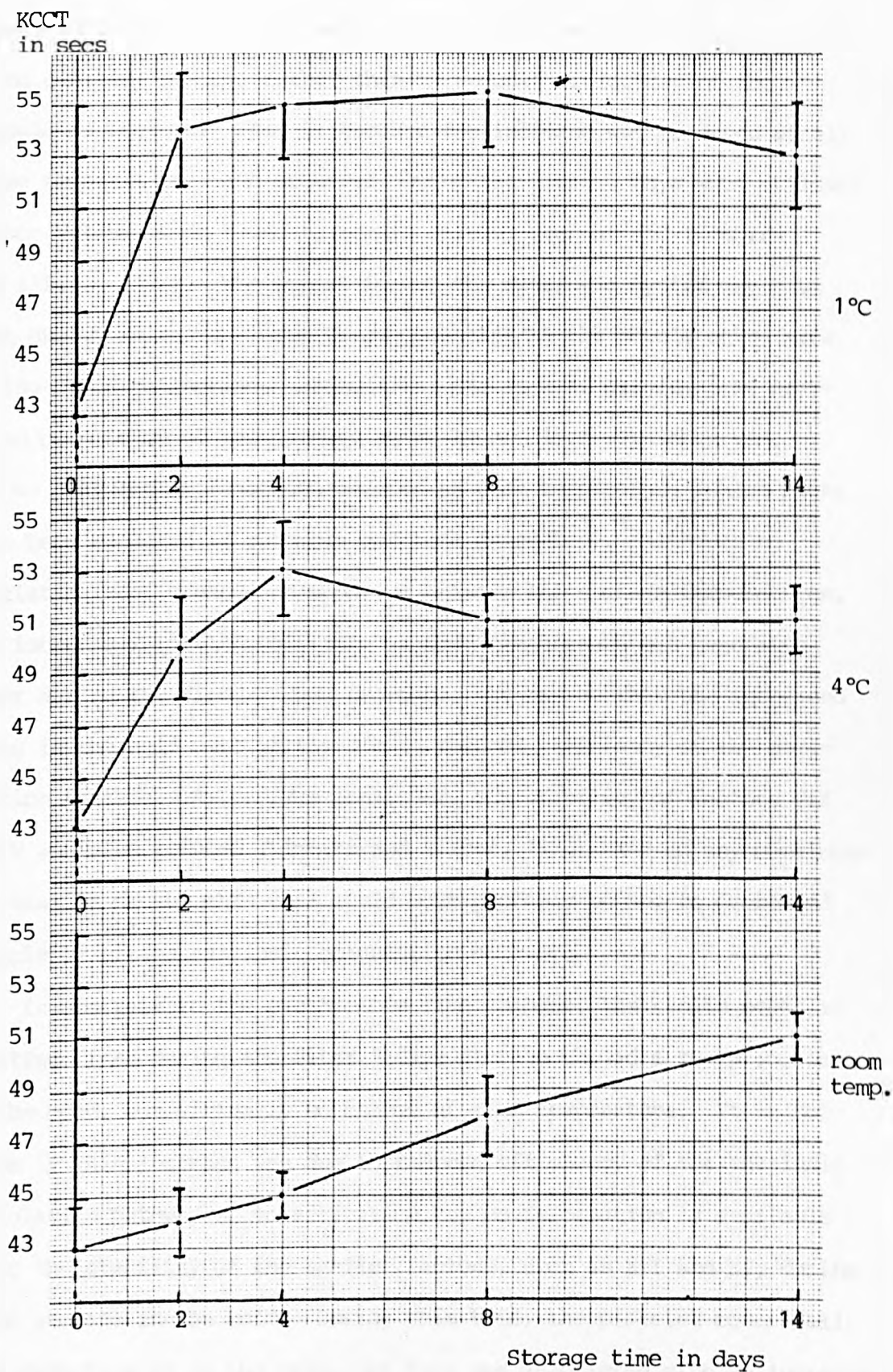|		Storage Time in Days					Platelet Count on Blood Collection ($\times 10^9 . L^{-1}$)
		0	2	4	8	14	
a)	1°C	41.5	52.5	52.5	53	51	177
		43	46.5	50	50	47	179
		40	54	56	56.5	57	254
		40	63	65	65	61	229
		46	56.5	57	57	52	211
		47	53.5	52	52	50	
	mean	43	54	55	55.5	53	
	± SEM	1.2	2.2	2.2	2.2	2.1	
b)	4°C	43	49.5	53.5	51	51.5	
		42.5	54.5	55	49.5	52.5	
		40	45	57	48	51	142
		43	50	51	52	47	245
		42	47	47	50	55	218
		47	55	55	54	50	
	mean	43	50	53	51	51	
	± SEM	1.1	2.0	1.8	1.0	1.3	
c)	Room	41.5	42	43	47	49	
	Temperature	39	40	46	43	52	142
		45	46.5	47	50	53.5	
		43	46	47	48	51	245
		42	44	43	49	48	218
		47	44.5	44	50	51	
	mean	43	44	45	48	51	
	± SEM	1.6	1.3	0.9	1.5	0.9	

Figure 26 CHANGES IN THE KAOLIN/CEPHALIN CLOTTING TIME ($\pm$ SEM)
DURING BLOOD STORAGE

between the three storage temperatures. It is possible that the
increased prothrombin time was related to activation of the extrinsic
blood clotting system, rather than to reduced efficiency of the
coagulated factors. This is because the increase was observed at all
three temperatures, and occurred during the first 2 days when at least
factor VII has been found to remain stable, even at 4°C (Limbird
and Silver, 1974). The activation of the extrinsic system may have
been due to released tissue thromboplastins at the needle entry site
during blood collection. Presumably, the lack of calcium ions prev-
ented completion of the pathway as no blood clots were observed,
and no platelet aggregation occurred at room temperature (which might
have been expected if thrombin had been generated). Although no
statistical difference was apparent between the storage temperatures,
the increase in prothrombin time at room temperature was generally
lower during the first 2 days storage. It may be that the increased
times in the cold were partly due to reduced efficiency of the coag-
ulation factors. As storage continued, the stability of factors VII
and V would be reduced (Limbird and Silver, 1974), and so any continued
increase in prothrombin time would most probably be due to decreased
efficiency of the extrinsic coagulation pathway.

In contrast to the prothrombin time changes, the kaolin cephalin
clotting times during the first 2 days storage showed a sharp increase
in the cold, but virtually no change at room temperature. It is not
known if this increase was due to reduced efficiency of the intrinsic
coagulation pathway or to activation, as no information is available
as to the stability of the earlier factors, such as XII and XI, during
blood storage in the cold. During this time, the platelet count fell
more dramatically in the cold, but this was associated with an increase

in platelet aggregation which was very apparent at 1°C (See Chapter 3). Platelets which are stimulated (as during aggregation), are known to become more effective blood clot promoting agents than unstimulated ones (Ehrman et al., 1978). They also have multiple sites of action in coagulation including the enhancement of activation of factor IX (Walsh, 1973). Factor IX is a zymogen, and its activation is thought to be its conversion to serine protease (Di Scipio et al., 1978). It has been suggested that the effect of an aggregating agent may be activation of a unique serine protease on the outer platelet membrane (Aoki et al., 1978), and because many coagulation factors are associated with the platelet membrane (See Chapter 1), it is possible that there is a relationship. This supports the hypothesis that platelet aggregation and blood coagulation are interdependent (Ardlie and Han, 1974; Akbar and Ardlie, 1978).

The apparent levelling off of the kaolin cephalin clotting time after 2 to 4 days blood storage at 1°C and 4°C suggests that either the coagulation factors had become totally unstable during this period and that no further increase in time is possible, or that all the available factors had become activated in the intrinsic coagulation pathway. The gradual increase in kaolin cephalin clotting time at room temperature at which no measurable platelet aggregation occurred, suggests a slow decline in coagulation efficiency.

Although it is probable that intrinsic blood coagulation was activated by platelet aggregate formation in the cold, and the extrinsic pathway by tissue thromboplastins, no blood clots were observed. This suggests that any coagulation pathway activation was incomplete possibly due to the lack of calcium ions, despite their release from platelets during aggregation. Also, blood coagulation is a system

- 152 -

which is delicately balanced and operates most efficiently in conditions which suit the enzyme catalysed sequence (Seegers, 1973). This includes temperature which is known to influence blood coagulation in-vitro - the colder it is, the longer the coagulation times (Dreher and Sutor, 1978). Therefore, blood clot formation in blood stored at 1°C or 4°C is likely to be a long process.

A GENERAL DISCUSSION OF THE FACTORS INVESTIGATED AND THEIR RELATION-
SHIPS WITH PLATELET AGGREGATION IN STORED BLOOD

A review of platelets and platelet aggregation has been presented.
Platelet aggregation occurs during the storage of blood in the cold
and the aggregates have been implicated in the aetiology of respiratory
distress syndrome following massive blood transfusions. Many sub-
stances have been shown to be able to aggregate blood platelets in-
vitro and in-vivo, but this investigation has examined some of those
factors that were thought most likely to influence platelet aggregation
during blood collection and storage. Although cold per se, is known to
be a potential platelet aggregating agent, the investigation was begun
at what was thought to be a storage temperature of 4°C, because this
is considered to be the most suitable temperature for the long term
storage of blood by the Blood Transfusion Service. Platelet aggregation
was measured in blood samples by the SFP technique, and it was sub-
sequently observed that a considerable difference in the rate and extent
of platelet aggregation occurred, which depended upon the storage temp-
erature. At 1°C, platelet aggregation was observed to be very rapid,
whilst at 4°C only a gradual increase in the SFP was generally measured
and this varied from donor to donor. A similar difference between
1°C and 4°C was observed in the fall in platelet counts. No measurable

platelet aggregation occurred at ambient room temperature (14°C to 21°C).

It was thought that a comparison of the concentrations of the measured parameters at the three storage temperatures might indicate their possible roles in platelet aggregation during blood storage. For logistical reasons and because several experiments had been carried out at 1°C, it was not possible to make all the comparisons between the three temperatures in any one experiment. Several experiments were performed in which a factor was measured at two of the storage temperatures, and several other experiments in which the same factor was measured at the remaining temperature. Because of the variation in the results between individual donors, it was not possible to compare the absolute results and so it was the change between consecutive mean results that were compared between the storage temperatures. It was considered that a minimum of six experiments would be adequate to permit comparisons on a relative basis between the means of the results. The changes at any one temperature for any one measured parameter were analysed for statistical significance using the Students paired 't' test.

It should be noted, however, that by using a small sample population, data for only one individual donor needed to show a different pattern to data from the other donors to make any mean increase or decrease insignificant. For several parameters, there were more results at one temperature than another and also, mainly in earlier experiments, some measurements were not made as frequently as in later ones. As a consequence, the comparison of changes between temperatures was made using the Students unpaired 't' test. For both

types of statistical analysis, significance was assumed at the 5%
probability level.

Parametric tests assume that the samples are random and are normally
distributed. The central limit theorem was applied to test for normal
distribution in each set of data. In this test, the mean of each
set of data was subtracted from each result in turn and divided by
the standard deviation for the set. 95% of the answers should be within
$\pm$ 1.96 if the distribution is normal. Application of the test showed
that the sets of data were normally distributed.

The parameters examined in this thesis and their relationships
with platelet aggregation have been discussed in each chapter. The
following serves as a summary of the more important observations and
comparison between the parameters.

Plasma adrenaline and noradrenaline concentrations were measured
in blood samples taken at the beginning and end of routine collections
of 450 ml of blood. The concentrations were found to vary considerably
between individuals, but the noradrenaline concentration was often
higher at the end of blood donation than at the beginning. The results
showed that the collection procedure and the blood volume reduction
had little effect on the concentrations of catecholamines in peripheral
venous blood, and the absolute values were well below those required
for the potentiation of platelet aggregation in-vitro by ADP. It is
possible that a more sensitive method for the measurement of plasma
catecholamines might have shown greater differences in the concentrations
of noradrenaline and adrenaline at the beginning and end of blood
collection. Recently, a kit has been made available in the U.S.A.
with a   sensitivity for plasma adrenaline down to 1 $pg.ml^{-1}$
(Upjohn), and a gas chromatography/mass spectrometry assay gave a

sensitivity limit of 2 pg.ml^{-1} for all three catecholamines (Ehrhardt and Schwartz, 1978). However, the latter assay gave plasma catecholamine concentrations in supine healthy volunteers which were very similar to the concentrations recorded at the beginning of blood collection in this thesis. The volunteers in this study all had normal blood pressures, but a future study could concentrate on hypertensive volunteers, who usually have an associated raised plasma catecholamine concentrations (Lancet, 1977b), to see if there were any increase in the rate of platelet aggregation in stored blood. Total plasma catecholamine concentrations were measured at intervals during blood storage. An increase in the concentrations was observed at all three temperatures but was most apparent at 1°C and least so at room temperature. It is not known to what extent the difference in concentration between temperatures was due to catecholamine degradation. Although catecholamines are not thought to be rapidly degraded in plasma or taken up into erythrocytes (see Chapter Two), it is probable that these would occur more readily at room temperature than in the cold. It seems unlikely that the difference in catecholamine concentration between the temperatures was entirely due to different degradation rates. It is more probable that the differences were due to the extent of platelet release or degradation. However, the catecholamine concentrations in plasma during 8 days of blood storage were below those that have been shown to influence platelet aggregation in-vitro.

In contrast to the plasma catecholamine concentrations, which continued to increase during blood storage, the measurement of plasma 5-HT showed an alternating increase and decrease in concentration during the first 4 days of blood storage, followed, generally, by a

gradual decrease. Once again, the change in plasma 5-HT concentration was most apparent at 1°C, though there was little apparent difference between the changes at 4°C and room temperature. The possible reasons for the fluctuating plasma 5-HT concentrations have been discussed (Chapter Four). The measured concentrations of plasma 5-HT were below those that have been shown to influence platelet aggregation. It seems that the measurement of plasma 5-HT was not suitable for monitoring platelet release and/or degradation, because of the alternate increase and decrease in concentration. The measurement of plasma catecholamine concentrations appeared to be more suitable for monitoring platelet release, but the inclusion of another parameter might have been useful. The concentration of a lysosomal enzyme such as acid phosphatase has been used as a marker of platelet destruction in stored blood (Polasek and Kubisz, 1968), though it may be released from other cell types. A platelet specific protein, such as β-thrombo-globulin has been suggested as being a suitable marker for the platelet release reaction (Ludlam et al., 1975; Pepper, 1977). A radio-immunoassay kit is now available for the measurement of plasma β-thromboglobulin concentrations (Radiochemical Centre, Amersham) and could be included in future investigations.

ADP is known to be a potent platelet aggregating agent and like catecholamines and 5-HT, is stored in platelets and released during the platelet release reaction (see Chapter One). It is also thought that ADP released from platelets may be the mediator of platelet second phase aggregation by several aggregating agents. A possible exception is the aggregating effect of cold (see Chapter Three). However, because of the probable importance of released ADP in platelet

aggregation, particular attention was paid to its possible role in platelet aggregation during blood storage. ADP concentrations were measured both in plasma and in whole blood and the cellular concentration calculated from these results. The mean plasma ADP concentration showed a decrease after 24 hours storage which was most apparent at room temperature and least so at 1°C. This was probably due to the rate of degradation in plasma which was found to be rapid, particularly at room temperature. This decrease in plasma ADP concentration was similar to that of plasma 5-HT. The plasma catecholamine concentration was not measured after 24 hours so it is not known how this varied during the storage period. The SFP had reached its measurable maximum of 600 mmHg after 24 hours blood storage at 1°C, and was often well above the normal range at 4°C. Because of the decrease in plasma 5-HT and ADP concentrations, this suggests that platelet release is not involved in the early stage of platelet aggregation in stored blood, an observation supported by Kattlove and Alexander (1971). It was possible that, if released, some of the ADP and 5-HT may have been bound to the platelet membranes and would, therefore, not have been measured in the plasma fraction. However, it is unlikely to have accounted for all released ADP and 5-HT, and had the release reaction occurred concomitant with the rapid increase in SFP at 1°C after 24 hours storage, then a nett increase in plasma 5-HT and ADP concentrations might have been expected.

Calculation of the mean cellular ATP concentration showed a marked decrease after 24 hours blood storage at all three temperatures, but particularly at 1°C. Since at this temperature, the SFP increased most rapidly, then as proposed in Chapter Five, it seems probable that a part of the energy consumed during the first 24 hours blood storage

in the cold was for platelet aggregation. If the energy requiring

release reaction was not involved during the first 24 hours of blood

storage, then energy may have been utilised in platelet contractile

processes during primary aggregation. This occurs before the release

reaction and secondary aggregation (see Chapter One). Primary platelet

aggregation is thought to consist of the formation of reversible,

loosely bound aggregates, and the electron micrographs of 24 hour old

blood samples showed these, (Figures 12 and 13, Chapter Four), though

the reversibility was not obvious from the SFP results. However, some

preliminary experiments were carried out adding chlorpromazine in a

final concentration of 2.8×10^{-4}M after 24 hours blood storage. At

1°C, no reversal in SFP measurements was observed, although because

600 mmHg was the measurable maximum, it is not known what might

have occurred if a higher pressure had been measurable. At 4°C

platelet aggregation was sometimes partly reversed, even after 48 hours

blood storage (Table 20). Nevertheless, these results do not suggest

that platelet aggregation in the first 24 hours of blood storage was

spontaneously reversible. Further evidence for the release reaction

occurring after the onset of aggregation during blood storage was

shown with the mean plasma 5-HT, catecholamine and ADP concentations

being increased after 48 hours storage particularly at 1°C. Since

5-HT and catecholamines are carried mainly in platelets, it suggests

that their increased concentration in plasma was due to either the

platelet release reaction or platelet degradation. However, the contin-

ued increase in plasma catecholamine concentrations up to 4 days blood

storage and the second increase in plasma 5-HT concentrations at 4 days,

suggest that the platelet contents were released as a consequence of

general degradation later than 2 days. Inhibition of ADP using apyrase

TABLE 20

THE CHANGE IN SFP UPON ADDITION OF CHLORPROMAZINE AT 2.8×10^{-4} M

TO BLOOD AFTER 48 HOURS STORAGE AT 4°C

0	1	2	3	4	8	14	(days)
26	120	190	90	85	65	40	
50	65	112	70	108	205	600	
48	140	180	105	145	200	530	
42	133	600	600	600	600	600	
52	67	345	600	600	600	600	

(50 μg.ml^{-1}) reduced the rate of platelet aggregation in stored blood at 1°C, but appeared to have no effect on blood stored at 4°C. This suggested that ADP did play a role in platelet aggregation at 1°C. It had been suggested that ADP released from erythrocytes may be important for platelet aggregation in blood in-vivo (Born et al., 1976) and this possibility was examined using chlorpromazine at 2.8 x 10^{-5}M. This concentration is thought to diminish or prevent ADP leakage from erythrocytes and was found to reduce the rate of platelet aggregation at 1°C but not at 4°C. It seems, therefore, that the onset of platelet aggregation in blood stored in the cold takes place without the mediation of platelet released ADP, 5-HT or catecholamines. Platelet aggregation at 1°C may occur more rapidly than at 4°C, because of ADP released from erythrocytes. The measurement of plasma haemoglobin concentrations during blood storage showed a marked increase at 1°C compared with blood stored at 4°C. This observation added weight to the probable involvement of erythrocytes in platelet aggregation at 1°C.

It was observed that particularly with plasma 5-HT concentrations, and to a lesser extent plasma catecholamines and plasma ADP concentrations, that platelet release at room temperature showed a similar pattern to that in the cold. It might, therefore, have been expected that platelet aggregation would have occurred, albeit relatively slowly, in blood stored at ambient room temperature. Although platelet aggregation at room temperature has been observed by other workers (Solis et al., 1975b) no platelet aggregation was measured at room temperature in this project using the SFP technique (Chapter Three). It therefore suggests that either the aggregating stimulus was not

present, or that aggregation was inhibited at room temperature. ADP is very rapidly degraded at room temperature and Born and Cross (1963) have shown that the products of ADP catabolism, adenosine and AMP, are potent inhibitors of platelet aggregation. Also, the pH of blood stored at room temperature decreased rapidly compared with blood stored in the cold. A reduced pH is associated with a reduced ability of platelets to aggregate. However, though the products of ADP catabolism and the reduced pH would probably have reduced platelet aggregation in blood stored at room temperature, the apparent lack of aggregation early in blood storage suggests that at room temperature, the initial aggregation stimulus was not present.

In an experiment with dog's blood, stored at 4°C, Wright (1975) showed that platelet aggregation was less marked in arterial blood than in venous blood. In another experiment, Wright (unpublished data) compared platelet aggregation in human arterial and venous blood samples. In this study, arterial blood was collected from the ante-cubital vein after the arterio-venous shunts in the forearm had been opened during submersion of the arm in hot water (45°C). In three volunteers, only one showed a marked difference between venous and arterial blood samples stored for 7 days at 4°C. The author wished to continue these studies but had difficulty in finding volunteers. However, two experiments were carried out in which arterial blood (450 ml) was collected from the radial artery in the wrist. In both experiments, similar SFP results were obtained as had been observed in venous blood from other blood donors at all three storage temperatures (Table 21a). It seems that in man, there is no advantage in collecting arterial blood so far as platelet aggregation is concerned, though in

TABLE 21

THE CHANGE IN SFP AND THE PLASMA CATECHOLAMINE

CONCENTRATION DURING THE STORAGE OF ARTERIAL BLOOD

a)

SFP (mm/Hg)	0	1	2	3	4	8	14	(days)
1°C	50	600						
	45	600						
4°C	50	35	55	75	110	360	600	
	45	68	420	600	600	600	600	
Room Temperature	50	50	62	55	48	42	44	
	45	53	55	50	45	46	46	

b) Plasma Catecholamines ($pg.ml^{-1}$)

		0	2	4	8	(days)
1°C		405	357	479	805	
		395	1133	1133	1776	
	mean	400	745	806	1291	
4°C		405	307	415	641	
		395	656	854	1277	
	mean	400	482	635	959	
Room Temperature		405	391	429	824	
		395	363	570	1134	
	mean	400	377	470	979	

view of the observations made with dog's blood, this may require further evaluation. It is interesting that the plasma catecholamine concentrations measured in both packs immediately after arterial blood collection seemed to be higher than the catecholamine concentrations in the majority of venous blood samples. However, there were similar increases in the plasma catecholamine concentration during arterial blood storage at 1°C, 4°C and room temperature as in venous blood (Table 21b [cf. Table 3, Chapter Two]).

The two basic blood coagulation tests for the intrinsic and extrinsic pathways were measured during blood storage. There was no apparent difference in the increased prothrombin times (extrinsic) between any of the three storage temperatures. However, the rapid increase in the kaolin-cephalin clotting time in the cold, particularly at 1°C, suggested a possible involvement of the intrinsic coagulation pathway in platelet aggregation during blood storage. It is unlikely, though, that during the early stages of blood storage in the cold, that the enzyme activated blood coagulation cascade would influence the extent of platelet aggregation. If the increased clotting time were due to factor activation rather than reduced efficiency, it is possible that only an early factor (perhaps factor XII) was activated so that subsequent measurement showed a longer clotting time. It is thought that fibrin may be involved in later stages of platelet aggregation in stored blood (Chapter Six). Disintegrating platelets may possibly be involved at this stage, by releasing polyunsaturated fatty acids which may become peroxidized (Gutteridge and Stocks, 1976). Peroxidized polyunsaturated fatty acids have been shown to have a marked effect on thrombin production in-vitro (Barrowcliff et al., 1975).

The evidence from the parameters measured in this investigation has shown that platelet aggregation during blood storage is induced in the cold and that blood storage at room temperature would be more suitable to reduce or prevent platelet aggregate formation. Although neither the release of catecholamines during blood collection, nor the release of catecholamines, 5-HT or ADP from platelets during blood storage seemed to have influenced the onset of platelet aggregation, it is possible that these factors may contribute to platelet aggregation later in blood storage. Individually, it would probably be only a small contribution, since their measured plasma concentrations were often too low to influence in-vitro aggregation, and the rate of platelet aggregation induced by ADP and adrenaline begins to decrease about 2 hours after venepuncture (Warlow et al., 1974). However, as discussed in Chapter One, it is thought that very low concentrations of many platelet aggregating agents can act synergistically. The combined effect of these agents was not examined in this investigation but may be of interest in a future study. Since at room temperature, there was also an increase in the plasma concentrations of 5-HT, ADP and the catecholamines, their synergistic effects might have been expected to induce platelet aggregation during blood storage at room temperature. However, it is likely that the catabolic products of ADP and the rapidly reduced pH, as mentioned earlier in the chapter, would have inhibited any such aggregation. Several other factors may contribute to platelet aggregation in stored blood. These include vasopressin, which has been considered (see Chapter One) and prostaglandins. It has been discussed (Chapter One) that prostaglandins may play an important role in the mechanisms of platelet aggregation, and may possibly be the link between the aggregating stimulus and platelet aggregation. In a recent

paper, Moretti and Abraham (1978) reported that various heme contain-ing compounds stimulated the synthesis of PGH_2 from PGG_2. In the presence of aromatic amines (which include catecholamines) these compounds produced an even greater stimulation of overall prostaglan-din synthesis. However, although both plasma haemoglobin and plasma catecholamine concentrations have been shown to increase rapidly in blood stored in the cold, platelet aggregation at 1°C occurred very rapidly. This suggests that the initial aggregating stimulus would have had to be present either on blood collection or within the first few hours of blood storage. However, an increased concentration of prostaglandins in the circulation has only been observed in severe circulatory shock. and are mainly of the PGE_1 type which have been shown to powerfully inhibit platelet aggregation (Bridenbaugh and Lefer, 1976). Therefore, it is most unlikely that platelet aggregating prostaglandins are released during blood collection, and because cold would inhibit or reduce prostaglandin synthesis, it is unlikely that prostaglandins play any significant role in platelet aggregation during blood storage.

Another factor which may have contributed to platelet aggregation in stored blood is platelet aggregating factor (PAF). PAF is a low molecular weight phospholipid released from IgE sensitized basophils. PAF may stimulate platelets to secrete, in a non-cytotoxic manner, their granular contents, and also initiate in-vitro platelet aggre-gation by a mechanism not thought to involve ADP (Henson and Oades, 1976). Though basophils may degrade quickly during blood storage (Lachman, 1977 - personal communication), it is difficult to imagine what would stimulate PAF release during blood collection, and, if

released, why it should not effect platelets at room temperature.

Protein denaturation is another possible cause of platelet
aggregation. Proteins may be denatured by contact with an interface
between air and blood. The denaturation occurs when strong electrostatic
forces exert a polarizing effect that results in the orientation of a
monomolecular layer of globular protein molecules on the interface.
Because of the molecular configuration of the protein molecules, the
polarizing forces disrupt the sulphydryl and/or hydrogen bonds which
stabilize the secondary and tertiary structure of the protein molecule
(Lee et al., 1961). These denatured protein molecules can adsorb
to the surface of erythrocytes and possibly platelets, and intra-
molecular aggregation may then ensue (Lee and Hairston, 1971).
Although it is possible to visualize these events occurring during
blood collection and storage, it is unlikely to be extensive and would
take place at all storage temperatures.

Platelet aggregation by complement activation may also be con-
sidered, particularly as Clq receptors have been demonstrated on human
platelets (Wautier et al., 1976). However, platelets are not thought
to be aggregated by conventional complement activation (Henson, 1969;
Lachman, 1977 - personal communication), and as with the coagulation
pathway, complement activation, is thought to be an enzyme dependent
cascade, which would be reduced at low temperatures.

There may be many factors that contribute to platelet aggregation during blood storage. Some of these factors have been examined, and others have been considered in the introduction and in the previous section. None of the examined factors have been found to be the initiator of platelet aggregation during blood storage, though ADP released from erythrocytes most probably contributed to platelet aggregation in blood stored at 1°C. These, and other factors, acting synergistically, may have extended platelet aggregation during blood storage. Platelet aggregation, as measured by the SFP technique, occurred only in the cold, and cold has been shown by other workers to initiate platelet aggregation. It is, therefore, possible that the blood storage temperature may be the basic criterion for platelet aggregation. The possible mechanism by which cold induces platelets to become sticky and aggregate spontaneously, have been discussed in Chapter Three. Kattlove and Alexander (1971) concluded that this type of aggregation depended upon the intactness of the platelet metabolic pathways, and was not mediated by ADP released from platelets. The results in this thesis lend support to these observations by showing that the onset of platelet aggregation in cold stored blood is possibly an energy dependent process, and that it may occur in the absence of the platelet release reaction. Cold may also have an indirect effect by reducing normal platelet metabolism and consequently reducing the decrease in pH. This is thought to be an important requirement for platelet aggregation; aggregation being gradually inhibited as the pH is reduced. It may be the lower pH of ACD

anticoagulated blood, compared with CPD, which accounted for the obser-
ved reduced platelet aggregation in ACD blood at 4°C (Wright and Sand-
erson, 1974; Solis et al., 1974b). Cold may also reduce the rate of
breakdown of ADP to its platelet aggregation inhibitory products, AMP
and adenosine.

The great majority of donor blood, however, is stored as whole blood
in the cold, and in situations were massive blood transfusions are
needed, the microfiltration of stored blood is widely accepted so that
the potential risk of transfusing platelet aggregates is reduced.
However, blood filters are expensive, and filter efficiency and filter
induced haemolysis is seemingly inversely correlated with blood flow
rates (Kennedy et al., 1977). So in the absence of fresh blood, when
large amounts of blood are rapidly required, filter selection must
balance the decisions regarding the risk of microembolization against
the need for rapid flow properties. Ideally, blood for transfusion
should contain viable cells and not require filtration. Additives
such as adenine and inosine have for some time been known to improve the
viability of platelets stored at 4°C (Baldini et al., 1960), and in
Sweden, adenine is regularly added to the anticoagulant to maintain
2,3 DPG, ATP and the post transfusion viability of erythrocytes in cold
stored blood (Kreuger et al., 1975). Prostaglandin E_1, a potent plate-
let aggregation inhibitor, when added to freshly collected blood per-
mitted 75% of viable platelets to be isolated from blood stored for 3
days at 4°C (Aster et al., 1976), though this degree of success was not
found in all studies (Valeri et al., 1972). Also, acetyl salicylic
acid when added to freshly collected blood reduced subsequent platelet
aggregation by 50% during storage at 4°C (Gervin et al., 1975).
However, the addition of any additives to stored blood is probably

better avoided because of their unknown effects. Continuous agitation of blood packs during storage at 4°C reduces platelet aggregation, though this may be a consequence of platelet destruction (Gervin et al., 1978). The storage of platelet concentrates at room temperature is already widely practised because of better maintained platelet viability and the results in this thesis have demonstrated that platelet aggregation did not occur in blood stored at room temperature. Also, the plasma catecholamine concentrations increased and platelet counts decreased more slowly at room temperature than in the cold, suggesting that platelet integrity was better maintained at room temperature. It would therefore seem that blood stored at room temperature would eliminate the need for filtration, and better maintain platelet viability. Considerable work would need to be undertaken to confirm that other cells and functions were better maintained in whole blood stored at room temperature. However, from the studies conducted in this thesis, it has been shown that the intrinsic blood clotting system is probably better maintained at room temperature than in the cold. Also, the measurement of plasma haemoglobin concentrations have shown that erythrocyte integrity is better maintained at room temperature. It has been shown by McCullough et al., (1978) that granulocyte chemotaxis is better preserved at room temperature than in blood stored between 1°C and 6°C.

Perhaps the most serious potential disadvantage of blood storage at room temperature is the increased risk of bacterial contamination and proliferation (Katz and Tilton, 1970; Murphy and Gardner, 1975). Although blood collection and storage is a closed system, the major risk of contamination, apart from carelessness and damage, is the skin plug that may enter the blood pack during phlebotomy. Bacteria that have

been found in blood packs include Escherichia coli, Staphylococcus epidermides, Enterobacter cloacae, and Pseudomonas aeruginosa (Kahn and Syring, 1975). All these proliferate at room temperature. However, a study by Silver et al., (1970), isolated no organisms from cultures from 40 platelet concentrates stored at 30°C, and sampled at intervals for 4 days. This suggests that the risk of bacterial contamination is minimal.

Another disadvantage of blood storage at room temperature is the rapid decrease in pH, due to lactic acid production and CO_2 retention. A fall in pH is one of the principal causes of gradual damage to erythrocytes (Mendonca et al., 1978) and at a pH of 6, platelet viability is lost (Murphy and Gardner, 1975). Although the permeability of plastic bags to gases means the blood can be maintained at a higher pH by controlling O_2 and CO_2 tensions in the ambient air, this presents practical difficulties. However, in this thesis, the pH of blood stored at room temperature for 14 days never decreased to a pH at which platelet viability would be lost. Nevertheless, despite the lack of platelet aggregation at room temperature, because of the unknown effects of long term room temperature blood storage, and because of the risk of bacterial proliferation, cold storage of blood will probably continue. An extensive study would be necessary, comparing blood cell functions at a variety of storage temperatures to elucidate the optimum storage temperature. However, the results in this thesis have demonstrated considerable differences in the behaviour of platelets and erythrocytes in blood stored at 1°C and at 4°C. It would therefore seem highly recommendable not to maintain a storage temperature below 4°C.

REFERENCES

ADAM, H. (1962)

Methods in Enzymatic Analysis, 1st Edition

VERLAG CHEMIE I 539

ADAM, H. (1970)

Methods in Enzymatic Analysis, 2nd Edition

VERLAG CHEMIE II 2051

AKBAR, H., ARDLIE, N. G. (1978)

Further evidence for the role of thrombin in the platelet release

reaction caused by various agents, and the nature of biphasic platelet

aggregation.

BRIT. J. HAEMATOL. 38 381 - 390

AKKERMAN, J. W. N. (1978)

Regulation of carbohydrate metabolism in platelets. A review.

THROMBOS. HAEMOSTAS. 39 712 - 724

ALAGILLE, D., SOULIER J-P. (1957)

Le test d'activite plaquettaire du serum

REV. FRANC. ETUDES CLIN. ET BIO. 2 231 - 246

ALEDORT, L. M., PUSZKIN, S., PUSZKIN, E., HANSON, J., KATZ, A. M. (1973)

Control of platelet contraction

MICROVASC. RES. 6 246

ALEXANDER, R. W., COOPER, B., HANDIN, R. I. (1978)

Characterisation of the human platelet ∝-adrenergic receptor.

J. CLIN. INVEST. 61 1136 - 1144

ANDEN, N. E., MAGNUSSON, T. (1967)

An improved method for the fluorimetric determination of 5-HT in tissues.

ACTA PHYSIOL. SCAN. $\underline{69}$ 87 - 94

ANTON, A. H., SAYRE, D. F. (1962)

A study of the factors affecting the Al_2O_3 - THl procedure for the

analysis of catecholamines.

J. PHARM. EXP. THER. $\underline{138}$ 360 - 375

AOKI, N., NAITO, K., YOSHIDA, N. (1978)

Inhibition of platelet aggregation by protease inhibitors, possible involvement of proteases in platelet aggregation.

BLOOD $\underline{52}$ 1 - 12

ARDLIE, N. G., HAN, P. (1974)

Enzymatic basis for platelet aggregation and release: the significance

of the "platelet atmosphere" and the relationship between platelet function and blood coagulation

BRIT. J. HAEMATOL. $\underline{26}$ 331 - 356

ARONOW, W. S., HARDING, P. R., DEQUATTRO, V., ISBELL, M. (1973)

Diurnal variation of plasma catecholamines and systolic time intervals.

CHEST $\underline{63}$ 722 - 726

ARRINGTON, P., MCNAMARA, J. J. (1974)

Mechanism of microaggregate formation in stored blood

ANN. SURG. $\underline{179}$ 146 - 148

ARRINGTON, P. J., MCNAMARA, J. J. (1975)

Effect of agitation of platelet aggregation and microaggregate formation

in banked blood.

ANNALS. SURG. $\underline{181}$ 243 - 244

ASTER, R. H., BECKER, G. A., FILIP, D. J. (1976)

Studies to improve methods of short term platelet preservation.

TRANSFUSION 16   4 - 7

BAILEY, D. N., BOVE, J. R. (1975)

Chemical and hematological changes in stored CPD blood

TRANSFUSION 15   244 - 249

BALDINI, M., COSTEA, N., DAMESHEK, W. (1960)

The viability of stored human platelets.

BLOOD 16   1669 - 1692

BALDINI, M. G., STEINER, M., KIM, B. K. (1976)

Permeability changes in the plasma and granular membrane of stored

platelets. Their prevention for improved platelet preservation.

TRANSFUSION 16   17 - 19

BARRER, M. J., ELLISON, N. (1977)

Platelet Function

ANAESTH. 46   202 - 211

BARRETT, J., DE JONGH, D. S., MILLER, E., LITWIN, M. S. (1976)

Microaggregate formation in stored human packed cells.

ANN. SURG. 183   109 - 113

BARRETT, J., TAHIR, A. H., LITWIN, M. S. (1978)

Increased pulmonary arteriovenous shunting in humans following blood

transfusion.

ARCH. SURG. 113   947 - 950

BARROWCLIFFE, T. W., GUTTERIDGE, J. M. C., DORMANDY, T. L. (1975)

The effect of fatty acid autoxidation products on blood coagulation.

THROMBOS. DIATHES. HAEMORRH. 33 271 - 277

BARTHEL, W., MARKWARDT, F. (1974)

Aggregation of blood platelets by biogenic amines and its inhibition by

·antiadrenergic and antiserotoninergic agents.

BIOCHEM. PHARMACOL. 23 37 - 45

BAUMGARTNER, H. R. (1969)

5-HT, uptake and release in relation to aggregation of rabbit platelets.

J. PHYSIOL. 201 409 - 423

BAUMGARTNER, H. R. (1970)

Effects of 4-hydroxytryptamine on platelet aggregation.

TRANSACTIONS OF XVIIIth ANNUAL SYMP. ON BLOOD 21 - 35

BAUMGARTNER, H.R., BORN, G. V. R. (1969)

The relation between the 5-HT content and aggregation of rabbit platelets.

J. PHYSIOL. 201 397 - 408

BECKER, G. A., ASTER, R. H. (1972)

Platelet transfusion therapy

MED. CLINICS. OF N. AMER. 56 81 - 94

BECKER, G. A., KUNICKI, T., ASTER, R. H. (1974)

Effect of prostaglandin E_1 on harvesting of platelets from refrigerated

whole blood.

J. LAB. CLIN. MED. 83 304 - 309

BENESCH, R., BENESCH, R. E.   (1969)

Intracellular organic phosphates as regulators of oxygen release by

haemoglobin.

NATURE 221 618 - 622

BEN-JONATHAN, N., PORTER, J. G.   (1976)

A sensitive radioenzymatic assay for dopamine, norepiniphrine and

epiniphrine in plasma and tissue.

ENDOCRINOLOGY 98 1497 - 1507

BENNER, K. U., BRUNNER, R. (1973)

Cold induced platelet aggregation in-vivo and its inhibition by a non-

ionic surface active substance.

THROMB. RES. 2 331 - 342

BENNET, B., RATNOFF, O. D. (1972)

The normal coagulation mechanism

MED. CLINICS OF NORTH AMERICA 56 95 - 104

BENNET, S. H., GEELHOED, G. W., AARON, R. K., SOLIS, R. T., HOYE, R. C.

(1972)

Pulmonary injury resulting from perfusion with stored bank blood in baboon

and dog.

J. SURG. RES. 13 295 - 306

BLAKNEY, G. B., DINWOODIE, J. J. (1975)

A spectrophotometric scanning technique for the rapid determination of

plasma haemoglobin.

CLIN. BIOCHEM. 8 96 - 102

BLUNDELL, J. (1818)

Experiments of the transfusion of blood by the syringe.

MEDICO-CHIRURGICAL TRANS. 56 - 92

BOHLEN, P., STEIN, S., DAIRMAN, W., UNDENFRIEND, S. (1973)

Fluorometric assay of proteins in the nanogram range

ARCH. BIOCHEM. BIOPHYS. 155 213 - 220

BOOYSE, F. M., GUILIANI, D., RAFELSON, M. E. (1973)

Cyclic adenosine 3'5' monophosphate dependent protein kinase of human

platelets: membrane phosphorylation and regulation of platelet function.

MICROVASC. RES. 6 244

BORN, G. V. R. (1956)

ATP in blood platelets

BIOCHEM. J. 62 33

BORN, G. V. R. (1962)

Aggregation of blood platelets by ADP, and its reversal

NATURE 194 927 - 929

BORN, G. V. R. (1965)

Uptake of adenosine and of adensine diphosphate by human blood platelets.

NATURE 206 1121 - 1122

BORN, G. V. R. (1967)

Possible mechanisms of platelet aggregation by ADP and of its inhibition.

THROMB. DIATH. HAEMORRH. 26 173 - 174

BORN, G. V. R., BERGQUIST, D., ARFORS, K. E. (1976)

Evidence for inhibition of platelet activation in blood by a drug

effect on erythrocytes.

NATURE 259 233 - 235

BORN, G. V. R., CROSS, M. J. (1963)

The aggregation of blood platelets.

J. PHYSIOL. 168 178 - 195

BORN, G. V. R., CROSS, M. J. (1964)

Effects of inorganic ions and of plasma proteins on the aggregation

of blood platelets by adenosine diphosphate.

J. PHYSIOL. 193 405 - 418

BORN, G. V. R., MILLS, D. C. B., ROBERTS, G. C. K. (1967a)

Potentiation of platelet aggregation by adrenaline.

J. PHYSIOL. 191 43 - 44P

BORN, G. V. R., DAY, M., STOCKBRIDGE, A. (1967b)

The uptake of amines by human erythrocytes in vitro.

J. PHYSIOL. 193 405 - 418

BRIDENBAUGH, G. A., LEFER, A. M. (1976)

Influence of humoral shock factors on in-vitro aggregation of dog

platelets.

THROMBOS. RES. 8 599 - 606

BRITTON, B. J., HAWKEY, C., WOOD, W. G., PEELE, M. (1974)

Stress - a significant factor in venous thrombosis?

BRIT. J. SURG. 61 814 - 820

BROEKMAN, M. J., HANDIN, R. I., COHEN, P. (1975)

Distribution of fibrinogen and platelet factor 4 and XIII in

subcellular fractions of human platelets.

BRIT. J. HAEMATOL. 31 51 - 54

BROWN, CH. H. LEMUTH, R. F. HELLUMS, J. D., LEVERETT, L. B., ALFREY,

C. P. (1975)

Response of human platelets to sheer stress.

TRANS. AMER. SOC. ARTIF. INT. ORGANS 21 35 - 39

CALLINGHAM, B. A. (1975)

Catecholamines in blood.

HANDBOOK OF PHYSIOL. 6 ENDOCRINOLOGY 437 - 445, Edt. by BLASCHKO, H.,

SAYERS, G., & SMITH, A. P. - AM. PHYSIOL. SOC.

CALLINGHAM, B. A., BARRAND, M. A. (1976)

Catecholamines in blood.

J. PHARM. PHARMAC. 28 356 - 360

CARRUTHERS, M. (1975)

Biochemical responses to stress in the environment.

PROC. ROY. SOC. MED. 68 429 - 430

CARRUTHERS, M., TAGGART, P., CONWAY, N., BATES, D., SOMERVILLE, W. (1970)

Validity of plasma catecholamine estimations.

LANCET ii 62 - 67

CHESNEY, C., SAMBERG, E., HARPER, E., COLMAN, R. W. (1972)

Human platelet collagenase: an enzyme liberated during the release reaction.

BLOOD 40 953

CHIEN, S. (1967)

Role of the sympathetic nervous system to haemorrhage.

PHYSIOL. REV. 47 214 - 288

CHIGNARD, M., VARGAFTIG, B. B. (1976)

Dog platelets fail to aggregate when they form aggregating substances upon stimulation with arachidonic acid.

EUROP. J. PHARMACOL. 38 7 - 18

CHOULIS, N. B. (1967)

Separation and quantitative determination of adrenaline using thin
layer chromatography.

J. PHARMACEUT. SCI. <u>56</u> 196 - 199

COHEN, G., HOLLAND, B., GOLDENBERG, M. (1958)

The stability of epiniphrine and arterenol (norepiniphrine) in

plasma and serum.

ARCH. NEUR. PSYCHIAT. <u>80</u> 484 - 487

COLLER, B. S., FRANZA, B. R., GRALNICK, H. R. (1976)

The pH dependence of quantitative Ristocetin induced platelet aggre-

gation: Theoretical and practical implications - a new device for

maintenance of platelet rich plasma pH.

BLOOD <u>47</u> 844 - 854

COLLINS, J. A. (1974)

Problems associated with the massive transfusion of stored blood.

SURGERY <u>75</u> 274 - 295

CONNELL, R. S., SWANK, R. L. (1973)

Pulmonary microembolism after blood transfusion: an electron

microscopic study.

ANNALS. SURG. <u>177</u> 140 - 150

CONNELL, R. S., SWANK, R. L., WEBB, M. C. (1973)

Secondary microembolism after transfusion: an electron microscopy study.

BIORHEOLOGY 10 561 - 564

COPLEY, A. L., WITTE, S. (1976)

On physiological microthromboembolization as the primary platelet function. Elimination of invaded particles from the circulation and its pathogenic significance.

THROMBOSIS RES. 8 251 - 262

COYLE, J. T., HENRY, D. (1973)

Catecholamines in foetal and newborn rat brain.

J. NEUROCHEM. 21 61 67

CRITCHLEY, J. A. J. H., WEST, C. P. (1977)

Peripheral venous plasma catecholamine estimations.

LANCET i 8023, 1203

CROSBY, W. H., FURTH, F. W. (1956)

A modification of the benzidine method for the measurement of hemoglobin in plasma and urine.

BLOOD 11 380 - 386

DACIE, J. V., LEWIS, S. M. (1975)

Practical Haematology, 5th Edition

CHURCHILL LIVINGSTONE, LONDON

DAWES, J., SMITH, R. C., PEPPER, D. S.   (1978)

The release, distribution and clearance of human β-thromboglobulin and

platelet factor 4.

THROMB. RES. 12 851 - 861

DAWIDSON I.   (1977)

Microfiltration of stored blood - present status and clinical indica-

tions

REPORT TO THE 16th SESSION OF THE SUBCOMMITTEE OF SPECIALISTS ON BLOOD

PROBLEMS COUNCIL OF EUROPE, FRANKFURT

DAWIDSON, I., BARRETT, J. A., MILLER, E., LITWIN, M. S.   (1975)

Pulmonary microembolism associated with massive transfusion.

ANN. SURG. 181 51 - 57

DAY, J. H., HOLMSEN, H., HOVIG, T.   (1969)

Subcellular particles of human platelets.

SCAND. J. HAEMATOL. SUPPL. 7

DE CHAMPLAIN, J., FARLEY, L., COUSINEAU, D., VAN AMERINGEN, M-R. (1976)

Circulating catecholamine levels in human and experimental hypertension.

CIRC. RES. 38 109 - 114

DEISSEROTH, A., WOLFE, S. M., SHULMAN, N. R.   (1970)

Platelet phosphorylase activity in the presence of activators and

inhibitors of aggregation.

BIOCHEM. BIOPHYS. RES. COMM. 39 551 - 557

DETWILER T. C., FEINMAN, R. D. (1973)

The platelet release reaction: kinetics of thrombin induced release of ATP

FED. PROC. <u>32</u> 220

DIAMANT, J., BYERS, S. O. (1975)

A precise catecholamine assay for small plasma samples.

J. LAB. CLIN. MED. <u>85</u> 678 - 693

DI SCIPIO, R. G., KURACHI, K., DAVIE, E. W. (1978)

Activation of human factor IX (Christmas factor).

J. CLIN. INVEST. <u>61</u> 1528 - 1538

DOCUMENTA GEIGY SCIENTIFIC TABLES (1962)

6th Edition, Ed. K. Diem, GEIGY, MANCHESTER

DREHER, A., SUTOR, A. H. (1978)

Influence of temperature on blood coagulation in-vitro.

BLUT <u>36</u> 231 - 238

DRUMMOND, A. H., GORDON J. L. (1975)

Specific binding sites for 5HT on rat blood platelets.

BIOCHEM. J. <u>150</u> 129 - 132

D'SOUZA L., GLUECK, H. I. (1977)

Measurement of nucleotide pools in platelets using high pressure liquid
chromatography.

THROMBOS. HAEMOSTAS. <u>38</u> 990 - 1001

EHRHARDT, J. D., SCHWARTZ, J. (1978)

A gas chromatography - mass spectrometry assay of human plasma catechol-amines.

CLIN. CHIM. ACTA. __88__ 71 - 79

EHRMAN, M., TOTH, E., FROJMOVIC, M. (1978)

A platelet procoagulant activity associated with platelet shape change

J. LAB. CLIN. MED. __92__ 393 - 401

EIK^A, C. (1973)

Anticoagulant and platelet aggregating activities of heparin.

THROMB. RES. __2__ 349 - 360

ENGELMAN, K., PORTNOY, B. (1970)

A sensitive double isotope derivative assay for norepiniphrine and epiniph-rine.

CIRC. RES. __26__ 53 - 57

ESTES, J. W., MCGOVERN, J. J., GOLDSTEIN, R., ROTA, M. (1962)

Stability of certain coagulation factors and metabolic activities of platelets in stored blood.

J. LAB. CLIN. MED. __59__ 436 - 444

FANTUS, B. (1938)

Blood preservation technic.

THERAPEUTICS __III__ 317 - 321

FEINBERG, H., BELAMARICH, F., BORN, G.V.R (1973)

Transphosphorylation in human and rabbit platelet suspensions.

FED. PROC. __32__ 220

FELIX, A. M., JIMENEZ, M. H. (1974)

Usage of fluorescamine as a spray reagent for TLC.

J. CHROMATOGRAPHY 89 361 - 364

FIESSLY, R., LUDIN, H. (1949)

Microscopie par contrastes des phases. III - Application à l'hematologie

REVUE HÉMAT. 4 481 - 501

FILIP, D. J., ASTER, R. H. (1978)

Relative hemostatic effectiveness of human platelets stored at 4° and 22°C

J. LAB. CLIN. MED. 91 618 - 624

FLUCK, D. C., SALTER, CHERYL. (1973)

Effect of tilting on plasma catecholamine levels in man.

CARDIOVASC. RES. 7 823 - 826

FOWLE, A. S. E. (1968)

The physiology of shock

BRIT. J. HOSP. MED. 1 307 - 311

FRANZEN, F., EYSELL, K. (1969)

Biologically active amines found in man.

PERGAMON PRESS, OXFORD

FRANZEN, F., GROSS, H. (1965)

Tryptamine. N,N.dimethyltryptamine, N,N,dimethyl 5 hydroxytryptamine

and 5 methoxytryptamine in human blood and urine.

NATURE 206 1052,

FUKAMI, M. H., SALGANICOFF, L.   (1977)

Human platelet storage organelles

THROMBOS. HAEMOSTAS. 38 963 - 970

GAARDER, A., JONSEN, J., LALAND, S., HELLEM, A., OWREN, P. A.   (1961)

Adenosine diphosphate in red cells as a factor in the adhesiveness of

human blood platelets.

NATURE 192 531 - 532

GARNER, R.   (1973)

Haematological changes in dogs subjected to extracorporeal circulation

of blood or to endotoxin induced shock.

M.Sc. THESIS, UNIVERSITY OF KEELE

GERRARD, J. M., WHITE, J. G., RAO, G. H. R., KRIVIT, W., WITKOP, C. J.

(1975)

Labile aggregation stimulating substance (LASS): The factor from

storage pool deficient platelets correcting defective aggregation and

release from aspirin treated normal platelets.

BRIT. J. HAEMATOL. 29 657 - 665.

GERVIN, A. S., MASON, K. G., WRIGHT, C. B. (1974a)

Microaggregate volumes in stored human blood

SURG. GYNAECOL. & OBSTET. 139 519 - 524

GERVIN, A., MASON, K., GIRARD, C. HALL, L.   (1974b)

Source of microaggregates in stored human blood

CLIN. RES. 22 177

GERVIN, A. S., MASON, K. G., BUCKMAN, R. F.   (1975)

The source and removal of microaggregates in aged human blood and

human blood components.

SURG. GYN. OBSTET.   141   582 - 586

GERVIN, A. S., MASON, K. G., BUCKMAN, R. F. (1978)

The effect of agitation of stored human blood on microaggregate form-

ation.

TRANSFUSION 18   73 - 78

GIORDANO, J., ZINNER, M., HOBSON, R. W., GERVIN. A. (1976)

The effect of microaggregates in stored blood on canine pulmonary

vascular resistance.

SURGERY 80   617 - 623

GORDON, J. L., BOWYER, D. E., EVANS, D. W., MITCHINSON, M. J. (1973)

Human platelet reactivity during stressful diagnostic procedures.

J. CLIN. PATH.   26   958 - 962

GRAND, M. G., DOWNING, S. E. (1970)

Metabolic and reflex influences on pulmonary vasomotion.

AM. J. PHYSIOL.   218   654 - 661

GRYGLEWSKI, R. J., KORBUT, R., OCETKIEWICZ, A. (1978)

Generation of prostacyclin by lungs in vivo and its release into the

arterial circulation

NATURE 273   765 - 767

GUTTERIDGE, J. M. C., STOCKS, J. (1976)

Peroxidation of cell lipids.

MED. LAB. SCI. _33_ 281 - 285

HAGGENDAL, J. (1963)

An improved method for fluorimetric determination of small amounts of

adrenaline and noradrenaline in plasma and tissues.

ACTA PHYSIOL. SCAN. _59_ 242 - 254

HARADIN, A. R., WEED, R. I., REED, C. F. (1969)

Changes in physical properties of stored erythrocytes relationship to

survival in-vivo.

TRANSFUSION _9_ 229 - 237

HARDEMAN, M. R., HEYNENS, C. J. L., (1974a)

Storage of human blood platelets. The serotonin uptake and hypotonic

shock response as in-vitro viability tests.

THROMB. DIATH. HAEMORRH. _32_ 405 - 416

HARDEMAN, M. R., HENENS, C. J. L. (1974b)

The active serotonin uptake and the hypotonic shock response of human

blood platelets.

THROMBOS. DIATHES. HAEMORRH. _32_ 391 - 404

HARDISTY, R. M., INGRAM, G. I. (1965)

Bleeding disorders. Investigation and management

1st ED. BLACKWELL OXFORD.

HARP, J. R., WYCHE, M. Q., MARSHALL, B. E., WURZEL, H. A.   (1974)

Some factors determining rate of microaggregate formation in stored

blood.

ANAESTH. 40 398 - 400

HARRISON, M. J. G., MITCHELL, J. R. A.   (1966)

The influence of red blood cells on platelet adhesiveness

LANCET ii 1163 - 1164

HASLAM, R. J. (1964)

Role of adenosine diphosphate in the aggregation of human blood

platelets by thrombin and by fatty acids.

NATURE 202 765 - 768

HASLAM, R. J., DAVIDSON, M. M. L., McCLENAGHAN, M. D. (1975)

Cytochalasin B, the blood platelet release reaction and cyclic GMP

NATURE 253 455 - 457

HASLAM, R. J., MILLS, D. C. B.   (1967)

The adenylate kinase of human plasma, erythrocytes and platelets

in relation to the degradation of ADP in plasma.

BIOCHEM. J. 103 773 - 784

HASLAM, R. J., LYNHAM, J. A. (1973)

Activation and inhibition of blood platelet adenylate cyclase by

adenosine or by 2 chloradenosine

LIFE SCIENCES 11 1143 - 1154   .

HASLAM, R. J., McCLENAGHAN, M. D. (1974)

Effects of collagen and aspirin on the concentration of cyclic GMP

in human blood platelets.

BIOCHEM. J. 138 317 - 320

HASLAM, R. G., ROSSON, G. M. (1971)

Effect of vasopressin on human blood platelets.

J. PHYSIOL. 219 36P - 38P

HENSON, P. M. (1969)

Role of complement and leukocytes in immunologic release of vasoactive

amines from platelets.

FED. PROC. 28 1721 - 1728

HENSON, P. M., OADES, Z. G. (1976)

Activation of platelets by platelet activating factor derived from IgE

sensitized basophils. 11. The role of serine proteases, cyclic nucleo-

tides, and contractile elements in PAF induced secretion.

J. EXP. MED. 143 953 - 968

HEPTINSTALL, S. (1976)

The use of a chelating ion-exchange resin to evaluate the effects of

the extracellular calcium concentration on adenosine diphosphate

induced aggregation of human blood platelets.

THROMBOS. HAEMOSTAS. 36 208 - 220

HIRSH, J. (1977)

Clinical effects of platelet function suppressing drugs.

FROM PLATELETS AND THROMBOSIS, ED. MILLS, D. C. B., PARETI, F. I.,

ACADEMIC PRESS, LONDON

HOLME, S., VAIDJA, K., MURPHY, S. (1978)

Platelet storage at 22°C: Effect of type of agitation on morphology viability and function in-vitro.

BLOOD 52 425 - 435

HOLMSEN, H., DAY, H. J., STORMORKEN, H. (1969a)

The blood platelet release reaction.

SCAND. J. HAEMATOL. 1 Suppl. 8

HOLMSEN, H., DAY, H. J., STORM EVA (1969b)

Adenine nucleotide metabolism of blood platelets - VI - subcellular localization of nucleotide pools with different functions in the platelet release reaction.

BIOCHIM. BIOPHYS. ACTA. 186 254 - 266

HOLMSEN, H. (1977a)

Platelet energy metabolism in relation to function

FROM PLATELETS AND THROMBOSIS, ED. MILLS, D. C. B., PARETI, F. I. ACADEMIC PRESS, LONDON.

HOLMSEN, H. (1977b)

Prostaglandin endoperoxide - thromboxane synthesis and dense granule secretion as positive feedback loops in the propagation of platelet responses during "The basic platelet reaction"

THROMBOS. HAEMOSTAS. 38 1030 - 1041

HOLMSEN, H. (1974)

Are platelet shape change, aggregation and release reaction tangible manifestations of one basic platelet function?

FROM PLATELETS - PRODUCTION, FUNCTION, TRANSFUSION AND STORAGE. ED. M. G. BALDINI & S. EBBE. GRUBE & STRATTON - NEW YORK.

HORTNAGL, H., BENEDICT, C. R., GRAHAME-SMITH, D. G., MCGRATH, B. (1977)

A sensitive radioenzymatic assay for adrenaline and noradrenaline in

plasma.

BRIT. J. CLIN. PHARMAC. 4 553 - 558

HOVIG, T. (1974)

The ultrastructural basis of platelet function.

FROM: PLATELETS: PRODUCTION, FUNCTION, TRANSFUSION AND STORAGE. ED.

BALDINI, M. G., EBBE, S., GRUNE AND STRATTON, NEW YORK.

IZRAEL, V., ZAWILSKA, K., JAISSON, F., TOLEDANO, S. L., CAEN, J. (1974)

Effect of a fast removal of plasmatic ADP by creatine phosphate (CP)

and creatine phosphokinase (CPK) system on human platelet function

in-vitro.

PLATELETS - PRODUCTION, FUNCTION, TRANSFUSION AND STORAGE, ED. BALDINI,

M. G., & EBBE, S., GRUNE AND STRATTON, NEW YORK.

JAMIESON, G. A. (1974)

Interaction of platelets and collagen

PLATELETS - PRODUCTION, FUNCTION, TRANSFUSION AND STORAGE. ED.

BALDINI, M. G., & EBBE, S. - GRUNE & STRATTON, NEW YORK.

JAMIESON, G. A. (1977)

The platelet.

FROM MAMMALIAN CELL MEMBRANES III ED. JAMIESON, G. A. & ROBINSON, D. M.

BUTTERWORTHS, LONDON.

JOIST, J. H. (1977)

Platelets and fibrinolysis

THROMBOS. HAEMOSTAS. 38 955 - 962

JONES, C. T., ROBINSON, R. O. (1975)

Plasma catecholamines in foetal and adult sheep.

J. PHYSIOL. 248 15 - 33

KAHN, R. A., SYRING, R. L. (1975)

The fate of bacteria introduced into whole blood from which platelet

concentrates were prepared and stored at 22 or 4°C.

TRANSFUSION 15 363 - 367

KARPATKIN, S. (1978)

Heterogeneity of human platelets VI. Correlation of platelet function

with platelet volume.

BLOOD 51 307 - 316

KATTLOVE, H. E., ALEXANDER, B. (1971)

The effect of cold on platelets. 1. Cold-induced platelet aggregation

BLOOD 38 39 - 48

KATZ, A. J., TILTON, R. C. (1970)

Sterility of platelet concentrates stored at 25°C

TRANSFUSION 10 329 - 330

KENNEDY, P. S., SOLIS, R. T., SCOTT, M. A., WILSON, R. K. (1977)

An evaluation of several blood transfusion filters.

TRANSFUSION 17 563 - 568

KINLOUGH-RATHBONE, R. L., PACKHAM, M. A., MUSTARD, J. F. (1977)

Synergism between platelet aggregating agents: the role of the

arachidonate pathway

THROMB. RES. II 567 - 580

KLEIN, B., STANDAERT, F. (1976)

Fluorometry of plasma amino nitrogen with use of fluorescamine.

CLIN. CHEM. 22 413 - 416

KOBAYASHI, I., DIDISHEIM, P. (1973)

Collagen induced release of adenine nucleotides from platelets in-vivo

FED. PROC. 32 220

KREUGER, A., AKERBLOM, O., HOGMAN, C. F. (1975)

A clinical evaluation of citrate-phosphate-dextrose adenine blood.

VOX. SANG. 29 81 - 89

KUSAJIMA, K., OZDEMIR, A., WEBB, W. R., WAX, S. D., PARKER, F. B. (1974)

Role of serotonin and serotonin antagonist on pulmonary haemodynamics

and microcirculation in haemorrhagic shock.

J. THORAC. CARDIOVASC. SURG. 67 908 - 914

LAMBERTH, E. L., WARRINER, R. A., BATCHELOR, E. D. (1974)

Effect of metabolic acidosis and alkalosis on human platelet aggregation

induced by epiniphrine and ADP

PROC. SOC. EXP. BIOL. MED. 145 743 - 746

LANCET, (1976)

PGX: a natural antithrombotic substance?

LANCET ii 1005

LANCET (1977a)

Prevention of thrombosis

LANCET i 127 - 128

LANCET (1977b)

Catecholamines in essential hypertension

LANCET i 1088 - 1090

LAUDAHN G. (1959)

Enzymatic activity and concentration of metabolic intermediate products

in the blood, in liver and heart diseases.

KLIN. WCHSR. 37 850 - 858

LEE, W. H., HAIRSTON, P. (1971)

Structural effects on blood proteins at the gas-blood interface.

FED. PROC. 30 1615 - 1620

LEE, W. H., KRUMHAAR, D., FONKALSRUD, E. W., SCHJEIDE, O. A.,

MALONEY, J. V. (1961)

Denaturation of plasma proteins as a cause of morbidity and death

after intracardiac operations.

SURGERY 50 29 - 39

LEWES, G. H. (1859)

Physiology of common life,

BLACKWOOD, EDINBURGH

LEWIS, J. J. (1965)

Introduction to Pharmacology. IIIrd edition.

LIVINGSTONE, EDINBURGH

LIM, R. C., OLCOTT, C., ROBINSON, JEAN, A. BLAISDALL, W. F. (1973)

Platelet response and coagulation changes following massive blood re-

placement.

J. TRAUMA 13 577

LIMBIRD, T. J., SILVER, D.   (1974)

Sequential changes in blood preserved with citrate-phosphate dextrose.

SURG. GYN. OBST. 138 401 - 405

LOVENBERG, W., JEQUIER, E., SJOERDSMA, A. (1968)

Tryptophan hydroxylation in mammalian systems.

ADV. PHARMACOL. 6 21 - 36

LUCY, J. A. (1970)

The fusion of biological membranes

NATURE 227 815 - 817

LUDLAM, C. A., MOORE, S., BOLTON, A. E., PEPPER, D. S., CASH, J. D.

(1975)

The release of a human platelet specific protein measured by RIA

THROMB. RES. 6 543 - 548

LUNDBERG, A. S. (1970)

DMSO - as a cryoprotective agent for blood platelets.

FED. PROC. 29 717

LÜSCHER, E. F. (1977)

New insights into the structure and function of platelet membranes

PLATELETS AND THROMBOSIS, ED. MILLS, D. C. B., PARETI, F. I.,

ACADEMIC PRESS, LONDON

McCAMAN, R. E. (1965)

Microdetermination of catechol - o - methyl transferase in brain.

LIFE SCIENCES 4 2353 - 2359

McCULLOUGH, H. (1968)

Semi-automated method for the differential determination of plasma

catecholamines.

J. CLIN. PATH. 21 759 - 763

McCULLOUGH, J., BENSON, S. J., YUNIS, E. J., QUIE, P. G. (1969)

Effect of blood bank storage on leucocyte function

LANCET ii 1333 - 1337

McCULLOUGH, J., WEIBLEN, B. J., PETERSON, P. K., QUIE, P. G. (1978)

Effects of temperature on granulocyte preservation.

BLOOD 52 301 - 309

MACFARLANE, D. E., MILLS, D. C. B. (1975)

The effects of ATP on platelets: evidence against the central role

of released ADP in primary aggregation.

BLOOD 46 309 - 320

McNAMARA J. J., BOATRIGHT, D., BURRAN, E. L., MOLOT, M. D., SUMMERS, E.,

STREMPLE, J. R. (1971)

Changes in some physical properties of stored blood.

ANN. SURG. 174 58 - 60

McNAMARA, J. J., BURRAN, E. L., LARSON, E., OMIYA, G., SUCHIRO, G.,

YAMASE, H. (1972)

Effect of debris in stored blood on pulmonary microvasculature

ANNALS THORACIC SURG. 14 133 - 137

MAGILLIGAN, D. J., SCHWARTZ, S. I.

Platelet response to regional and systemic shock.

SURGERY 77 268 - 274

MAICKEL, R. P., MILLER, F. P. (1966)

Fluorescent products formed by reaction of indole derivatives and

O-phthalaldehyde.

ANALYTICAL CHEM. 38 1937 - 1938

MANGAN, G. F., MASON, J. W. (1958)

Fluorimetric measurement of exogenous and endogenous epiniphrine

and norepiniphrine in peripheral blood.

AM. J. PHYSIOL. 194 476 - 480

MARKLEY, K., HORAKOVA, Z., SMALLMAN, E. T., BEAVEN, M. A. (1975)

The role of histamine in burn, tourniquet and endotoxin shock in mice.

EUR. J. PHARMACOL. 33 255 - 265

MARSHALL, B. E., ELLISON, N., WURZEL, H. A., NEUFELD, G. R., SOMA L. R.

(1975)

Microaggregate formation in stored blood II. Influences of anti-

coagulants and blood components.

CIRCULATORY SHOCK 2 185 - 196

MASSINI, P. (1977)

The role of calcium in the stimulation of platelets.

IN PLATELETS AND THROMBOSIS, ED. MILLS, D. C. B., PARETI, F. I.,

ACADEMIC PRESS, LONDON

MAYNERT, E. W., ISAAC, L. (1968)

Uptake and binding of serotonin by the platelet and its granules.

ADV. PHARMACOL. 6 113 - 122

MENDONCA, M. de, APPEL, M., BIDAULT, J., MASSON-PIERRE, D., TERRIER, E.
(1978)

Maintenance of 2,3,DPG levels in blood stored for 30 days at a con-

stant pH obtained by variation of CO_2

VOX. SANG. <u>35</u> 184 - 192

MICHEL, H., CAEN, J. P., BORN, G. V. R., MILLER, R., D'AURIAC, G. A.,

MEYER, P. (1976)

Relation between the inhibition of aggregation and the concentration

of cAMP in human rat platelets

BRIT. J. HAEMAT. <u>33</u> 27 - 38

MILLER, L. D., PESKIN, G. W., (1963)

Effect of serotonin on adrenal catecholamine release: possible role in

serotonin-induced syndromes.

SURG. FORUM <u>14</u> 50 - 52

MILLS, D. C. B. (1966)

The breakdown of adenosine diphosphate and of adenosine triphosphate

in plasma

BIOCHEM. J. <u>98</u> 32P - 33P

MILLS, D. C. B., ROBB, I. A., ROBERTS,G. C. K. (1968)

The release of nucleotides, 5HT and enzymes from human blood platelets

during aggregation.

J. PHYSIOL. <u>195</u> 715 - 729

MILLS, D. C. B., ROBERTS,G. C. K. (1967)

Effects of adrenaline on human blood platelets

J. PHYSIOL. <u>193</u> 443 - 453

MISHLER, J. M., DARLEY, J. H., CEDERHOLM-WILLIAMS, S., WRIGHT, G.
(1978)
Whole blood storage in citrate and phosphate solutions containing
half-strength trisodium citrate: cellular and biochemical studies.
J. PATH. 124 125 - 139

MIYATA, T., SCHWARTZ, A., WANG, C. L., RUBIN, A. L., STENZEL, K. H.
(1976)
Deposition of platelets and fibrin on chemically modified collagen
hollow fibres.
TRANS. AMER. SOC. ARTIFIC. INT. ORGANS. 22 261 - 268

MOLLISON, P. L., (1972)
Blood Transfusion in clinical medicine.
BLACKWELL, OXFORD.

MONCADA, S., KORBUT, R., BUNTING, S., VANE, J. R. (1978)
Prostacyclin is a circulating hormone.
NATURE 273 767 - 768

MOORE, F. D. (1965)
The effects of hemorrhage on body composition.
NEW ENG. J. MED. 273 567 - 577

MOORE, S. (1974)
Organ damage due to platelet aggregates.
THROMB. DIATH. HAEMORRH. 60 59 - 66

MORLEY, A. (1969)
A platelet cycle in normal individuals.
AUSTRALIAN ANNALS. MED. 18 127 - 129

MORETTI, R. L., ABRAHAM, S. (1978)

Stimulation of microsomal prostaglandin synthesis by a vasoactive material isolated from blood plasma

PROSTAGLANDINS 15 603 - 622

MOSELEY, R. V., DOTY, D. B. (1970)

Death associated with multiple pulmonary emboli soon after battle injury.

ANNALS SURG. 171 336 - 346

MOSKOWITZ, J., HARWOOD, J. P., REID, W. D., KRISHNA, G. (1971)

The interaction of norepiniphrine and prostaglandin E_1 on the adenyl cyclase system of human and rabbit blood platelets.

BIOCHIM. BIOPHYS. ACTA   230 279 - 285

MOURAD, N. (1968)

A simple method for obtaining platelet concentrates free of aggregates

TRANSFUSION 8 48

MURPHY, S., GARDNER, F. H. (1969)

Platelet preservation - effect of storage temperature on maintenance of platelet viability.

NEW ENG. J. MED. 280 1094 - 1098

MURPHY, S., GARDNER, F. H. (1975)

Platelet storage at 22°C: role of gas transport across plastic containers in maintenance of viability.

BLOOD 46 209 - 218

MURPHY, S., GARDNER, F. H. (1976)

Room temperature storage of platelets.

TRANSFUSION 16 2 - 3

MUSTARD, J. F., PACKHAM, M. A., (1970)

Factors influencing platelet function, adhesion, release and aggregation

PHARMACOL. REV. 22 98 - 187

MUSTARD, J. F., PACKHAM, M. A., KINLOUGH-RATHBONE, R. L., PERRY, D. W.

REGOECZI, E. (1978)

Fibrinogen and ADP - induced platelet aggregation.

BLOOD 52 453 - 466

MUSTARD, J. F., ROWSELL, H. C., MURPHY, E. A. (1966)

Platelet economy (platelet survival and turnover)

BRIT. J. HAEMAT. 12 1

NIEWIAROWSKI, S. (1977)

Proteins secreted by the platelet

THROMBOS. HAEMOSTAS. 38 924 - 938

NIKODEJEVIC, B., SENOH, S., DALY, J. W., CREVELING, C. R. (1970)

Catechol-o-methyltransferase II. A new class of inhibitors of catechol-
o-methyltransferase; 3,5,dihydroxy4-methoxybenzoic acid and related
compounds.

J. PHARMACOL. EXP. THERAPEUTICS 174 83 - 93

NOUR-ELDIN, F. (1967)

Blood coagulation simplified.

BUTTERWORTHS, LONDON

NURDEN, A. T., CAEN, J. P. (1976)

Role of surface glycoproteins in human platelet function

THROMBOS. HAEMOSTAS. 35 139 - 150

OAKES, R. A., WRIGHT, G., SANDERSON, J. M. (1978)

Temperature and platelet aggregation during storage of whole blood.

LANCET ii 314 - 315

O'BRIEN, J. R., (1955)

The platelet-like activity of serum.

BRIT. J. HAEMATOL. 1 223 - 228

O'BRIEN, J. R. (1962)

Platelet aggregation, some effects of the adenosine phosphates, thrombin

and cocaine upon platelet adhesiveness.

J. CLIN. PATH. 15 446 - 455

O'BRIEN, J. R. (1964a)

Variability in the aggregation of human platelets by adrenaline.

NATURE 202 1188 - 1190

O'BRIEN, J. R. (1964b)

A comparison of platelet aggregation produced by seven compounds and

a comparison of their inhibitors

J. CLIN. PATH. 17 275 - 281

O'BRIEN, R. A., SPECTOR, S (1975)

Determination of platelet serotonin by RIA and comparison with the

spectrophotofluorometric method.

ANALYTICAL BIOCHEM. 67 336 - 338

PEPPER, D. S. (1977)

Measurement of platelet release reaction in-vivo and in-vitro - a pos-

sible approach to the prediction and diagnosis of thromboembolic disease.

THROMBOEMBOLISM - A NEW APPROACH TO THERAPY ED. BY J. R. A. MITCHELL,

J. G. DOMENET, ACADEMIC PRESS (LONDON)

PHILLIPS, D. R. (1973)

Thrombin induced alterations in the surface structure of the human

platelet plasma membrane

MICROVASC. RES. 6 243

PLETSCHER, A. (1968)

Metabolism, transfer and storage of 5HT in blood platelets.

BRIT. J. PHARMAC. CHEMOTHER. 32 1 - 16

POLASEK, J. (1968)

Particulate Nature of acid phosphatase released from thrombocytes

THROMB. DIATH. HAEM. 38 466 - 473

POLASEK, J., KUBISZ, P. (1968)

Acid phosphatases during coagulation and storage of blood.

ACTA HAEMAT. 40 48 - 54

POWIS, G. (1975)

The binding of catecholamines to human serum proteins.

BIOCHEM. PHARMACOL. 24 707 - 712

PROST-DVOJAKOVIC, R. J., SAMAMA, M. (1973)

Clot-promoting and platelet aggregating effects of fatty acids.

HAEMOSTASIS 2 73 - 84

PUSZKIN, S., PUSZKIN, E., HANSON, J., ALEDORT, L. M. (1973)

Effect of ADP on the superprecipitation of human platelet thrombosthenin

FED. PROC. 32 220

RAO, G. H. R., WHITE, J. G., JACHIMOWICZ, A. A., WITKOP, C. J. (1976)

An improved method for the extraction of endogenous platelet serotonin

J. LAB. CLIN. MED. 87 129 - 137

RAWLINSON, W. A. L., LOACH, A. B., BENEDICT, C. R., (1978)

Changes in plasma concentration of adrenaline and noradrenaline in

anaesthetized patients during sodium nitroprusside-induced hypotension

BRIT. J. ANAESTH. 50 937 - 943

REUL, G. J., BEALL, A. C., GREENBERG, S. D. (1974)

Protection of the pulmonary microvasculature by fine screen blood

filtration.

CHEST 66 4 - 9

REUL, G. J. GREEN BERG, S. D., LEFRAK, E. A. (1973)

Prevention of post traumatic pulmonary insufficiency fine screen filt-

ration.

ARCH. SURG. 106 386 - 394

RHYS-WILLIAMS, A. T. (1976)

Clinical chemistry and fluorescence

LAB. EQUIP. DIGEST. 14 73 - 96

ROBISON, G. A., ARNOLD ANITA , HARTMAN, R. C. (1969)

Divergent effects of epiniphrine and PGE_1 on the level of cAMP

PHARMACOL. RES. COMM. 1 325 - 332

RODMAN, N. F., PENICK, G. D. (1972)

The effect of pH on platelet aggregation responses

BLOOD 40 953,

ROGERS, A. B. (1972)

The effect of pH on human platelet aggregation induced by epiniphrine and ADP.

PROC. SOC. EXP. BIOL. MED. 139 1100 - 1102

ROSEMAN, S. (1970)

The synthesis of complex carbohydrates by multiglycolsyltransferase systems and their potential function in intercellular adhesion.

CHEM. PHYS. LIPIDS 5 270 - 297

SALZMAN, E. W., STEAD NANCY, DEYKIN, D. (1973)

Interrelations of platelet PG synthesis and cyclic AMP metabolism

ABSTRACTS OF THE IV INTERNAT. CONGRESS ON THROMBOSIS & HAEMOST. 78

SANDERSON, J. H., GOLDSTONE, A. H. (1977)

Platelets in Health and Disease

HOSPITAL UPDATE 3 249 - 258

SASAKAWA, S., TOKUNAGA, E. (1976)

Physical and chemical changes of ACD preserved blood: a comparison of blood in glass bottles and plastic bags.

VOX. SANG. 31 199 - 210

SAUERBIER, I., VON MAYERSBACH, H. (1977)

Circadian variation of catecholamines in human blood

HORM. METAB. RES. 9 529 - 530

SCHICK, P. K., KURICA, K. B., CHACKO, G. K. (1976)

Location of phosphatidylethanolamine and phosphatylserine in the human platelet plasma membrane.

J. CLIN. INVEST. 57 1221 - 1226

SCHMID-SCHONBEIN H., WELLS, R., GOLDSTONE, J. (1969)

Influence of deformability of human red cells upon blood viscosity

CIRC. RES. 25 131 - 143

SCHNEIDER, W. H. G. (1974)

Regulation of energy metabolism in human blood platelets by cyclic AMP

IN PLATELETS - PRODUCTION, FUNCTION, TRANSFUSION AND STORAGE. ED.

M. BALDINI, & S. EBBE, GRUNE & STRATTON, NEW YORK

SEAMAN, G. V. F. (1976)

Electrochemical features of platelet interactions.

THROMB. RES. 8 Suppl. II 235 - 246

SEEGERS, W. H. (1973)

Blood coagulation: a cybernetic system

MICROVASC. RES. 6 251 - 252

SEVER, P. S., BIRCH, M., OSIKOWSKA, B., TUNBRIDGE, R. D. G. (1977)

Plasma noradrenaline in essential hypertension

LANCET i 1078 - 1081

SHARMAN, D. F., (1975)

The metabolism of circulating catecholamines.

HANDBOOK OF PHYSIOLOGY - ENDOCRINOLOGY. AM. PHYSIOL. SOC. 6

SHARP, A. A. (1958)

Viscous metamorphosis of blood platelets: a study of the relationship

to coagulation factors and fibrin formation

BRIT. J. HAEMAT. 4 28 - 37

SHELLENBERGER, M. K., GORDON, J. H. (1971)

A rapid, simplified procedure for simultaneous assay of noradrenaline, dopamine, and 5HT from discrete brain areas.

ANALYTICAL BIOCHEM. 39 356 - 372

SHIVELY, J. A., GOTT, C. L., DEJONGH, D. S. (1970)

The effect of storage on adhesion and aggregation of platelets.

VOX. SANG. 18 204 - 215

SIGGERS, D. C., SALTER, C., TOSELAND, P. A. (1970)

A double isotope dilution method for differential determination of adrenaline and noradrenaline in plasma.

CLIN.CHIM. ACTA. 30 373 - 376

SIH, C. J., TAKEGUCHI, C., FOSS, P.   (1970)

Mechansim of PG biosynthesis III - catecholamines and 5HT as coenzymes

J. AM. CHEM. SOC. 92 6670

SILVER, H., SONNENWIRTH, A. C., BEISSER, L. D. (1970)

Bacteriologic study of platelet concentrates prepared and stored without refrigeration

TRANSFUSION 10 315 - 316 ·

SKILLMAN, J. J., HEDLEY-WHYTE, J., PALLOTTA, J. A. (1971)

Cardiorespiratory, metabolic and endocrine changes after haemorrhage

ANNALS SURG. 174 911 - 922

SMITH, J. B., WILLIS, A. L. (1970)

Formation and release of PG's by platelets in response to thrombin.

BRIT. J. PHARMACOL. 40 545P - 546P

SMITH, J. B., INGERMAN, C., KOCSIS, J. J., SILVER, M. J. (1973)

Formation of prostaglandins during aggregation of human blood platelets

J. CLIN. INVEST. 52 965

SMITH, J. B., INGERMAN, C. M., KOCSIS, J. J., SILVER, M. J. (1974a)

Studies on platelet aggregation: synthesis of PG's and effects of

synthetase inhibitors.

PROSTAGLANDING SYNTHETASE INHIBITORS ED. ROBINSON H. J., VANE, J. R.

RAVEN PRESS, NEW YORK

SMITH, J. B., INGERMAN, CAROL, KOCSIS, J. J. SILVER, M. J. (1974b)

Formation of an intermediate in PG synthesis and its associaation with

the platelet release reaction.

J. CLIN. INVEST. 53 1468 - 1472

SMITH, J. B., SEDAR, A. W., INGERMAN, C. M., SILVER, M. J. (1977)

Prostaglandin endoperoxides: platelet shape change, aggregation and

the release reaction.

IN PLATELETS AND THROMBOSIS ED. MILLS, D. C. B., PARETI, F. I.

ACADEMIC PRESS, LONDON

SNYDER, S. H., AXELROD, J., ZWEIG, M. (1965)

A sensitive and specific fluorescence assay for tissue serotonin.

BIOCHEM. PHARMACOL. 14 831 - 835

SOLIS, R. T., GIBBS, M. B. (1972)

Filtration of the microaggregates in stored blood.

TRANSFUSION 12 245 - 250

SOLIS, R. T., WRIGHT, C. B., GIBBS MARY, B., STEVENS, P. M. (1974a)

Quantitative studies of microaggregate formation in-vitro and in-vivo

CHEST 65 SUPPL. 4 44 - 47

SOLIS, R. T., GOLDFINGER, D., GIBBS, M. B., ZELLER, J. A., (1974b)

Physical characteristics of microaggregates in stored blood.

TRANSFUSION 14 538 - 550

SOLUM, N. O., HAGEN, I., GJEMDAL, T. (1977)

Platelet membrane glycoproteins and the interaction between factor

VIII related protein and human platelets.

THROMBOS. HAEMOSTAS. 38 914 - 923

SOMERVILLE, B., HINTERBERGER, H. (1975)

Levels of 5HT bound to platelets and free in plasma in jugular and fore-

arm venous blood as determined by a fluorescent-OPT (orthophthalaldehyde)

assay.

CLIN. CHIM. ACTA. 65 399 - 402

STEINER, M. (1974)

The role of membrane proteins in platelet aggregation.

IN PLATELETS: PRODUCTION, FUNCTION, TRANSFUSION AND STORAGE ED.

BALDINI, M. G., EBBE, S., GRUNE AND STRATTON, NEW YORK.

STRAMENTINOLI, G. (1978)

Catechol-o-methyltransferase (COMT) in human and rat platelets.

THROMBOS. HAEMOSTAS. 39 238 - 239

STROLIN-BENEDETTI, M., GUTTY, D., STROLIN, P. (1976)

A comparative study of the effect of oral contraceptives and cigarette

smoking on platelet adhesiveness

HAEMOSTASIS 5 14 - 20

SUN, F. F., CHAPMAN, J. D., McGUIRE, J. C. (1977)

Metabolism of prostaglandin endoperoxide in animal tissues.

PROSTAGLANDINS 14 1055 - 1074

SWANK, R. L. (1961)

Alteration of blood on storage: measurement of adhesiveness of "aging"

platelets and leukocytes and their removal by filtration.

NEW. ENG. J. MED. 265 728 - 733

SWANK, R. L., (1962)

Adhesiveness of platelets and leukocytes during acute exsanguination

AM. J. PHYSIOL. 202 261 - 264

SWANK, R. L., (1968)

Platelet aggregation: its role and cause in surgical shock.

J. TRAUMA 8 872 - 879

SWANK, R. L. (1971)

Microemboli and cardiopulmonary bypass: treatment and prevention.

PROC. OF A SYMP. ON SHOCK IN LOW AND HIGH FLOW STATES. EXERPTA MEDICA

SWANK, R. L., EDWARDS, M. J. (1968)

Microvascular occlusion by platelet emboli after transfusion and shock.

MICROVASC. RES. 1 15 - 22

SWANK, R. L., FELLMAN, J. H. (1967)

Blood cell aggregation and biological agents.

BIBL. ANAT. 9 98 - 103

SWANK, R. L., FELLMAN, J. H., HISSEN, W. W. (1963)

Aggregation of blood cells by 5HT

CIRC. RES. 13 392 - 400

SWANK, R. L., PORTER, G. A. (1963)

Disappearance of microemboli transfused into patients during cardio-

pulmonary bypass.

TRANSFUSION 3 192 - 197

TAKANO, S. (1975)

Mutual potentiation between noradrenaline and 5 hydroxytryptamine in

producing aggregation of canine platelets.

ACTA. HAEM. JAP. 38 292 - 298

TAKAORI, M., NAKAJO, N., ISHII, T. (1977)

Changes of pulmonary function following transfusion of stored blood.

TRANSFUSION 17 615 - 620

THOMAS, D. P. (1967)

Effect of catecholamines on platelet aggregation caused by Thrombin.

NATURE 215 298 - 299

THOMPSON, J. H., SPEZIA, C. A., ANGULO, M. (1969)

Ascorbic acid effects on serotonin fluorescence.

ANAL. BIOCHEM. 31 321 - 330

THOMPSON, J. H., SPEZIA, CH, A., ANGULO, M. (1970)

Fluorometric detection of 5HT using o-phthaldialdehyde: an improvement

EXPERIENTIA <u>26</u> 327 - 329

TSCHOPP, TH. B., BAUMGARTNER, H. R. (1976)

Enzymatic removal of ADP from plasma: unaltered platelet adhesion

but reduced aggregation on subendothelium and collagen fibrils.

THROMBOS. HAEMOSTAS. <u>35</u> 334 - 341

TURNER, S. R., TAINER, J. A., LYNN, W. S. (1975)

Biogenesis of chemotactic molecules by the arachidonate lipoxygenase

system of platelets.

NATURE <u>257</u> 680 - 681

UDENFREIND, S., STEIN, S., BOHLEN, P., DAIRMAN, W. (1972)

Fluorescamine: a reagent for assay of amino acids, peptides,

proteins and primary amines in the piconede range.

SCIENCE <u>178</u> 871 - 872

UDENFRIEND, S., WEISSBACH, H., CLARK, C. T. (1955)

The estimation of 5HT in biological tissues.

J. BIOL. CHEM. <u>215</u> 337 - 344

UMBREIT, W. W., BURRIS, R. H., STAUFFER, J. F. (1964)

Manometric techniques, 4th Edition.

BURGESS, MINNEAPOLIS.

VALERI, C. R., ZAROULIS, C. G., ROGERS, J. C., HANDIN, R. I.,

MARCHIONNI, L. D. (1972)

Prostaglandins in the preparation of blood components.

SCIENCE <u>175</u> 539 - 542.

VANABLE, J. W. (1963)

A ninhydrin reaction giving a sensitive quantitative fluorescence
assay for 5HT.

ANALYTICAL BIOCHEM. 6 393 - 403

VANE, J. R., (1957)

A sensitive method for the assay of 5HT

BRIT. J. PHARMACOL. 12 344 - 349

VANE, J. R. (1969)

The release and fate of vaso-active hormones in the circulation

BRIT. J. PHARMACOL. 35 209 - 242

VENDSALU, A (1960)

Studies on adrenaline and noradrenaline in human plasma.

ACTA. PHYSIOL. SCAND. 49 Suppl. 173.

WALSH, P. N. (1973)

Platelet coagulant activities: evidence for multiple different functions
of platelets in intrinsic coagulation.

MICROVASC. RES. 6 250.

WALSH, P. N. (1977)

Collagen - Platelet interaction in coagulation, haemostasis and throm-
bosis.

IN PLATELETS AND THROMBOSIS. ED. MILLS, D. C. B., PARETI, F. I.,
ACADEMIC PRESS, LONDON.

WANG, C. L., MIYATA, T., SCHLEAR, S., WEKSLER, B., RUBINL A. L.,
STENZEL, K. H. (1975)

Collagen and glomerular basement membrane effects on platelets.

TRANS. AMER. SOC. ARTIF. INT. ORGANS. 21 422 - 425

WARLOW, C., CORINA, A., OGSTON, D., DOUGLAS, A. S. (1974)

The relationship between platelet aggregation and time interval after

venepuncture.

THROMBOS. DIATHES. HAEMORRH. 31 133

WARLOW, C., OGSTON, D., DOUGLAS, A. S. (1976)

Platelet function after the administration of chlorpromazine to human

subjects.

HAEMOSTASIS 5 21 - 26

WAUTIER, J. L., TOBELEM, G. M., PELTIER, A. P., CAEN, J. P. (1976)

Cl and human platelets. II. Detection by immunological methods and role.

IMMUNOLOGY 30 459 - 465

WEIL-MALHERBE, H., BONE, A. D. (1954)

Blood platelets as carriers of adrenaline and noradrenaline.

NATURE 174 557 - 558

WEISS, A., BAENZIGER, N. L., ATKINSON, J. P., (1978)

Platelet release reaction and intracellular cGMP

BLOOD 52 524 - 531

WEISS, H. J. (1976)

Antiplatelet drugs, - a new pharmacologic approach to the prevention

of thrombosis.

AM. HEART. J. 92 86 - 102

WEISS, H. J., WILLIS, A. L., KUHN, D., BRAND, H. (1976)

Prostaglandin E_2 potentiation of platelet aggregation induced by LASS
endoperoxide: absent in storage pool disease, normal after aspirin
ingestion.

BRIT. J. HAEMATOL. 32 257 - 272

WESKLER, B. B., (1974)

The platelet in inflammation: interaction with complement to promote
chemotaxis.

IN PLATELETS: PRODUCTION, FUNCTION TRNASFUSION AND STORAGE: ED.
BALDINI, M. G., EBBE, S. GRUNE AND STRATTON, NEW YORK.

WHITE, J. G. (1974)

The ultrastructural basis of platelet function.

IN PLATELETS: PRODUCTION, FUNCTION, TRANSFUSION AND STORAGE: ED.
BALDINI, M. G., EBBE, S., GRUNE AND STRATTON, NEW YORK.

WHITE, J. G., GOLDBERG, N. D., ESTENSEN, R. D., HADDOX, M. K.,
RAO, G. H. R. (1973)

Rapid increase in platelet cyclic 3'5'guanosine monophosphate (cGMP)
levels.

J. CLIN. INVEST. 52 89a

WILLIS, A. L. (1974)

Prostaglandin biosynthesis - key to coronary thrombosis.

INFLO 7

WILSON, J. D., TASWELL, H. F. (1968)

Autotransfusion: historical review and preliminary report on a new
method.

MAYO CLIN. PROC. 43 26 - 35

WOLF, P. (1967)

Platelet products in plasma.

BRIT. J. HAEMATOL. 13 269- 288

WRIGHT, G. (1975)

Pathological effects of intra-arterial blood transfusions in dogs.

CARDIOVASC. RES. 9 685 - 690

WRIGHT, G., SANDERSON, J. M. (1974)

ACD and CPD blood preservation

LANCET ii 173

WU, V. Y., McCOY, L. E. (1977)

Platelet factor 3: quantitation and characterization

THROMB. RES. 11 581 - 593

ZIEGLER, M. G., LAKE, C. R., KOPIN, I. J. (1976)

Plasma noradrenaline increases with age.

NATURE 261 333 - 335

ZUCKER, M. B., BORRELLI, J. (1959)

Platelets as a source of serum acid nitrophenylphosphatase.

J. CLIN. INVEST. 38 148 - 154