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THE SECONDARY AGGREGATION REACTION

OF BLOOD PLATELETS

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M.D. Thesis

University of Edinburgh

1971.

I N D E X

III

Abbreviations

ADP	-	adenosine disphosphate
ATP	-	adenosine triphosphate
AMP	-	adenosine monophosphate
5H-T	-	5 hydroxy-tryptamine
PRP	-	platelet rich plasma
cit	-	citrated
PPP	-	platelet poor plasma
R.T.	-	room temperature
L.T.	-	light transmission
O.D.	-	optical density
μM	-	micro-moles per litre (refers to final concentration in the plasma)
RBC	-	red blood cell
WBC	-	white blood cell
10^3	-	thousand(s)
PA	-	Primary Aggregation
SA	-	Secondary Aggregation

SUMMARY

Blood platelets have the ability to stick avidly to one another and to damaged surfaces in blood vessels and by so doing form platelet aggregates. These aggregates form the initial obstruction to blood loss from a wound and blood flow through a thrombosing artery and vein.

Platelets are stimulated to aggregate by a specific chemical messenger, ADP, which apparently acts on receptor sites on the platelet surface. Platelets themselves contain large stores of ADP which are explosively released when platelet aggregation is promoted by thrombin, adrenaline, collagen or 5 Hydroxytryptamine. These promoters of platelet aggregation can be studied in vitro where the ADP they induce platelets to release produces a dose-dependent (primary) aggregation which is partially reversible.

This thesis reports the finding that some platelets undergo a further ('secondary') aggregation, much more marked than the primary one, to form large persistent aggregates. Secondary aggregation occurred because small amounts of ADP in the plasma were causing the platelets to release nearly all their stores of ADP and to show an exaggerated aggregation response to ADP.

Studies in man showed that there are two grades of secondary platelet response. Most platelet suspensions derived from normal healthy controls showed a self-limiting secondary aggregation, but 20% showed a powerful progressive 'chain-reactive' type of aggregation.

Studies carried out on 'sick' patients in the 'hyperthrombotic' period following acute illness showed that the incidence of the 'chain reaction' response had risen to 70%.

In animal studies most mammals failed to show any secondary aggregation reaction. A self-limiting response was observed in blood derived from some baboons and guinea pigs. Only the cat showed a consistent 'chain reaction' response. This is the only animal which develops arterial thrombo-embolic disease in its natural environment.

This association between secondary aggregation and arterial thrombosis, and the high incidence of the chain-reactive response during the 'hyper-thrombotic' state following acute illness, suggests that secondary platelet aggregation may be a significant factor in the pathogenesis of thrombotic disease in man.

Furthermore, inhibition of this reaction by newly available drugs may offer a new approach to the prevention and perhaps treatment of thrombotic and embolic disease.

CHAPTER ONE

REVIEW

HISTORICAL ASPECTS

The first microscope which gave sufficient definition for viewing bodies of 2-3μ, and so blood platelets, was developed by Lord Lister's father in about 1826. Over the next few years shed blood was examined with increasing care and many small particles were described and drawn. From their reports it seems likely that by 1846 blood platelets had been seen independently by Gulliver, Addison, Arnold and Zimmermann (for review see Robb-Smith 1967), although none of these workers realised the significance of these small bodies. Donné (1841) is sometimes credited with the discovery of the blood platelets for his description of 'les globulins du chyle', however, as dilution of the blood with water increased the number of 'les globulins', he may easily have seen what he thought he saw, namely chylomicrons.

T. W. Jones FRS (1851), working in London with the frog foot preparation, was the first person to notice the accumulation at sites of injury of cells from the blood; but amphibian platelets are both nucleated and larger than mamalian platelets and so a cross correlation between the two was not made. The next advance was made by Sir William Osler in 1871. He made an in vivo study of the mesenteric blood vessels of a mammal and saw, in addition to red and white blood cells, small disk shaped bodies which he correctly correlated with the blood platelets in shed blood from man. He also noted that platelets had the ability to form clumps of a 'granular' appearance.

Finally, the correlation between vessel injury and platelet 'granular' mass formation was made by Bizozzero (1881) and was confirmed and amplified by Eberth and Schimmelbusch (1886). Like Osler, these workers studied
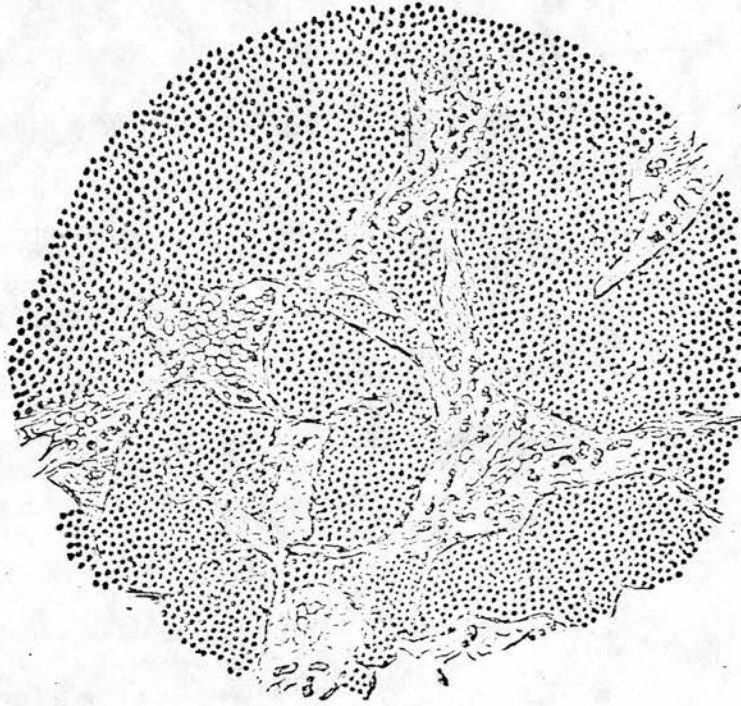
**From section of half-hour thrombus produced by forcible ligature of femoral artery of dog (see text).
Shows masses of blood plates containing bands of fibrin and leucocytes.   Zeiss D. ocular 3.**

Fig. 1.1.

Section of thrombus drawn by Welch 1887.

mammalian mesenteric blood vessels, but with the additional manoeuvre of vessel injury. They found that a mass of blood platelets formed at the injury site. The individual platelets in this mass developed a granular appearance and then the platelets appeared to fuse into a granular mass, certainly their individual outlines could no longer be distinguished by light microscopy.

Welch (1887) extended the applicability of this sequence of changes to the thrombotic situation, by observing the changes which occurred when the femoral artery of a dog was injured by tying a tight ligature around it. After the ligature was released platelets adhered and aggregated at the site of injury, forming large, irregular masses. Then, between five and fifteen minutes after the injury, thick fibrin strands formed between the platelet masses, and white blood cells migrated into the thrombus (Fig. 1.1.).

Welch followed the progress of these platelet-fibrin thrombi, and found that, two days later, they had a homogeneous appearance to light microscopy and a response to staining which was 'indistinguishable from fibrin'. This progression from platelets to 'fibrin' lead him to postulate that the so-called 'fibrin thrombi' found in man, were the later stages of a platelet-fibrin ('white') thrombus, and did not arise de novo as fibrin deposits. The electron microscope had shown that he was right (vide infra).

Eberth and Schimmelbusch (1886) used the term 'viscose metomorphose' to describe the development of a granular appearance in individual platelets in a platelet aggregate. Their name has since given rise to confusion and controversy, for it has been subsequently applied to granular changes in a platelet aggregate ('viscous metamorphosis' Wright and Minot 1917) and to the biochemical aspects of the platelet release reaction (vide infra). In the authors opinion the confusion caused by the use of this term has hindered understanding of platelet aggregation, and its use should be abandoned.

Thus by the end of the 1880's the initial events, of platelet adhesion
to injured vessels followed by platelet aggregation, with later strengthening
by fibrin strands, were known.   No important further advances were made in
this field until the advent of the electron microscope and of improved
biochemical methods.

PLATELET ADHESION

Adherence of blood platelets to injured vessel walls is the first
event in the formation of a haemostatic plug or a thrombus.   The platelets
do not adhere to normal vascular endothelium subjected to a laminar blood
flow.   They are able to adhere when only minor endothelial damage is present
(French et al 50), but only temporarily (5).   It is the exposure, by damage,
of vessel wall connective tissue (collagen) which produces a firm base for a
platelet-fibrin mass, for platelet adherence to collagen is rapid and firm
(Boumameaux 20, Roskam 156, Kjarheim and Hovig 96a, Johnson et al 86).

The platelet-collagen reaction is a highly specific one, as is shown by
its abolition by a collagenase (Hovig 78) and by altering the helical
structure of the collagen molecule (Nosel et al 135, 185).   It differs from
platelet to platelet aggregation, in that calcium, which is essential for
aggregation, is not required for adhesion (Spaet and Zucker 154, Spaet and
Lejnieks 172).

PLATELET AGGREGATION

Platelet aggregation (platelet adhering to platelet) is the property
of blood platelets which allows them to form the main mass of a haemostatic
plug and a white thrombus;   it has the effect of supplying the 'bricks'
and part of the 'mortar' (the remainder of the 'mortar' is provided
subsequently by thick fibrin threads).

Until the 1960's, our knowledge of platelet aggregation was limited
to morphological observations, and to the demonstration that thrombin would
induce platelets to aggregate (Sharp 165).   In 1960, Hellem (72) reported
that a substance released during the boiling of red blood cells was a
potent platelet aggregating agent.   Analysis of the red cell extract showed
that the active substance was the adenine nucleotide, adenosine disphosphate
(ADP) Gaarder et al (54).   Closely related adenine nucleotides (adenosine
triphosphate or ATP, adenosine monophosphate or AMP, and adenosine) did not
produce aggregation.

Before the discovery by Gaarder et al (54) of it's platelet aggregating
properties, ADP had been known mainly for its part in the ATP intracellular
energy transfer process.   One preceding piece of information, however, gave
special interest to the ADP story.   In 1958 Professor Born (11) had shown
that blood platelets themselves contained a very high (almost uniquely so)
concentration of adenine nucleotides.   This raised the possibility that
the platelets themselves and not red blood cells (which had after all to be
boiled) could be the source of the ADP.   Subsequent work with labelled
adenine nucleotides (Holmsen 73, 74, 75, Ireland 84) showed that there
were two roughly equal pools of adenine nucleotides in platelets.   One
was metabolically active, and is presumed to be concerned with ordinary
intracellular energy transfer;   the other apparently served no metabolic
function, and had an unusually high concentration of ADP, indicating that
a specialised storage process was occurring.   This stored adenine nucleotide
pool with a high ADP content, is now known to be released when the platelet
is stimulated by certain platelet aggregating agents (Holmsen 73, 74, 75,
Ireland 84, Mills et al 116, 117).
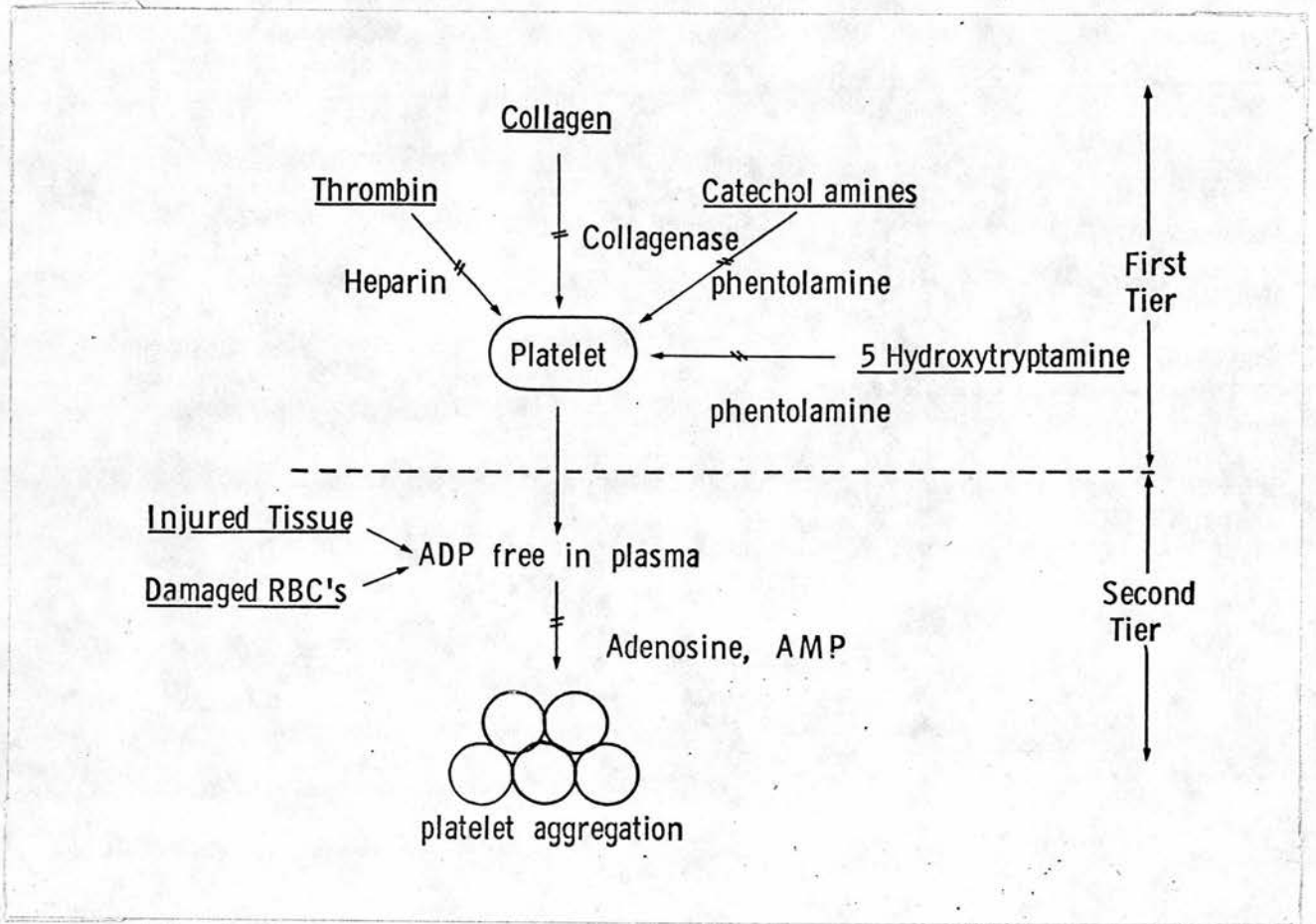
Fig. 1.2.

The 'two-tier' hypothesis of platelet aggregation. ADP is first released into the plasma from platelets (by thrombin, collagen, 5H-T) or by damage to 'cells' (first tier), and this free ADP then stimulates platelets to aggregate (second tier). The inhibitors of the platelet aggregating action of each stimulator are given beside the appropriate arrow (⇥).

Since 1962 many new platelet aggregating substances have been discovered in addition to the original ones, thrombin and ADP (for reviews see Marcus 112, Michal and Firkin 113). The ones likely to be of physiological (and pathological) importance are the catechol amines (adrenaline and noradrenaline, Mitchell and Sharp 121, O'Brien 138), collagen (in addition to stimulating adhesion, Hovig, 78, 79) and 5-Hydroxytryptamine or serotonin (O'Brien, 139). Thrombin (84) collagen (73) and the catechol amines (117) induce platelets to release the adenine nucleotides of their metabolically inactive pool; 5 hydroxytryptamine (5H-T) is suspected also to induce a release (139, Haslam, 69), but it is at most a small one (139).

The release of ADP from platelets by aggregating agents such as thrombin, collagen, and adrenaline suggests a 'two tier' action (Fig. 1.2.). The first 'tier' consists of the release of ADP, and the second, of the action of the released ADP on the platelets to produce aggregation. There is other evidence for the two-tier hypothesis. Inhibitors of aggregation are known which act on individual aggregating agents (e.g. heparin on thrombin, collagenase on collagen etc. see Fig. 1.2.) without at the same time inhibiting the aggregation produced by other agents or ADP, but inhibitors of ADP induced aggregation (adenosine, 2 chloroadenosine (13)) inhibit the aggregation of all aggregating agents. Even more convincing is Haslams demonstration (68, 69), that the enzyme system pyruvate kinase-phosphoenol pyruvate (PK - PEP), which removes ADP rapidly from plasma, prevents thrombin and adrenaline from producing aggregation, even although the ADP-release induced by these substances is still occurring.

There are two exceptions to this two tier system, the first phase of adrenaline aggregation (vide infra), and the phenomenon of Secondary Aggregation of blood platelets, which is the subject of this thesis.

Secondary Aggregation (S.A.) was first described by Macmillan (106)
in 1966 and has since been confirmed by several groups of workers (Mills
et al 118, 116, Zucker and Peterson 196, 197, O'Brien 142, 143, Hardisty
et al 64, 65).   S.A. is a marked, almost maximal, aggregation response in
platelet rich plasma induced by low concentrations of ADP.   It is associated
with the release by the platelets of their ADP stores.   This ability of ADP
to induce the release of more ADP from platelets means that it is performing
both first and second 'tier' actions.   It thus can produce a self-
propagating platelet aggregation/platelet ADP release reaction.   S.A. will
be described fully in the results section and its possible implications will
be discussed at the end of this thesis.   Zucker and Peterson (196) have shown
that platelet factor 3 activity is released when ADP induces S.A. (but not in
the absence of S.A.), indicating that S.A. also promotes coagulation.

As was mentioned above, the catechol amines occupy a unique position.
O'Brien (138, 139, 140) showed that adrenaline produced two phases of
aggregation, in contrast to the one phase occurring with other aggregating
agents,   Whereas in all ADP mediated aggregation platelets change from their
in vivo shape of disks (Osler 146, Algire 2) to irregular spheres (Zucker and
Borelli 195, Macmillan and Oliver 109, O'Brien 141), in the first of the two
phases of adrenaline aggregation the aggregating platelets retain their in vivo
disk shape (O'Brien 141, 144 Macmillan 106).   This suggests that ADP is not
involved in the first phase of adrenaline aggregation, a suggestion which is
supported by the demonstration that concentrations of the adrenaline
inhibitor phentolamine, too small to affect ADP induced aggregation, cause
complete disaggregation of the adrenaline first phase, (Macmillan 106,
Mills et al 117).   The second phase of adrenaline aggregation, like the
aggregation induced by other aggregating agents, is mediated through ADP

released by the platelets (Mills et al 117, O'Brien 139, Macmillan 106, Haslam 69).

There is a strong association between the release of the platelet stores of ADP and degranulation of the platelets (Hovig 78,79, Mills et al 116, Marcus 112). Amitriptyline inhibits S.A. while leaving the P.A. response to ADP unaltered (Mills et al 118), and the platelets in aggregates formed in amitriptyline treated plasma do not show degranulation (Mills et al 116). These observations suggest that the nucleotide 'pool' is in one of the granules, as does the general principle that it would be difficult otherwise for the cell to maintain a store of adenine nucleotides with a high ADP content.

The way in which platelets are induced to aggregate by ADP has not yet been discovered, nor has the nature of the bridge between platelets. It is however reasonably certain that ADP acts on specific receptors sites on the platelet surface, because concentrations far too small (10^{-8}M) to form a monolayer around the platelet (about 10^{-3}M) produce aggregation, and because adenine nucleotides closely related to ADP inhibit aggregation (Born and Cross 15, Mustard and Packham 133), suggesting the presence of a competitive inhibition mechanism.

In 1964 Gaarder and Laland (54a) suggested that ADP may be both stimulating platelets to aggregate and forming part of the bridge between them. These workers noted that the only other adenine nucleotide which caused platelet aggregation, a synthetic substance adenosine tetra-phosphate, was also the only other nucleotide which (like ADP) had an uneven number of negative charges. Aggregation is known to be calcium dependent (111), and these workers hypothesised that ADP became fixed to platelet receptor sites and the odd negative charges on adjacent platelets were then joined through a calcium bridge. It is now known that ADP does not become firmly fixed to

platelets (Born 14, Salzman et al 159, 170).   Also, in view of the transient nature of much platelet aggregation, it is unlikely that a strong link, such as a co-valent calcium one, is present.

Several workers have subsequently offered explanations of how ADP may alter the function of the platelet membrane as a prelude to aggregation. Spaet and Lejnieks (172) suggested that conversion of ADP to AMP provided energy for aggregation, but this conversion can be prevented by KCN (Salzman et al 159) while leaving platelet aggregation unimpaired.   Salzman has suggested (160) that the ADP is producing 'product inhibition' of the action of a platelet membrane ecto-ATPase, but again there are several objections to this theory (Michal and Firkin 113).   The most interesting suggestion is the recent one by Packham et al (149) and Mills and Smith (119), that since platelet aggregation inhibitors of widely varying structure have the common link of affecting the cyclic-AMP system in the platelet membrane, the ADP 'message' may be being transmitted to the rest of the cell through cyclic AMP, the recently discovered intracellular 'messenger' substance (198).

At the other end of the process, the inter-platelet bridge, there has been an as yet unsubstantiated suggestion of a combination between the two portions (actin and myosin) of the contractile protein thrombosthenin situated on adjacent platelets (Booyse and Rafelson 9a, b, c,).   It will be interesting to see the reports of follow up studies on this possible mechanism.

After platelet aggregates have formed they often break up (disaggregate), sometimes to a marked degree.   Born and Cross (17) suggested that this might be due to the inhibitory action of ADP degradation products (AMP and adenosine). Subsequent work has shown, however, that this degradation is relatively slow (Ireland and Mills 85), and disaggregation is in fact occurring while about 80% of the ADP dose is still present.   Since AMP and adenosine are weak inhibitors of ADP mole for mole (15), the platelets are disaggregating inspite

of a persisting aggregation-inducing stimulus. O'Brien (139) showed that
a grossly pharmacological dose of ADP (100μM/L) in incubated platelet-rich
plasma, made the platelets refractory to ADP. This refractory response
mechanism was found still to occur with 15μM ADP/L by Rosenberg and Holmsen
(154), and by the author (108) to be still present at the physiological dose
range of ADP of 0.5-1.5μM/L. It also occurred following the addition of low
concentrations of thrombin and 5H-T. It is thus likely that, following the
aggregation-prone response to ADP, the platelets pass into a refractory
state. This refractory state may be the reason for disaggregation.

The work described so far has been concerned with the in vitro
situation. Several workers have shown correlations between the results
in vitro and the effects of aggregating agents in experiments on intact animals
Systemic infusions of ADP into cats (Born and Cross 16,) rabbits (Busfield and
Tomich 23) and man (Davey and Lander 36) all produced a fall in platelet count.
In the rabbit, a preceding infusion of the ADP inhibitor adenosine protected
the animal from thrombocytopenia to a subsequent ADP infusion (152).

Mustard et al (128, 129) investigated the pathological changes which
occurred when ADP and thrombin were infused into pigs. Both these substances
produced platelet aggregates in small vessels. The ADP induced aggregates
broke up within a few minutes and the thrombin induced ones persisted for
upwards of an hour. This showed that in the pig ADP induced aggregation is
a transitory phenomenon. An interesting finding in Mustard's work on ADP
infusion was the occurrence of cardiac arrhythmias after the platelet
aggregates had dispersed.

Another experimental model which has been used, is rabbit cortical
vessels exposed by craniotomy. When these vessels are injured by artery
forceps a small injury ('minor') produced no change and a large ('major')
one induced platelet aggregation ('white body' formation) at the site of the

damage (Honour and Ross-Russell 76a). Honour and Mitchell (76) showed
that the application of platelet aggregating agents to 'minor' injury
sites resulted in white body formation at this normally quiescent site.
In vitro inhibitors of the aggregating agents, both specific and general
(adenosine), were equally inhibitory in this in vivo situation. These
workers later showed (18) that systemic infusion of the ADP inhibitor
adenosine prevented 'major' injuries from inducing 'white body' formation.
This finding suggests that the stimulus causing platelets to aggregate
onto a damaged vessel wall in vivo is ADP.

FUNCTIONS OF PLATELETS

The prime role of the platelet is in haemostasis (vide infra).

Platelets also provide an essential part of the intrinsic coagulation system, a lipid material (Platelet Factor 3). It's precise chemical nature is not known but it certainly contains phosphatides similar to, or a mixture of, phosphatidyl ethanolamine, phosphatidylserine and inosital phosphatides (176, 6).

There is increasing evidence that platelets play an important part in most responses of the blood to injury (148). Platelets adhere to bacteria both in vivo and in vitro (77, 101). They phagocystose foreign material, and the platelet count is reduced during anaphylactic reactions (89) and after injections of bacterial endotoxins (157, 175). In rats, the platelets seem to play an essential part in the production of anaphylactic shock (56), but the relevance of this finding to man is not yet known.

One further effect of platelets is their endothelial supporting function (Johnson et al 86). The absence of platelets allows petechial and purpuric haemorrhages to develop and their presence prevents this escape of red cells from vessels. The mechanism of this protective function of platelets, of maintaining 'vascular integrity', has not been elucidated.

Finally, platelets are an important constituent of thrombi (vide infra).

BLOOD PLATELETS AND HAEMOSTASIS

The work of Bizozzero (9) and Eberth and Schimmelbusch (40)
in the 1880's has been confirmed by subsequent studies using light
(Roskam et al 55, Jorgenson and Borchgrevink 91, 92) and electron
microscopy (Kjarheim and Hovig 96a). The sequence of events is now
known to be, vessel contraction, then platelet adhesion and aggregation
at the sites of the cut blood vessels, and following these two events
the formation of thick fibrin strands around and in the spaces in
platelet aggregates. These events produce a stable and impermeable
platelet-fibrin plug ('consolidated' plug) (for review see Roskam 156).
Coagulation alone can produce haemostasis in capillaries, but this is
not an important factor in achieving haemostasis (91, 92). Over the
two weeks or so following an injury the platelet-fibrin mass is
organised to scar tissue composed of new collagen.

Both normal platelets (quantitative and qualitative) and
normal coagulation, are necessary for the formation of a consolidated
plug. When the platelet component is defective, prolonged bleeding
occurs from small puncture wounds ('bleeding time' test). The bleeding
time is normal in patients with coagulation defects, because the
platelet plug and vessel contraction can arrest the bleeding, but
microscopy shows that the plug is friable and unstable (Jorgenson et al
93). It is therefore understandable that this friable plug can become
dislodged, allowing bleeding to recommence.

BLOOD PLATELETS AND THROMBOSIS

In a recent electron microscopy study on experimental thrombosis (in pigs), Mustard et al (130) have confirmed and extended Welch's observations (184) of 1887. The sequence of events found by Mustard and his co-workers was the same as that during the formation of haemostatic plugs. Platelets were found adhering to collagen and attached to these were aggregates of platelets encased in fibrin. In the two days following the injury the platelet-fibrin mass was invaded by mononuclear cells which part digested the platelets, and then phagocytosed the parts and whole platelets. By five days, many fibroblasts were seen and a little new collagen, and by fourteen days, the platelet-fibrin thrombus had been converted to an intimal scar which was rich in smooth muscle cells and collagen, and covered with normal endothelium.

This process, of repairing the injured artery to near its normal state, is an efficient one, and is a necessary one for maintaining the life of an animal. However, in man, an extension of this process, thrombo-atherosclerosis, is now the main cause of death in adults in industrialised countries, due to its effects on coronary and cerebral blood vessels.

The mechanism of the development of thrombo-atherosclerosis remains the subject of controversy to this day, and the purpose of this part of the review is to assess the part thrombosis is playing in this process, by describing the pathological evidence derived from man himself.

In 1949 Duguid (39) revived the 'encrustation' theory of atherosclerosis, when he demonstrated fibrin staining material on arterial walls which was being converted to fibrous plaques. This showed that at least some atherosclerotic plaques were formed in this way. The problem of assessing how many plaques are so formed, is

complicated by a 'tip of the iceberg' phenomenon. Since a thrombus
is organised in a few days or weeks into a fibrous plaque, the number
of thrombotic deposits seen at any one time is likely to only be a
very small percentage of the total number which are occurring.

The iceberg phenomenon is also operating in very diseased
arteries, as has been shown by Mitchell and Schwartz (122). These
workers examined the coronary artery vessels of patients dying after
a myocardial infarction. When the time between the infarction and
subsequent death was taken into account, they found a progression from
totally occluded coronary arteries in all patients dying in the first
forty-eight hours after the infarction, to stenotic fibro-atherosclerotic
plaques in patients dying at four months after the infarction. This
suggests that organisation of thrombi is a major factor in the
production of stenotic atherosclerotic plaques.

The same conclusion has been reached by the other workers in
this field (Crawford 30, Branwood and Montgomery 20a), because they
frequently found thrombus on atherosclerotic plaques, often layers of
thrombus. As Crawford (30) has said 'it will be appreciated that the
natural history of most of these cases (of atherosclerosis) is one of
recurring episodes of thrombosis. At an early stage when blood flow
is rapid and the lumen widely patent, the thrombi are small. They
become rapidly consolidated by the blood pressure and incorporated in
the wall. But with successive deposits stenosis builds up and there
is a tendency for larger thrombi to form and be less completely
incorporated in the vessel wall, until ultimately occlusive thrombosis
occurs.'

The reported incidence of occlusive thrombosis in patients
dying of recent myocardial infarction has varied widely, as reviewed

by Crawford (30).   Davenport found 60%, Branwood and Montgomery only 21% with recent mural thrombus in a further 38%.   But these workers did not separate their patients into forty-eight hours and increasing time periods, nor was the most informative technique of serial sectioning of all narrowed areas used.   Mitchell and Schwartz (122) finding of 100% (see above) would therefore appear to be nearer the true incidence.

Some occlusions are due to atheromatous debris and not white (platelet-fibrin) thrombus.   The most careful study to date is probably the one of serial sectioning of 40 occlusions of coronary arteries by Friedman and Byers (51).   These workers found an atheromatous deposit ('abscess') which had ruptured into the vessel lumen as the initial event in all the occlusions.   A platelet-fibrin thrombus was the cause of the block in 38 occlusions, with atheromatous debris the cause in the other two.   Constantinides (29) has reported parallel findings in occluded cerebral arteries.

In sudden death from ischaemic heart disease, there has been a lower incidence of occlusions (Crawford, Dexter and Teare, 31). Thrombotic occlusions were present in 50%, and fresh non-occlusive thrombosis in a further 20%.   The incidence of thrombosis was highest in the younger patients, especially those under forty years.   Those without occlusive thrombus had severe stenotic lesions of their coronary arteries, a finding which was also made by Branwood and Montgomery (20a). The deaths in those patients without a thrombotic occlusion have been attributed to cardiac arrhythmia, thought to be caused by ischaemia. An alternative cause of arrhythmia would be transient block of the stenotic vessels by platelet aggregates, like that shown to cause arrhythmia in animals (Mustard et al 130).   Thus some of these deaths could be due to a transient or subsequently dislodged platelet aggregate.
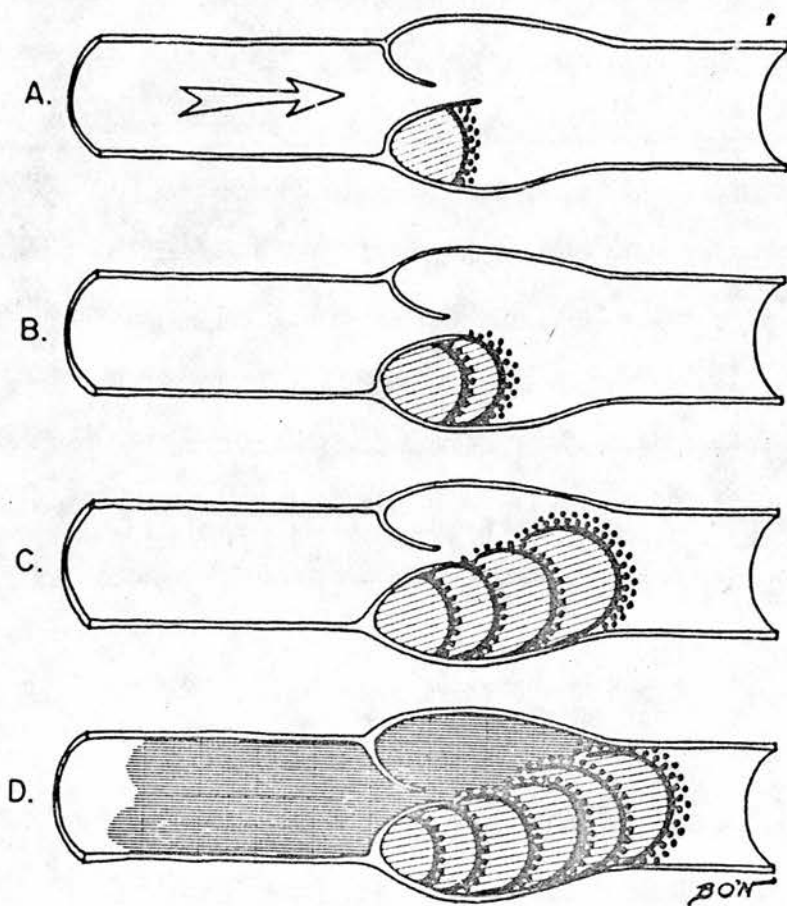
Fig. 4   *Diagram illustrating the propagation of deep vein thrombi from a nidus in a valve pocket (A) and the deposition of successive layers of fibrin, platelets, etc (B and C). Retrograde extension occurs when there is venous blockage from propagation (D).*

Fig. 1.3.

Diagram of the stages in the pathogenesis of deep vein thrombosis from Sevitt 1971.

There would seem to be a little doubt that platelet-fibrin thrombi and their organisation is the major mechanism in thrombo-atherosclerosis. What remains uncertain is how other markers of the atherosclerotic trait, such as hyperlipidaemia, obesity and cigarette smoking, are acting on this process. Are they responsible for atheromatous abscess formation, or for the bursting of these abscesses? Or do they promote thrombogenesis or prevent fibrinolysis, or otherwise impair the arterial walls ability to organise thrombi, or even increase the amount of scar tissue formed? No doubt future research will answer these questions, but the morphologic evidence, based on findings in man himself (not on animal experiments), leaves little doubt of the thrombotic pathogenesis of most atherosclerosis.

Platelet-fibrin thrombi are also the major event in the initiation of deep vein thrombosis, a cause of significant morbidity and mortality following acute illness. The major worker in this field has been Sevitt who recently reviewed his own and other people's work (163). Deep vein thrombi arise in the areas of turbulent blood flow around venous valves (162), as layered platelet-fibrin thrombi (see Fig. 1.3.) which eventually gives rise to a block of the vein. Red coagulation thrombosis then occurs, and parts of the red thrombus sometimes become dislodged and embolise to the lungs.

Layered white thrombi are also one of the sources of arterial emboli. They arise in areas of turbulent blood flow, such as fibrillating atria, and arterial aneurysms; and on areas of vascular damage, such as endocardial myocardial infarctions and eroded atherosclerotic plaques.

This short review only includes a part of the very large literature on platelets and the part they play in health and disease.

There have been many extensive reviews of different aspects of the literature in recent years of which the most helpful are, in the authors opinion, those by Marcus and Zucker 1965 (111), Kowalski and Niewiarowski 1967 (98), Johnson and Seegers 1967 (78), Marcus 1969 (112), Michal and Firkin 1969 (113) and Mustard and Packham 1970 (133).

SUMMARY OF REVIEW

1. _In vitro_ studies on man and animals have shown that the biochemical mechanism of platelet aggregation is centred on ADP. A store of ADP is present in platelets and is released by substances such as thrombin, collagen, adrenaline and 5 H-T. This released ADP then acts on specific receptor sites on platelets making them capable of aggregating with other platelets.

2. _In vivo_ studies suggest that _in vitro_ studies are relevant to platelet aggregation behaviour in the intact animal, and in one study in man.

3. Blood platelets are the major initial constituents of haemostatic plugs, of thrombi on atherosclerotic plaques, and of the white head of venous thrombi.

4. After adhesion of platelets to collagen in an injured vessel, a platelet mass is formed by the clumping of platelets to each other (platelet aggregation). The platelet mass is then strengthened by the formation of fibrin strands in it's spaces and around it.
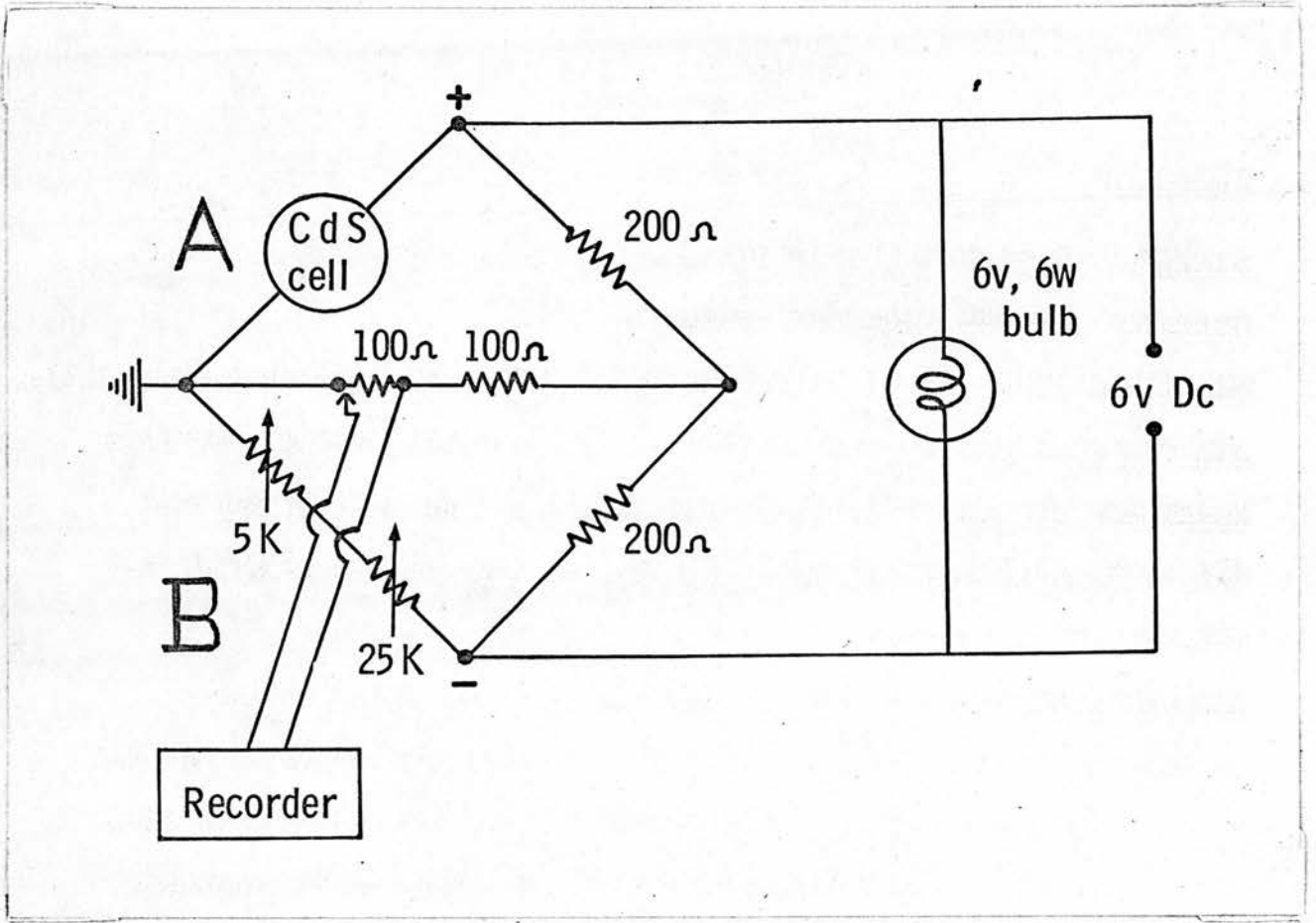
Fig 2.1   Circuit diagram of the turbidimetric method for measuring platelet aggregation

CHAPTER TWO

MATERIALS:

Needles: Disposable plastic and steel needles.   Sizes G20 and No.1.

Syringes: Plastic disposable syringes.

Glassware: All glassware was siliconised. (Siliclad, Clay-Adams Inc., N.Y).

Centrifuge Tubes: 50 ml. containers.   Siliconised glass or polythene

Micrometer syringes: Burroughs Wellcome Ltd.   One turn of the head (50 marks) delivered 0.01 mls.   The needles used for the delivery were G26 stainless steel.

Magnetic stirrers for plasma: Magnetised plastic strip (Eclipse, J. Neill & Co (Sheffield) Ltd.) was placed in a 3 mm. length of polythene tubing (NO.3 sterivac, Allen & Hanburys) and the ends were sealed with molten plastic.   They were checked for comparability in the aggregation apparatus.

Aggregation Apparatus:

1.   Modified Titrator (Evans Electro-selenium Ltd.).

   A cadmium sulphide photo-resistor cell (Mullard ORP 12) was substituted for the standard selenium photo-electric cell.   A red filter (EEL606) was placed in front of the cell.   The table for holding specimens was removed, and a plastic water jacket (Professor G. V. R. Born's Workshop, Royal College of Surgeons, London) was inserted.

2.   6v.D.C. stabilised supply (Cossor Instruments Ltd., England).

3.   Honeywell strip Chart recorder - 1mV full scale deflection, Type Y 153X18/V-II-III-/IV-/-R.

4.   **Circuit** Fig. 2.1.   The photo-cell (A) was situated in one arm of a Wheatstone bridge circuit.   Variable resistances (B) of 25K and 5K were placed in the opposite arm (allowing coarse and fine adjustment of the baseline).   A 6v DC stabilised supply was connected

across the balancing arm. Changes in the balance arm were recorded across a 100 ohm variable resistance (<u>sensitivity</u> control) by the recorder.

5. The stirrer motor of the EEL titrator revolved at 1300 rpm and was exactly followed by the magnetised stirrer in the plasma (both checked with a stroboscope).

<u>Centrifuge</u>: M.S.E. super minor with a head for four 50 ml. buckets.

<u>Tachometer</u> (Smiths Industries): Full scale reading on higher sensitivity 0 to 1000 and on lower sensitivity 0 to 5000.

<u>Water Bath</u>: Sensitive to $\pm$ 0.5^{o}C (Type SB2, Grants Instruments (Camb.) Ltd.).

<u>Microscope</u>: Reichert, Biozet, with phase contrast attachment.

<u>Parafilm</u>: (Lindsay and Williams Ltd., London).

<u>Cleaning of glassware and plastic</u>: After rinsing, the apparatus was soaked for one hour in Pyroneg solution. Then it was re-rinsed ten times. Distilled water was used for the last three rinses. The glass was dried in a hot air oven at 37°C.

REAGENTS

<u>Adenosine disphosphate</u> from equine muscle (Sigma Chemicals Ltd).
A stock solution (10^{-2}M in saline) was prepared and divided into
2 ml. aliquots.   These were stored at -20°C until used and a new
batch of aliquots was prepared every two months.

Other chemicals were prepared and stored in the same way.

<u>Adenosine, adenosine monophosphate, adenosine triphosphate</u>
(all Sigma Chemicals Ltd).

<u>Adrenaline acid tartrate</u> (Burroughs Wellcome Ltd).

<u>Thrombin</u> – bovine (Parke-Davis).

<u>Collagen</u> was prepared from human achilles tendon obtained postmortem
(method of Hovig 78).   It was stored at -4°C, and a batch was only
used for two weeks.

<u>Heparin</u> (Pularin, Evans) was dissolved in saline to give the required
strength and stored at -4°C.

<u>EDTA</u> Trisodium salt (Hopkin & Williams)

<u>Calcium Chloride</u> A 40mM stock solution in distilled water was prepared.

<u>Citrate anticoagulant</u> 3.8% Trisodium citrate was prepared in de-ionised
distilled water by the Pharmacy department.   It was stored at 4°C.

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METHODS

<u>Preparation of platelet rich and platelet poor plasma.</u>

Blood was collected by clean venepuncture and two syringe technique
into 20 ml. syringes which contained the anticoagulant.   The
anticoagulants were 2 mls. 3.8% citrate (final concentration in blood
0.38%);   2 mls. 30 mM EDTA (final concentration 3mM);   or 200 units
heparin in 0.2 mls. (final concentration 10u/ml).   The blood and
anticoagulant were mixed using a bubble of air drawn into the syringe.
The anticoagulated blood was discharged into a 50 ml. centrifuge tube
and incubated at 37°C for 3 mins.   It was then centrifuged at 150g. for
10 mins. at room temperature.   The platelet rich plasma was removed to
a level of about 0.5 cms. above the blood/plasma interface, and
transferred to a test-tube in a waterbath at 37°C where it was stored
until it was used.   The residue of blood and plasma was re-centrifuged at
460g., and the platelet poor plasma was removed to a level of about 0.5 cms.
above the plasma/blood interface.   It was stored at 37°C.

<u>Platelet aggregation</u>: theoretical and practical details are described
in Chapter Three.

<u>Platelet counts</u>:   Blood and plasma specimens were collected in sequestrine
plastic tubes and platelet counts were performed by the method of Dacie
(34), using phase contrast microscopy.

<u>Platelet shape estimations</u>:   Specimens were fixed in formalin solution
and examined and counted according to the wet preparation technique
described by Zucher and Borelli 1954 (189).   The specimens in this test
and tests of platelet morphology (see below) were coded before counting
in order to make the examination a 'blind' one.
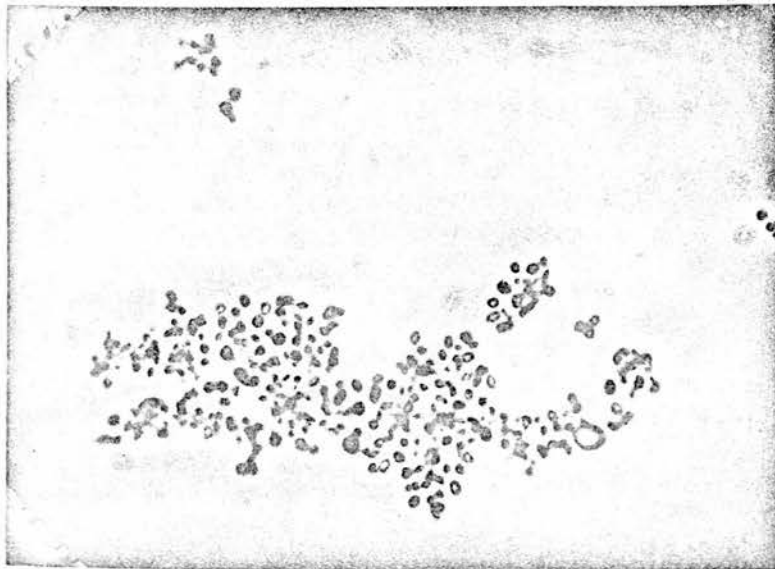
Fig. 2.2 Loose Adhesion of platelets in an aggregate. Nearly all platelets individually indentifiable.
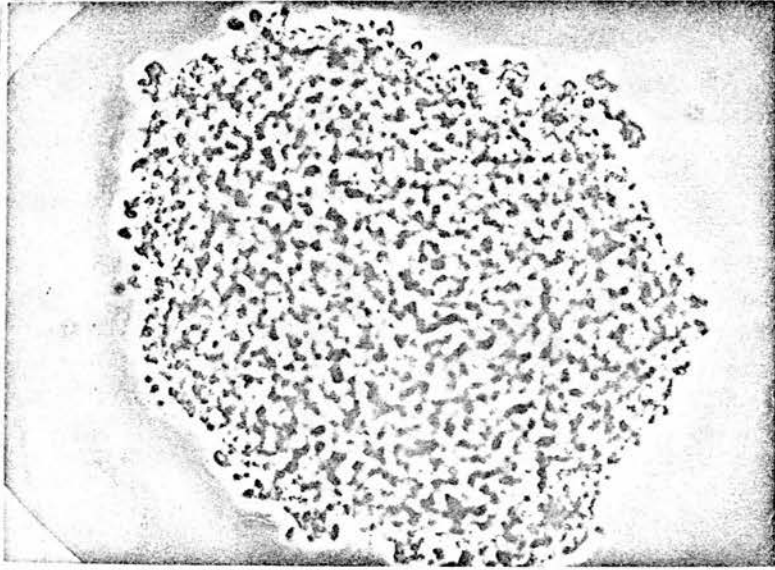
Fig. 2.3 Moderate Adhesion of platelets in an aggregate. Platelets individually seen at the periphery of the aggregate, but not in its centre.
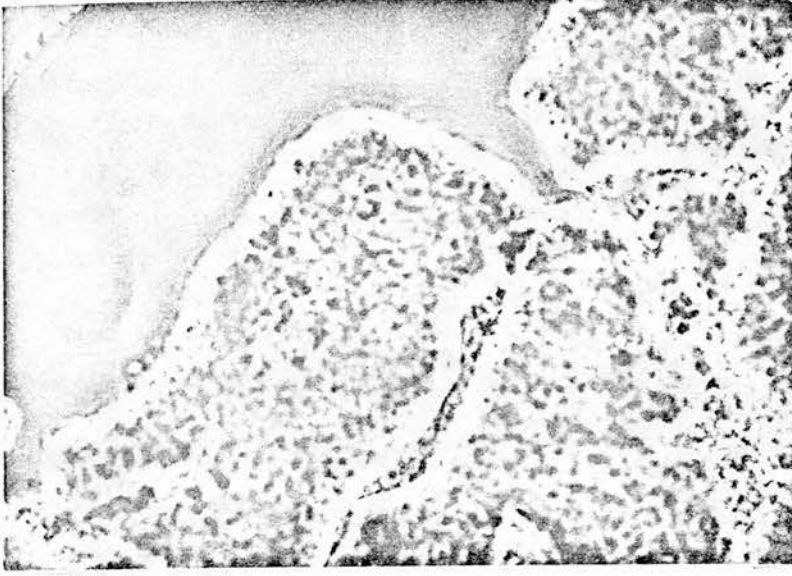
Fig. 2.4 Marked Adhesion of platelets in an aggregate. Individual platelets can no longer be seen at the edges of the aggregate.

Three categories of shape were counted:-

(a) Disks – platelets whose short diameter (as they turned over in the wet preparation) was 30 percent or less of the long diameter.

(b) Spheres – roughly spherical platelets whose shortest diameter was not less than 50 percent of the longest one.

(c) Others – shapes intermediate between disks and spheres, or degenerate shapes.

Platelet aggregate morphology: Specimens were fixed as for platelet shape.

(a) Sizes of aggregate. Aggregates were recorded in the size ranges of 2-10, 11-20, 21-50 platelets and then as 50 plus platelets to $\frac{1}{4}$ HPF (high power field 400x), $\frac{1}{4}$-1 HPF, 1-2 HPF, 2-5 HPF and 5 plus HPF's. The percentage of the total platelets in each of the aggregate size ranges was estimated visually by comparing identical dilutions of the original platelet rich plasma (PRP) and aggregated PRP with each other. The estimate was recorded as the percentage of the originally single platelets (i.e. 100%) present in the aggregates of each range of aggregate size. This method is semi-quantitative, however in a series of duplicates, examined in a blind experiment, the results in the pairs were similar.

(b) Morphology of larger aggregates. The aggregates were slowly and firmly squashed by pressing the slide against the coverslip (Sharp 166). The degree of separation of the platelets produced by the compression indicates the strength of inter-platelet bonds. Specimens were classified in the following categories:

(1) Loose adhesion – nearly all platelets in the aggregates individually identified (Fig. 2.2).

(2) Moderate adhesion – Individual platelets identified at the edges of the aggregate, but not at its centre (Fig. 2.3).

(3) <u>Marked adhesion</u> - Individual platelets not seen even at the
        edge of the aggregates (Fig. 2.4).

A specimen was classified on the adhesion category which was dominant.
Although the method is semi-quantitative, blind examination of duplicates
produced similar results.

<u>Measurement of the aggregating activity present in a PRP supernatant</u>

During platelet aggregation substances such as adenine nucleotides,
5 H-T and adrenaline may be released by the platelets and those
substances added to the PRP are altered by plasma and platelet metabolism.
Some are stimulators and some inhibitors of aggregation. One often
wishes to know the sum of the effect of their interaction on platelets,
but some of these substances may not yet have been identified and the
interactions of those already identified are complex. Their composite
effect has been measured in this thesis biologically - by adding the
supernatant to fresh PRP. The aggregation produced by the supernatant
was compared with that produced in the same PRP by known concentrations
of ADP.

In some experiments the total aggregating activity of the supernatant was
measured. In most only the ADP-like aggregating effect was estimated.
This was separated from the total by adding the adrenaline and 5 H-T
inhibitor, phentolamine, and the thrombin inhibitor, heparin, to the
test PRP (Macmillan 106).

<u>Method.</u>

Aggregated specimens of plasma were allowed to stand for 45 secs to allow
the larger aggregates to sediment (sedimentation and centrifugation of
aggregated specimens gave equivalent results). 0.25 mls of supernatant
was then drawn into a plastic 1 ml. syringe and injected at 60 secs after
the end of aggregation (i.e. 15 secs after the sedimentation period)
into a fresh specimen of plasma (calibration plasma). The aggregation

TABLE 2.1 Reproducibility of estimations of ADP-like activity in different calibration PRP

Dilution of Supernatant	ADP-like aggregating activity (μM/L)				Mean (percent)
	Plasma(1)	Plasma(2)	Plasma(3)	Plasma(4)	
100%	2.125 (2.05;2.2)	1.825 (1.95;1.7)	2.05 (2.05;2.05)	1.60 (1.55;1.65)	1.90 (100%)
60%	1.45 (1.5;1.4)	1.25 (1.2;1.05)	1.25 (1.15;1.3)	1.05 (1.05;1.05)	1.21 (63.5%)
30%	0.75 (0.7;0.8)	0.5 (0.5;0.5)	0.65 (0.65;0.65)	0.45 (0.45;0.45)	0.59 (31%)

produced by the supernatant in the calibration plasma was compared
with that produced by platelet poor plasma (PPP), prepared from the
plasma under test, to which known concentrations of ADP had been added.
The aggregating activity in the supernatant was expressed as its ADP
equivalent activity in μ moles per litre (μM).   When the calibration
specimen contained heparin and phentolamine the activity of a supernatant
was called it's ADP-like aggregating activity and was also expressed as
μ moles per litre.

Reproducibility.   A pool of supernatant was prepared from platelets
which had released aggregating activity in response to ADP (Secondary
aggregation, Macmillan 106).   The pool was stored at 4°C and four sets
of diluted specimens, each in duplicate, were prepared.   Each set
consisted of a specimen of undiluted supernatant, of pooled supernatant
diluted to 60 percent, and to 30 percent (by PPP from the same specimen)
and of PPP containing known quantities of ADP.   The preparation took
45 minutes.   The four sets were stored at -20°C.   The ADP-like
aggregating activity in the supernatant and it's dilutions was measured
using calibration plasma from four different subjects.   Calibration
plasma specimens contained phentolamine (4μM) and heparin (10 units/ml).
The experiment lasted a total of six hours.   The results are shown in
Table 2.1.

The average ADP-like aggregating activity in diluted supernatant matched
closely with the dilutions made i.e. 60% to 63.5% and 30% to 31%.   The
range of results were, mean $\pm$ 20% for 100% supernatant;   mean $\pm$ 25% for
60% dilution and mean $\pm$ 30% for 30% dilution.   The duplicate results in
each calibration plasma were close together whereas there was a wider
variation between the different calibration PRP's.

This showed that the range of the results of $\pm$ 20% to $\pm$ 30%, was due more to biological variations in the response of the calibrating plasma than experimental technique. The degree of variation found is of the order expected with biological materials. **Validity.** Direct comparisons were not made between aggregating activity and biochemical measurements of adenine nucleotides, 5H-T, and catecholamines in the same plasma. However the results obtained by this biological method are the same as those subsequently obtained when biochemists examined the same situation (Mills et al 116).

Definitions.

Platelet adhesion: Platelet adherence to non-platelet surfaces (eg. collagen and glass).

Platelet aggregation: Platelet adherence to other platelets to form platelet aggregates.

Platelet disaggregation: The breaking up of platelet aggregates, resulting in smaller aggregates or single platelets.

Platelet stickiness: An _in vitro_ phenomenon measuring the reduction in the platelet count produced by varying stimuli (usually glass) which cause platelet adhesion plus platelet aggregation, and possibly disruption of the platelets.

Primary aggregation (PA): An aggregation response to ADP, occurring in all platelet rich plasma, in which the aggregation process increased in extent gradually with increase in the added ADP concentration (P.A. was followed by disaggregation at up to, and including, the 10μM ADP dose).

Secondary aggregation (SA): A second wave of aggregation to ADP, occurring in only some platelet rich plasma. It produced an abrupt increase in aggregation of near maximal extent. No disaggregation followed for at least five minutes. The ADP concentration which first produced S.A. was called the S.A. point ADP concentration.

Three types of S.A. occurred:-

(1) <u>Biphasic S.A.</u>: The strongest S.A. It was able to reverse established disaggregation, and so produced a markedly biphasic aggregation tracing.

(2) <u>Non-phasic S.A.</u>: A weaker S.A. It was able to continue the primary aggregation process but did not reverse established disaggregation.

(3) <u>Level S.A.</u>: A non-phasic S.A. in which the increased aggregation reached a steady (level) state lasting at least five minutes.

<u>Chain-reactive S.A.</u>: An S.A. process which was able to extend it's aggregation-inducing potential beyond the plasma in which it occurred to further specimens of the plasma, so producing a marked aggregation <u>chain</u> reaction.

<u>Self-limiting S.A.</u>: An S.A. process which was unable to induce a marked aggregation chain-reaction.

Statistics

The question of which type of analysis to use in this thesis was discussed with Dr. Barr, Director of the Statistical Department, Oxford Regional Board. The measurements of the 'secondary aggregation' reaction of platelets were made by finding which of a <u>range</u> of concentrations of a chemical produced the reaction; also a significant, but quantifiable (see Chapter Seven), biological variation was present. Both these facts argued strongly for a conservative approach to the analysis. It was decided to use the less sensitive parametric range of tests which demand more experiments and greater agreement to achieve significance, and it was felt that confidence could be placed in the 5% ($p = 0.05$) probability level. The use of parametric tests allowed direct analysis of the experiments which gave the part qualitative, part quantitative data, inherent in studies of the secondary aggregation reaction of blood platelets.

The tests were performed according to the directions given by Langley 1968 (ref 100).

Asterisks are used in the tables to denote levels of significance, on the scale shown below (this will not be repeated for each table in the text).

* $p = < 0.05$ ** $p = < 0.01$ *** $p = < 0.002$ **** $p = < 0.001$

CHAPTER THREE

In vitro MEASUREMENTS OF PLATELET ADHESION AND AGGREGATION

1. INTRODUCTION

In vitro measurements of platelet adhesion and aggregation involve the obtaining of preparations containing a highly metabolically active 'cell', the platelet, and exposing them to an acute stimulus as comparable as possible to the ones occurring in vivo during haemostasis and thrombosis.

Obtaining platelet preparations means for practical purposes obtaining whole blood i.e. a plasma, red cell, white cell, platelet mixture. Unless the blood is immediately tested (Salzman (158), Busfield (23), it must be either cooled (which damages platelets (192), or anticoagulated (or it will clot). Thus, if separation of platelets from red cells is contemplated, one must either damage them with cold or alter their behaviour with anticoagulants. Anticoagulants are not necessarily a lesser upset than cooling, however they do allow easier handling of platelet preparations, more exact observation of platelet behaviour, and the use of more complex tests.

Platelet adhesion and aggregation studies are hampered by a problem which is common to much biological material from man - no absolute standards can be prepared. An additional problem is that the number of platelets in plasma varies over a wide range giving difficulties in quantitation.

In spite of these drawbacks much has been accomplished, as will be seen from the preceding review. But it is important to remember the degree of manipulation which occurs before testing, and the degree of comparability of the stimuli used to the in vivo situation it is

Table 3.1   Advantages and drawbacks of methods of measuring platelet adhesion and aggregation

Problems of all tests:
1. Either rapid testing, or anticoagulants or low temperature to prevent clotting
2. Changes in platelet response with temperature and through time
3. If centrifuging blood may be selecting an atypical population of platelets
4. No standard platelet preparation can be prepared

Test	Theoretical		Technical	
	Advantages	Drawbacks	Advantages	Drawbacks
Platelet Adhesion	1. Single process	1. Adhesion to glass differs from adhesion to collagen	1. Constant observation	1. Semi quantitative
Platelet stickiness	1. All elements blood present 2. Both adhesion and aggregation present	1. as above 2. So many variables that further more specific tests needed to find the cause of a change 3. Only two observation points, with unknown in between and afterwards	1. Simple apparatus	1. Technical difficulties and procedural pitfalls of platelet counting methods 2. Standardisation of apparatus very important and difficult 3. Technique requires constant contact time with stimulus and constant volume
Platelet aggregation (Borns Method)	1. Single process 2. Observe whole process	1. Centrifugation may give atypical platelet population 2. Red cell and most white cells absent	1. Constant observation 2. Many stimulants can be used in series or parallel 3. Sampling for biochemical or morphological studies 4. Work at body temperature	1. Larger sample (20 mls against 5 mls) 2. More complicated equipment

intended to mimic, when considering the possible relevance of _in vitro_ findings to the _in vivo_ situation.

2. PRINCIPLES OF TESTS OF PLATELET ADHESION AND AGGREGATION

There are three main groups of tests:-

(1) Adhesion tests - the adhesion of platelets to various substances, especially glass, is observed directly.

(2) Aggregation tests - platelets, separated from red cells and most white cells, are stimulated to aggregate, and the effects of the stimulus are followed in different ways; visually, by optical or ultrastructural microscopy, and automatically by recording the changes in the density of the plasma.

(3) Platelet stickiness tests - platelets are induced to adhere to a surface (usually glass), other platelets aggregate onto the adherent ones (142, 149) and yet other platelets form non-adhering aggregates; some platelets may disrupt. This is thus a composite test. The combined effects of these three processes are calculated from platelet counts taken before and again after the blood has been exposed to the stimulus.

3. CRITIQUE OF THE TESTS

The advantages and drawbacks of the three groups of tests are given in Table 3.1.

(a) Tests of adhesion involve microscopic study of blood or platelet rich plasma in contact with an adhesion-inducing surface. Collagen in injured tissue has been used (174), but the effects of this reaction was until recently only roughly quantifiable.

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Spaet et al (173) have now developed a technique which may solve this problem. It is easier to quantitate tests in which glass is the stimulus, but the mechanism of adhesion to glass shows differences from that of adhesion to collagen (20), suggesting that different platelet properties may be being measured.

(b) Tests of platelet stickiness have their earlier origins in Morowitz and Jurgens (124) experiments, in which they drew non-anticoagulated blood back and forth through a capillary tube. The modern era of this test was initiated by Helen Payling Wright (186). She constructed a vaseline coated, revolving bulb which allowed intermittent and moving contact between blood and a clean non-vaseline coated glass coverslip. The platelets were counted at zero time and again at intervals of 20 mins. The decrease in platelet count was taken to be an estimate of platelet adhesiveness. Many variations of this technique of prolonged contact with a small glass area have been developed since (111, 61). A new generation of tests with a short glass contact time was introduced by Moolten and Vroman (123) in 1949 and the technique was much improved by Hellem (72). In order to increase the area of glass Hellem used many small glass beads packed into a container. This represented a major advance in standardisation as he was able to pump blood through the beads at a standardised rate. He also described many of the technical and sample variations which affect the results, and which are now known to be crucial for worthwhile results. Others continue to be added, (61, 53, 55). Modifications are plentiful, with Busfields (22) the most attractive. Unfortunately it is not possible to work at body temperature with these methods.

The numerical basis of platelet stickiness tests is the platelet count. As Mitchell (55) has so aptly said, "only those who have tried to decide whether a blob in a counting chamber is, or is not, a platelet, can appreciate the difficulties of this laboratory procedure, and understand the ease with which observer bias may occur".

The place of this test in studies of platelets and their disorders has become clearer with the passage of time. Its greatest use is as a _clinical_ method for _screening_ patients and populations

It has the advantage of allowing interaction of all elements of the blood. However the corollary of this is that when changes are detected it is necessary to perform more specific tests to find out which component of whole blood is producing the change. Are the red cells releasing more (or less) ADP? (e.g. measure red cell fragility): or are the platelets varying in adhesion, or aggregation, or disintefgration?, or are the white cells or plasma (e.g. fibrinogen) responsible? Thus this test has a position analogous to that of the whole blood clotting time in coagulation work, any abnormality must be investigated further to find it's cause, and a negative result does not exclude an abnormality.

(c) _Tests of platelet aggregation_: In these tests the platelets are separated from the red cells, thus limiting the number of reactants. The act of separation introduces the possibility that one will be studying an atypical platelet population, and this possibility has been studied in this thesis. Also the red cells could theoretically affect the platelets by releasing ADP during the separation process, but there is no proof that this actually occurs.

These theoretical disadvantages are outweighed by the many advantages of being able to study a single process, aggregation. Initially platelet rich plasma was studied in test-tubes containing

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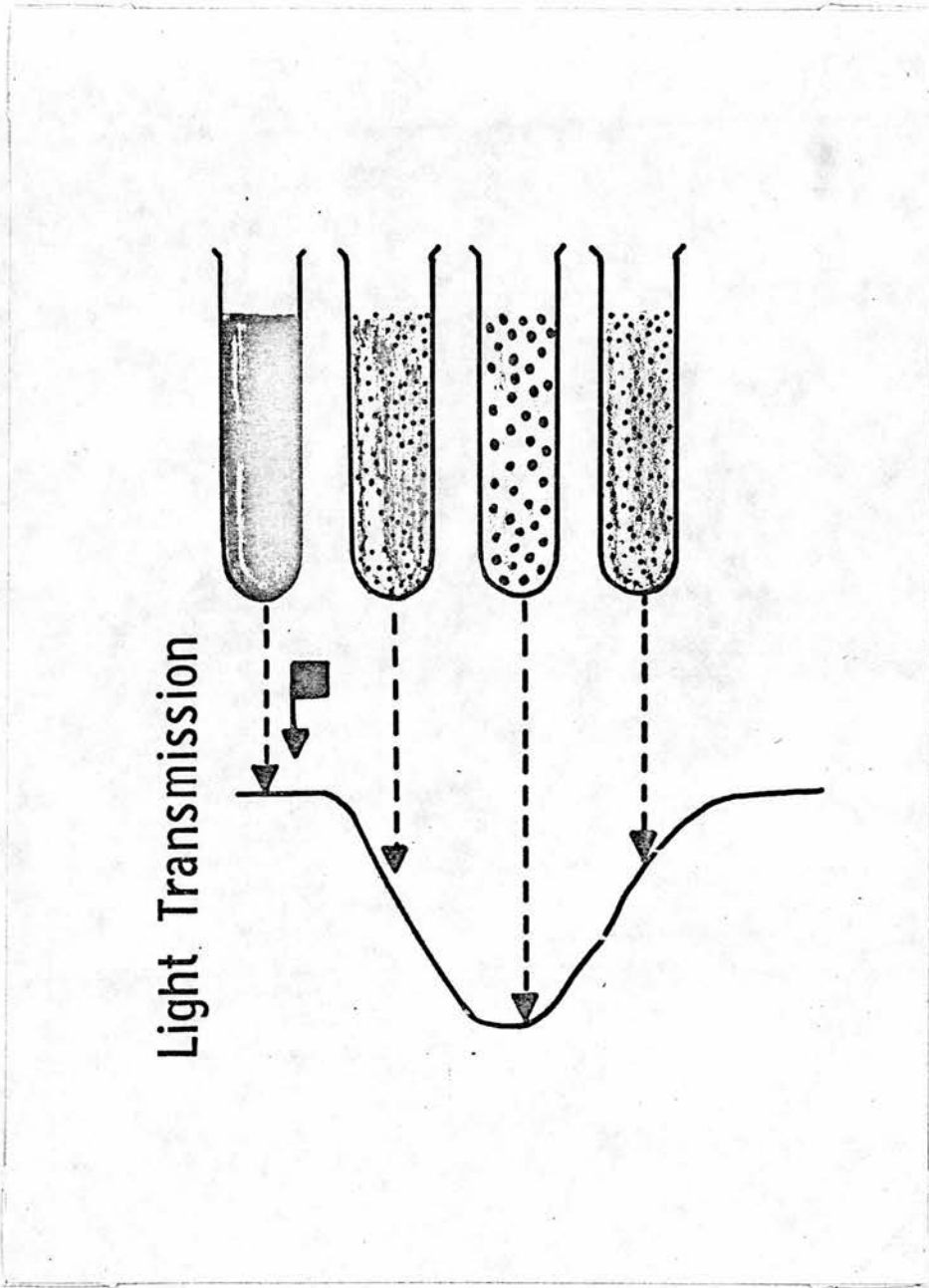
Fig. 3.1 Diagramatic representation of the correlation between plasma light transmission and the formation and subsequent breaking up of platelet-aggregates.

Borns Method

Light

Water jacket

Magnet

to recorder
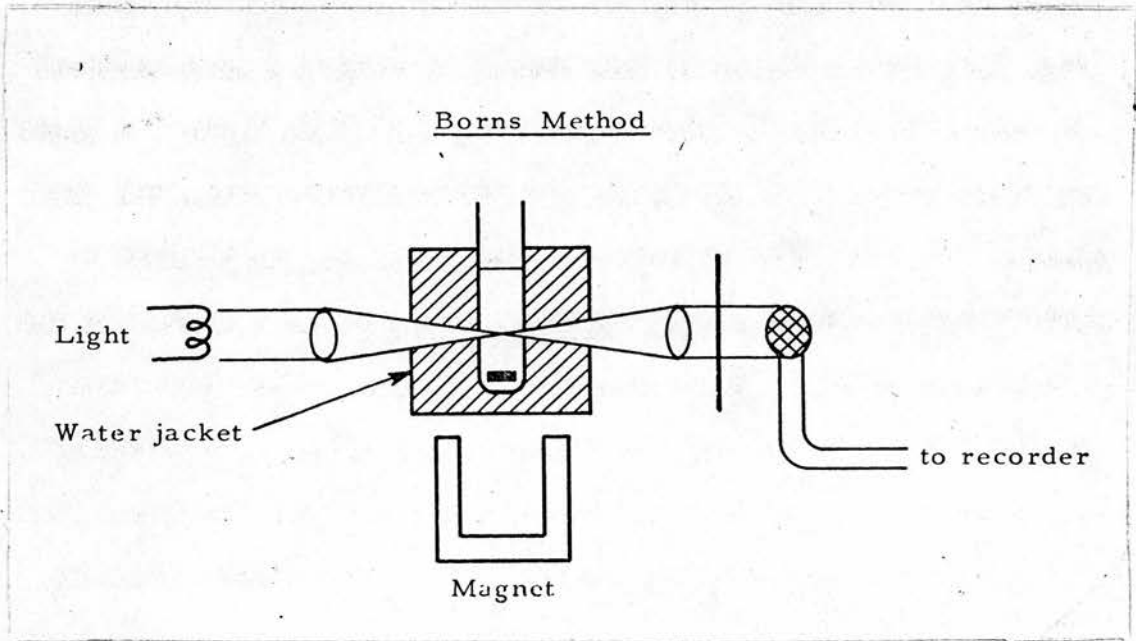
Fig. 3.2 Diagram of the apparatus of the
turbidimetric method of Born.

PRP which were shaken after the addition of aggregating agents
(Sharp, 165, 166).

Method of Born: Professor Born (12, 17) noted that as aggregates
formed in a test-tube of PRP the density of the plasma decreased
(Fig. 3.1) and he harnessed this change to develop a much improved
and automatic method of measuring aggregation (Fig. 3.2). A light
was shone through the plasma on to a photo-electric cell, and the
changes in plasma density were recorded by noting the changes in
light transmission. A continuous recording of the aggregation and
disaggregation of platelets was thus possible. Other advantages
of this method are that the stirring is carried out automatically
by a magnetic stirrer and revolving magnet, and that the tubes
containing the plasma can be enclosed in a water-jacket, allowing
work at body temperature.

This method is sensitive, for example even changes in platelet
shape can be detected (Macmillan et al 1965, 109; O'Brien 141, 144).

Specimens of plasma and aggregates can be removed, and so
correlations can be made between the tracings of light transmission
and microscopical or biochemical tests.

DESCRIPTION OF THE TURBIDIMETRIC METHOD USED IN THIS THESIS

1. Equipment: The types and specifications have been listed in the
   materials section of Chapter Two.

2. Preparation of the equipment:

   (a) The water-jacket was perfused with water at 37°C for 20 mins.
       before use.

   (b) The light source was centred on the lens system. This was
       achieved by moving the light source until inserting a glass
       tube containing saline into the curvette (saline blank)

reduced the light transmission by less than 3%. This centring of the light source was important as turbid solutions such as plasma were found to block differing percentages of the light transmission at different positions of the light source (unlike coloured solutions or coloured filters).

(c) The part of the L.T. scale required for an experiment was marked on to the recording paper. To do this a saline blank was inserted into the cuvette and previously calibrated blue filters were inserted into the light path.

(d) Micrometer syringes were filled with active chemical solutions, taking care to avoid air bubbles in the syringe and needle (G26). The needle was bent to a right angle in such a way that the bevel would face the side of the tube of plasma during an injection.

3. The technique of measuring platelet aggregation.

Using a disposable 1 ml syringe, 1 ml of plasma was transferred from the store of PRP in the waterbath to the tube used for testing. This tube was then incubated at 37°C for at least two minutes. It was wiped dry and placed in the cuvette, and alignment marks on the tube and the water-jacket were opposed.

The stirrer was added and recording started. After an interval of exactly 15 or exactly 30 seconds the injection of active agent was started. The injection took from two to five seconds (0.005 ml to 0.025 ml). After the injection the needle was wiped against the side of the tube.

The changes in plasma light transmission were recorded for three to ten minutes.

In many experiments the tracings produced by increasing concentrations of an aggregating agent were superimposed by backwinding the chart to a constant starting point. This made comparisons much easier.

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Light transmission, light extinction, density and optical density, and the tracings of platelet aggregation

Platelets vary in size and shape, and this variability is even greater in platelet aggregates. For this reason only descriptive scales can be strictly applied to the light changes in aggregating platelet rich plasma, and the 'light transmission' scale is therefore used. Density is **inversely** proportional to light transmission (0% L.T. = completely dense to light, 100% L.T. = no obstruction to light), and to avoid the mental gymnastics of this conversion the term light extinction which corresponds **directly** with density has been used by some (68). In this thesis the simpler word 'density' will be used. Density as a general term is not to be confused with 'optical density' which applies only to particles of 1µ or less. The optical density scale is used in some parts of platelet aggregation tracing interpretation (vide infra) but in the full knowledge that it is not strictly applicable.

The platelet aggregation tracings were recorded in such a way that the top of the tracings were positioned at a low light transmission (high density) and the bottom of the tracings at a high light transmission (low density). Thus a fall in the tracing indicated that the light transmission had increased i.e. the density had fallen; and vice versa.
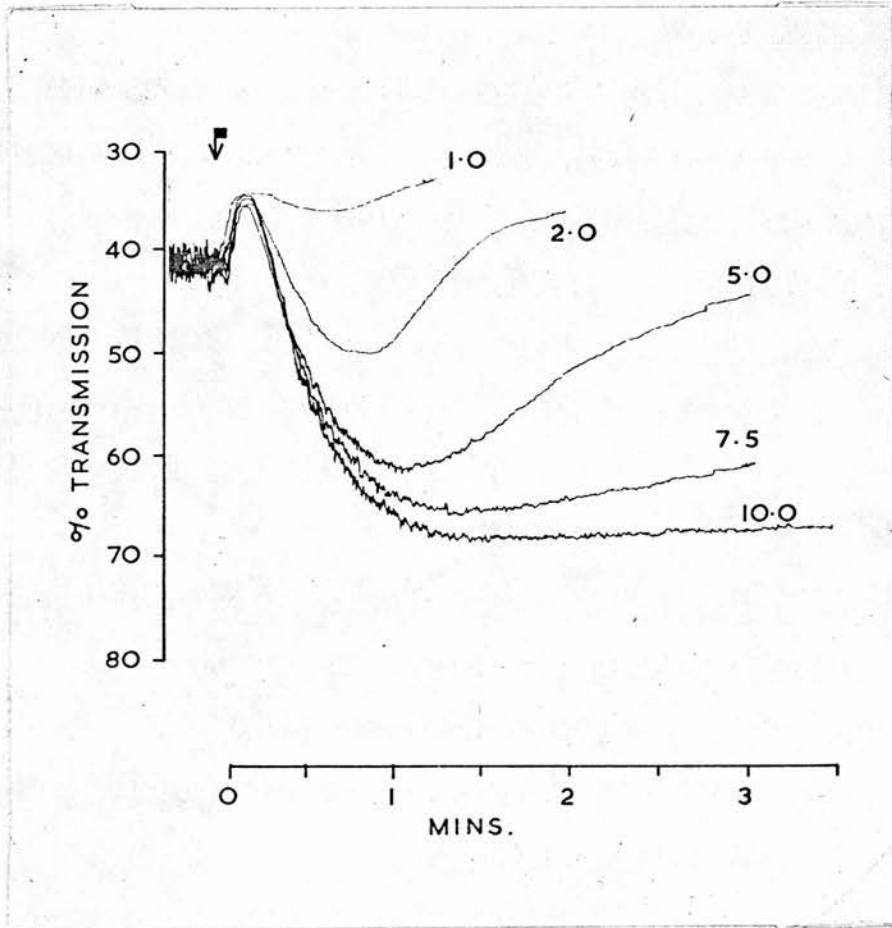
Fig. 4.1.

Photograph of Dose-response curve to ADP of platelet rich plasma

In this, and subsequent dose response curves, the plasma was stirred
for 15 or 30 seconds and then,at the point marked by an arrow, ADP
was added. The number beside each tracing is the final concentration
of ADP. The tracings produced by increasing concentrations of ADP
have been superimposed by back winding the chart. ADP was added to
this plasma in concentrations of from 1μM to 10μM. After a small
initial rise in the tracing, there followed a fall which increased
with the ADP concentration. Then the tracing showed a further rise
which decreased as the ADP concentration was increased.

PLATELET AGGREGATION-DISAGGREGATION (DOSE-RESPONSE CURVES) TO ADP IN HUMAN CITRATED PRP AT BODY TEMPERATURE (37°C).

THE DOSE-RESPONSE CURVE TO ADP

Previous work. Born (12, 17) and O'Brien (122) have described this response as one of increasing aggregation and decreasing disaggregation as the ADP concentration was increased. Born was working at room temperature, and O'Brien, using hot air, at 37°C.

Present Investigation. Fig. 4.1. shows a dose response curve to ADP of the type described by Born and O'Brien. The tracings which occurred with increasing concentrations of ADP have been superimposed to aid comparison (see methods).

Before the addition of ADP, small baseline oscillations were present. The addition of ADP was followed by three changes.

1. Oscillations ceased and the tracing <u>rose</u>.

2. A <u>fall</u> in the tracing with, at high ADP concentrations, a reappearance of oscillations.

3. A variable <u>return</u> of the tracing towards the baseline, which was of lesser degree as the fall increased.

MICROSCOPY OF SPECIMENS TAKEN DURING TRACING CHANGES.

Previous work. All investigators have confirmed Born's original observation of the presence of aggregates visible to the naked eye as the density of a plasma decreased (tracing falls), and of breaking up of the larger of these visible aggregates as the density subsequently increases (rise of tracing).

Present study. The initial rise in the tracing which followed the addition of ADP, was investigated by microscopy of samples removed from plasma undergoing this change. Then the microscopical appearance of the aggregation and disaggregation processes was investigated.

1. **Loss of baseline oscillations and rise of tracing.**

Previous work. In vivo blood platelets are disk shaped (2, 7, 9) and this in vivo disk shape is well maintained in citrated plasma at $37^{\circ}C$ (189). ADP has been shown to change the shape of platelets from disks to spheres coincident with aggregation (195).

O'Brien, 1965, (141) and Macmillan and Oliver, 1965, (109) noted that when plasma containing disk shaped platelets was stirred a swirling effect could be seen by the naked eye, presumably due to the disk shape of the platelets resisting stirring.

The light transmitted by this swirling plasma oscillated (141, 109). When the shape of the platelets was changed from disk to sphere by ADP the swirling ceased and the plasma density rose. The light transmission showed corresponding changes.

The experiment described below is an extension of the work reported in 1965.

Present investigation. Samples from the same pool of plasma were stirred for 5 mins. After 30 secs. stirring saline or low concentrations of ADP (0.1, 0.2, 0.4 μM) were added, (using these low concentrations the earliest stages of the 'rise' were exposed). Samples were taken for microscopical

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Fig. 4.2. Effect of low concentrations of ADP on PRP (correlated with microscopy of samples).

After 30 seconds stirring, saline (top specimen) and ADP 0.1, 0.2, 0.4 μM (lower specimens) was added (arrow with box). Samples for microscopy were taken (plain arrow) before the addition of the saline or ADP, and on four occasions after the addition. ADP 0.1uM produced a small rise in the tracing with a partial loss of oscillations - there was a partial fall towards the baseline and some return of oscillations over the five minutes. With 0.2μM the rise was sharper and oscillations ceased, and with 0.4μM ADP the rise was followed by a small fall and subsequent rise of the tracing. The results of the microscopy are given in the text.
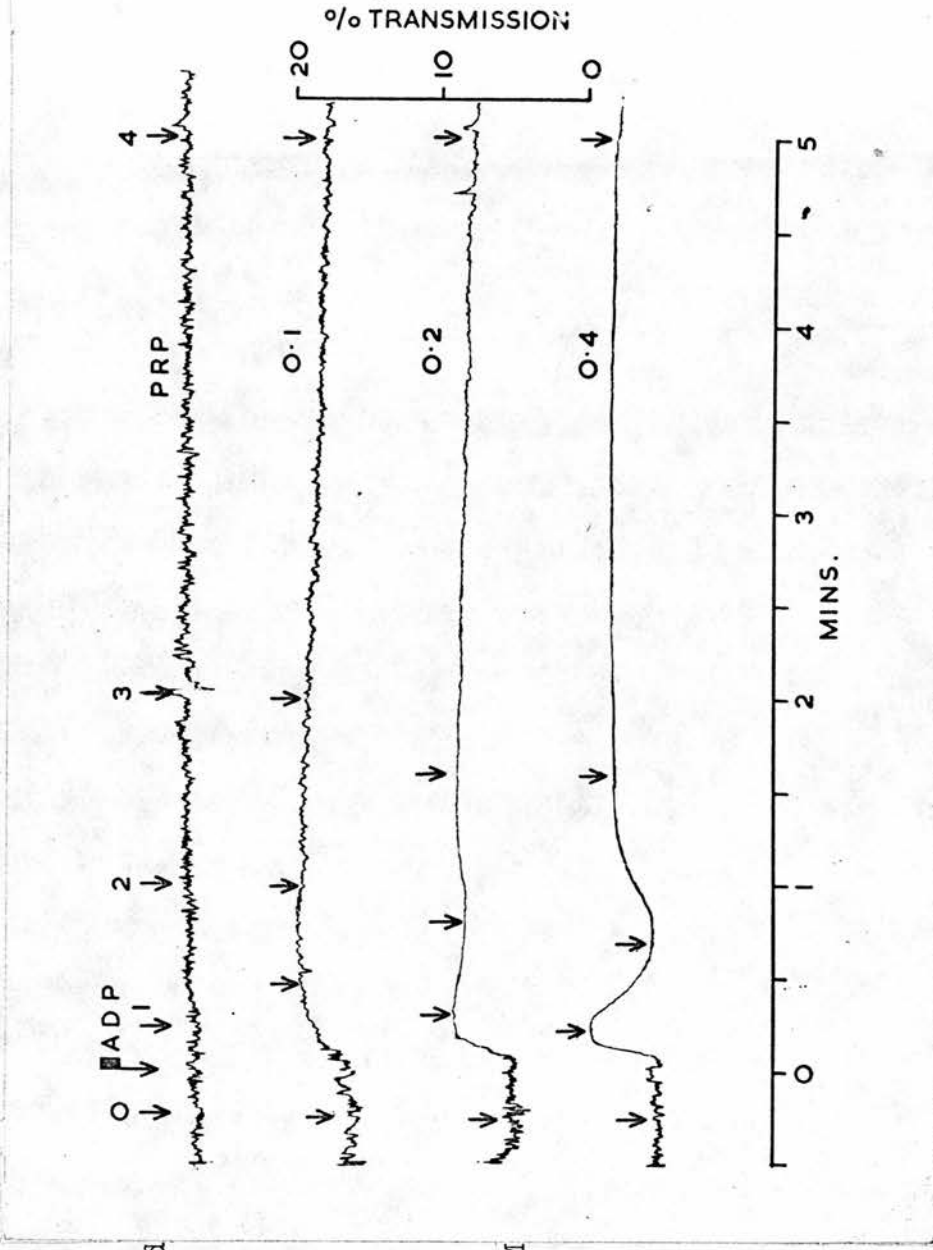
examination during the 'pre-addition' period of stirring and at four
points after the addition.   Three experiments were performed and they
gave the same results.

Results Fig. 4.2.

1.   PRP + saline.   Oscillation of the tracing persisted for the 5 mins.
    of the experiment.   The microscopy samples contained over 80% of
    'disk' shaped platelets throughout the 5 mins.   At the 'pre-addition'
    point most of the residual platelets were 'indeterminate' in shape.
    At 5 mins. about 50 percent of the residual platelets were 'sphered'.
    A few aggregates of disks (2-5 platelets) were observed.

2.   ADP 0.1 µM.   Coincident with the small rise in tracing and partial
    loss of oscillations induced by the ADP, the percentage of single disk
    platelets fell from 84% to 60% and of single spheres rose from 2 to 30%.
    Further spheres were present in aggregates of 2-10 platelets and an
    estimated 15% of the platelet population was in aggregates.   By
    5 mins, almost complete disaggregation had occurred and the percentage
    of disk platelets had risen to 77% and of spheres had fallen to 15%.

3.   ADP 0.2 µM.   The rise of the tracing was more abrupt and oscillations
    ceased.   By the top of the rise the percentage of disk shaped platelets
    had fallen from 84% to 20% with a corresponding rise in the percentage
    of spheres.   25% of the platelet population was in aggregates of
    spheres which varied in size from 2 platelets to 20 platelets.   Over
    the next 5 mins complete disaggregation occurred, and the percentage
    of the disk shaped platelets rose to 62%.

4.   ADP 0.4 µM.   After an abrupt rise in the tracing there followed a
    fall, and soon afterwards a further rise.   Subsequently the tracing
    fell towards the baseline.

        By the top of the rise only 5% of the platelets were still disk
    shaped (85% were spheres and the remainder 'indeterminate').   Eighty
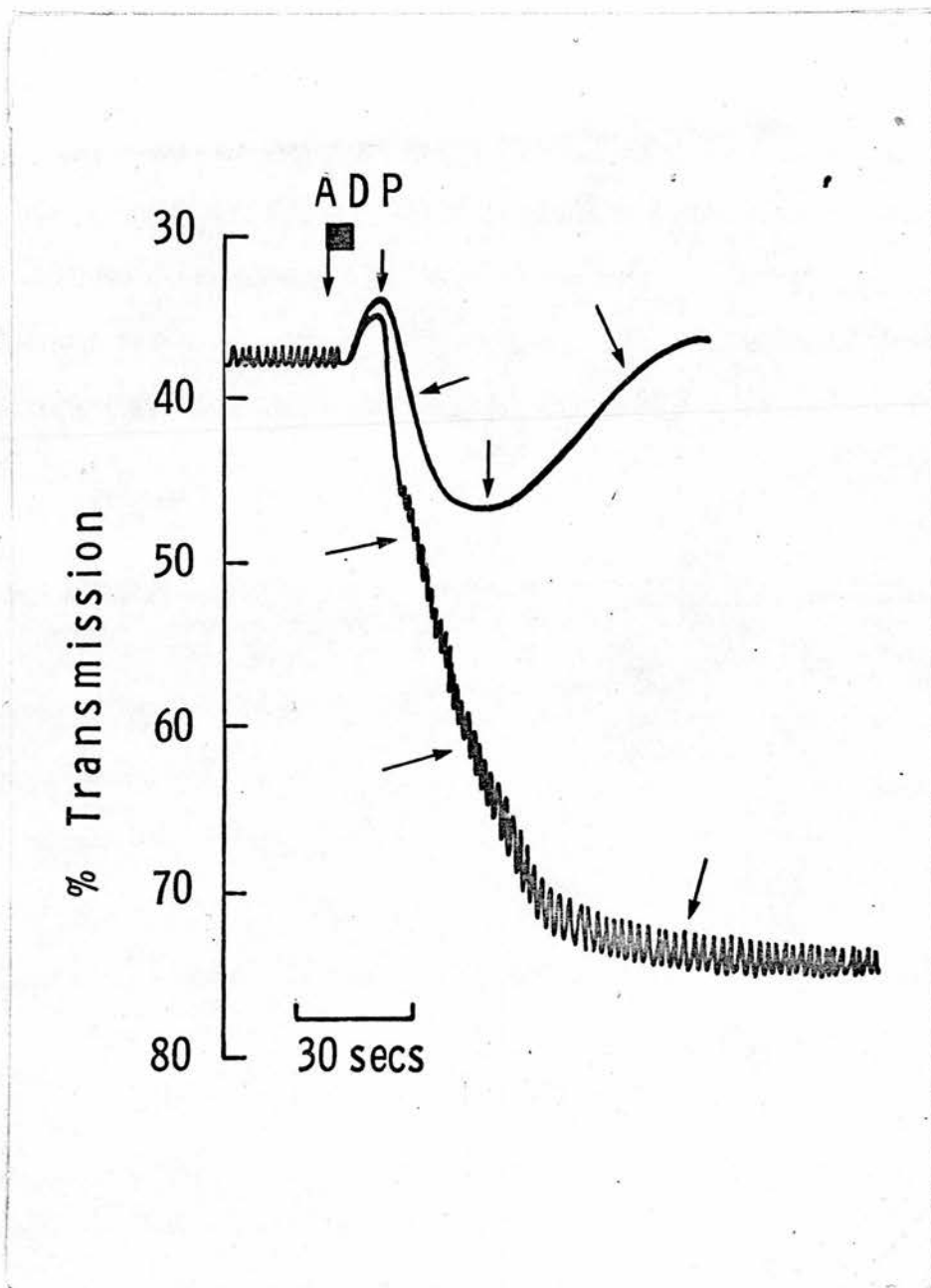
Fig. 4.3.

Diagram of the tracings used for tracing/microscopy correlations

ADP was added after 30 seconds stirring (arrow with box).
Samples were taken from the specimens at the points shown by plain arrows.

percent of the platelets were in aggregates, and the size of aggregates ranged from 2 platelets to 50 - $\frac{1}{4}$ H.P.F. (high power field, see methods). Over the 5 mins of observation over 80% of platelets became single. The remainder were in small aggregates (2 to 20 platelets). Over 40% of platelets resumed their previous shape of disks.

Comments

1. ADP produced sphering of disk shaped platelets and some spherical platelets formed aggregates. These changes were detected by the turbidimetric method of observing PRP. Over 5 mins a marked disaggregation and moderate regaining of the disk shape occurred.

2. There was a marked variation in the response of the platelets. Only some changed to spheres. Only some sphered platelets formed aggregates, and these aggregates varied in their size.

3. The variability of the platelet response was presumably due to the different ages of the platelet population of a PRP.

2. Aggregation (Fall of tracing).

Previous work. Born and Hume (19) have demonstrated a rapid disappearance of single platelets during the fall of the tracing produced by ADP-mediated platelet aggregation. These workers then studied the density of the larger aggregates paying particular attention to the changes in the density of aggregates during 'secondary' aggregation (106). This density was increased compared to that of 'primary' aggregates.

Present work. Specimens of plasma were removed for microscopical study at several points of a moderate aggregation response (25 - 35% of initial plasma O.D.) and of a very marked aggregation response ('secondary' aggregation). See Fig. 4.5. for diagramatic representation of the tracings and the sampling points. The samples were examined for the percentage distribution of the platelets in different sizes of aggregates,

Table 4.1. __Microscopy of specimens at different stages of ADP induced aggregation and of disaggregation.__

| | Percent of platelet population | | | | | | | | Internal Adhesion of larger aggregates |
| | | | | High Power field (x400) | | | | | |
	Single	2-10	10-50	$\frac{1}{16}-\frac{1}{4}$	$\frac{1}{4}-1$	1-2	2-5	5+	
1. Moderate fall									
a) Baseline	100	-	-	-	-	-	-	-	-
b) Top of rise	11	19	24	46	13	-	-	-	Loose
c) 50% fall	11	11	14	31	31	1	-	-	Loose
d) 100% fall	11	18	34	25	10	1	-	-	Loose
e) 80% disaggregation	49	28	18	9	-	-	-	-	Loose
2. Marked fall									
a) Baseline	97	3	-	-	-	-	-	-	-
b) Top of rise	13	19	21	25	15	9	-	-	Loose
c) 30% fall	10	10	9	10	24	25	16	5	Moderate
d) 60% fall	8	9	5	4	56	10	5	3	Marked
e) 100% fall	6	7	1	3	20	46	15	-	Marked

* Average of four experiments

and for the degree of inter-platelet adhesion in the larger aggregates
(as described under methods). Four experiments were performed at each
level of aggregation and the pattern of the changes was similar.

Results Table 4.1.

1. Moderate fall. As expected microscopy confirmed the presence of
    aggregates but the pattern of their formation was unexpected. By
    the top of the rise some of the aggregates were nearly as large as
    those present during the late stages of the aggregation process.
    The greatest involvement of platelets in large aggregates occurred
    at half-fall. The percentage of platelets in larger aggregates at
    the full fall point was less than that in larger aggregates at the
    top of the rise. At all points single platelets and platelet
    aggregates of all the sizes up to the maximum one occurring were
    present. Adhesion of platelets to each other in the aggregates was
    'loose' at all times.

2. Marked fall (S.A.). By the top of the rise many aggregates, some
    large, were present. The number of single platelets and aggregates
    of 2-10 was small at all times. During the 30% to 100% fall time
    period, aggregates of sizes 10 platelets to $\frac{1}{4}$ HPF almost disappeared,
    with almost all platelets forming into large aggregates. However
    the largest aggregates were present at the 30% fall position.

    The degree of internal adhesion of platelets in the aggregates
    increased steadily from 'loose' to 'marked' from the 30% to 100% fall
    position.

3. Disaggregation (subsequent rise of tracing). Microscopy of specimens
    taken while the tracing was rising confirmed the break-up of aggregates.
    But the degree of disaggregation was less marked than that suggested
    by the degree of return of the tracing to the baseline.

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When the tracing returned to the level of the top of the 'rise', disaggregation was microscopically incomplete (although it could become complete over the next 5 to 10 mins). After a tracing returned from a medium fall to the level of the oscillating baseline, upwards of 50% of the platelet population was still in small aggregates (up to 1/16 HPF).

Samples taken after the small disaggregation which follows some marked falls (vide infra, primary aggregation) followed for 10 mins showed 20% plus of platelets were single. But many aggregates of up to 1 HPF in size were still present, and these showed moderate adhesion of the platelets.

Summary of microscopy correlations with the tracings.

1. The light transmission from stirred human platelet rich plasma was an oscillating one and it correlated with a disk shape of the platelets.

2. When ADP was added to the plasma three changes occurred.

3. First, oscillations ceased and the tracing rose. This change correlated with a change of platelet shape from disk to sphere and with aggregation of some of these spheres.

4. Second, the tracing fell and this correlated with further platelet aggregation. A few aggregates near to the maximum size occurring to a particular ADP concentration were already present before the fall in tracing started. The largest aggregates were present at about the 50% fall position of the tracing.

5. Finally, the tracing rose. This rise correlated with macro- and microscopic disaggregation. The microscopic extent was less than that suggested by the degree of rise of the tracing.

6. Throughout the microscopic study there was considerable variation in the individual responsiveness of the platelets.

Only in the very marked aggregation falls, where aggregates
of sizes 10 to about 200 platelets ($\frac{1}{4}$ HPF) disappeared, was
this variation absent.   The variation would be expected
from a population of platelets of different ages.
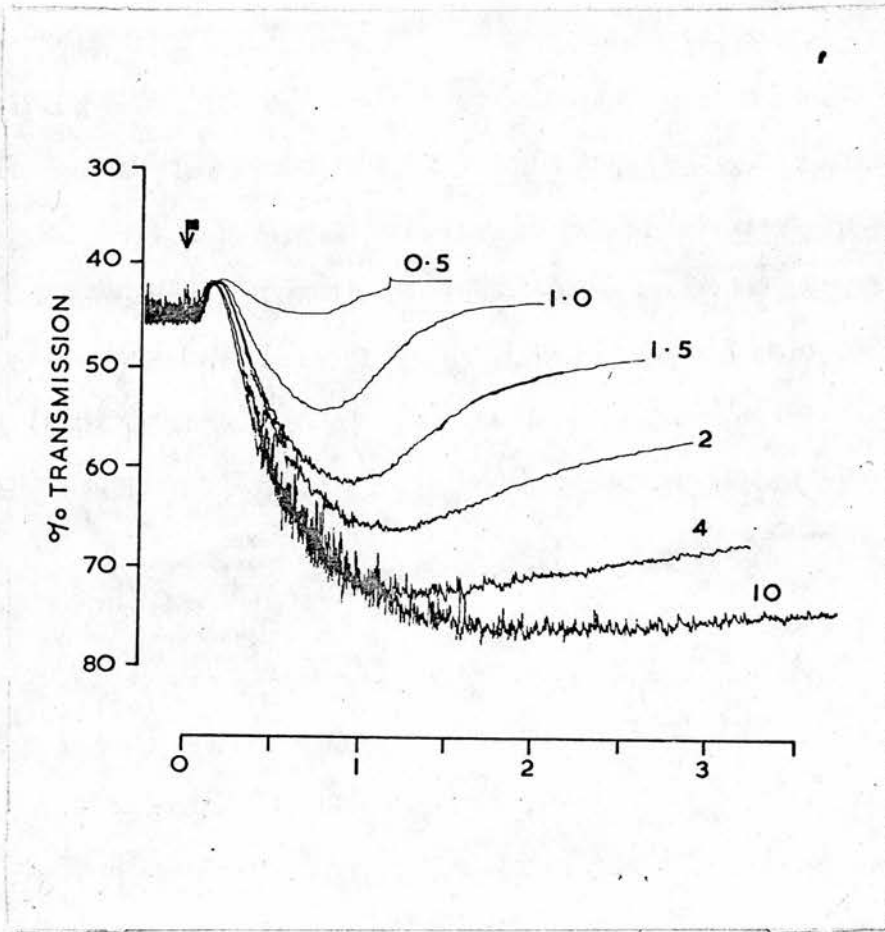
Fig. 4.4.

Dose response curve to ADP of a PRP which showed Primary Aggregation
(P.A.) only.

Increasing concentrations of ADP produced increasing aggregation. This
increase was initially rapid and then slower. Aggregation was followed
by disaggregation which decreased in extent as the ADP concentration
rose; but which was still just present at the 10μM ADP dose.

PRIMARY AGGREGATION (P.A.) TRACING

This type of response was found in all platelet rich plasma from man. A plasma showing this response is shown in Fig. 4.4. The lowest ADP concentration producing aggregation varied from 0.01 μM to 1.0 μM. The degree of aggregation increased in direct proportion to the ADP concentration, initially rapidly and later more slowly. The maximum concentration used was 10 μM, as this is at the upper limit of the (ADP) which platelets can release in their response to aggregating agents (116, 117, 74, 65).

Aggregation was followed by disaggregation. The degree of disaggregation decreased as the aggregation increased, but it was still present in slight degree after the 10 μM ADP dose. When the aggregated plasma was stirred for 10 mins further disaggregation occurred. In this 10 mins the tracing returned to the baseline level following ADP additions of up to about 3 μM but not after higher doses of ADP (Macmillan 106).

Addition of the pharmacological ADP dose of 100 μM produced very marked aggregation with little or no disaggregation during 10 mins of stirring.

Measurement of the P.A. process

The widely used (13, 67, 139) measurement for primary aggregation of the percentage fall (O.D. scale) in density produced by a fixed dose of ADP will be used. In this thesis 1 μM ADP was the fixed dose in most experiments.

Summary of primary aggregation (P.A.)

1. The primary aggregation (P.A.) response is one showing a gradual increase in aggregation directly proportional to the added (ADP). This was followed by disaggregation which was inversely proportional to the ADP concentration. However, slight disaggregation was still occurring after a 10 μM ADP addition.

2. P.A. will be characterised by the percentage fall in O.D. of a plasma produced by 1 μM ADP.
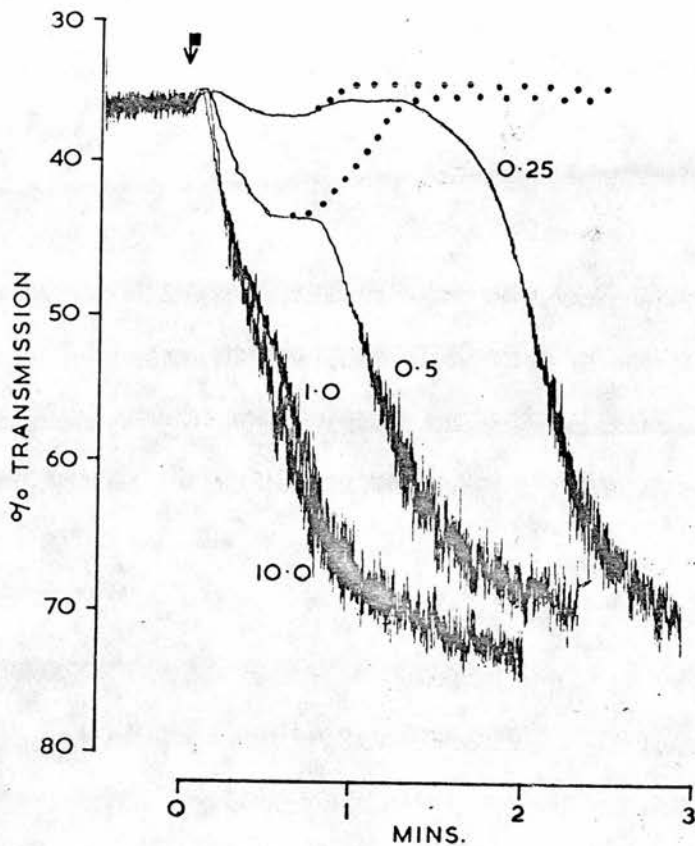
Fig. 4.5.

Dose-response curve to ADP of a PRP which, in addition to P.A., showed
biphasic Secondary aggregation to 0.25µM ADP

The 0.25µM ADP addition induced a small primary aggregation which was
followed by disaggregation. After the disaggregation was complete there
occurred a further, very marked, and near maximal aggregation
(•••••= expected P.A. response). This is Secondary aggregation (S.A.)
and the shape of the tracing of P.A. plus S.A. is biphasic. The
0.5µM ADP addition induced a larger primary aggregation which reached a
trough. Then instead of showing primary disaggregation (dotted line)
S.A. occurred. The 1.0µM addition produced a fusion of P.A. and S.A.
and a degree of aggregation which was not increased by ADP 10µM.

SECONDARY AGGREGATION (S.A.) TRACING.

Introduction

In most plasma from man the 'primary' aggregation response to added ADP was interrupted by a further very marked aggregation ('secondary' aggregation). The author's original description of the process and the subsequent confirmation of it's presence are detailed in the review (Chapter 1).

Present Study.

The stage of the P.A. process at which it was interrupted by S.A. varied widely in plasma from different subjects. Very rarely S.A. occurred after stirring alone. In the remainder of the plasma it occurred after ADP additions anywhere between 0.25 µM ADP and 10 µM ADP. In a few plasma S.A. was not detected.

Dose-response curves to ADP of plasma from patients and volunteers showing the range of S.A. responses are described below.

Results.

The S.A. process was most easily seen when it was very active, for it then converted a small P.A. response into a very marked aggregation (maximal or near maximal).

The most active form is shown by Fig. 4.5. In this plasma 0.25 µM added ADP produced the expected very small P.A. aggregation. This was followed by a small disaggregation, which was slightly impaired in degree (for expected, see Fig 4.5). Instead of proceeding to further disaggregation and a regaining of disk shape by the platelets (as expected, see Fig. 4.2), after about one minute of the disaggregation phase the tracing started to fall again. The speed of this fall gathered momentum and the oscillations of large aggregate formation started to appear. The process continued until very marked aggregation was present. Comparison of this tracing of the S.A. response with that induced by 10 µM ADP during P.A. (Fig. 4.4), showed that the degree of aggregation
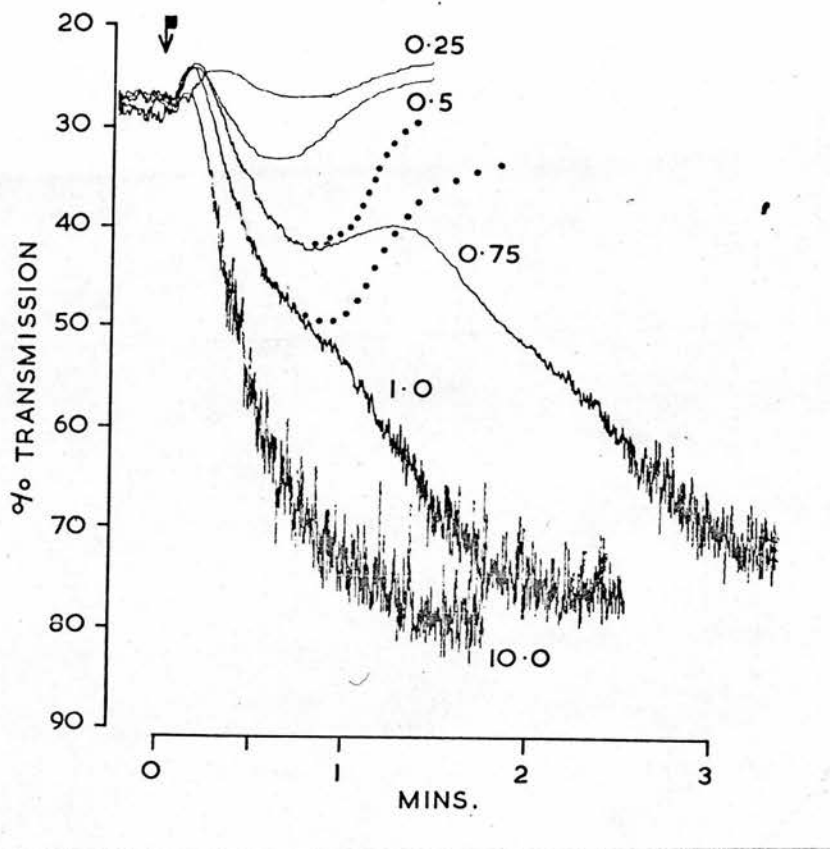
38

Fig. 4.6.

Dose response curve to ADP showing biphasic S.A. to 0.75µM

The addition of 0.25 to 0.75µM ADP induced P.A. The disaggregation
phase of the 0.75µM tracing was interupted by a secondary aggregation
which continued to a near maximal extent. 1.0µM ADP produced fusion
of P.A. and S.A. 10µM ADP only slightly increased the degree of
aggregation.

.... = expected P.A. response
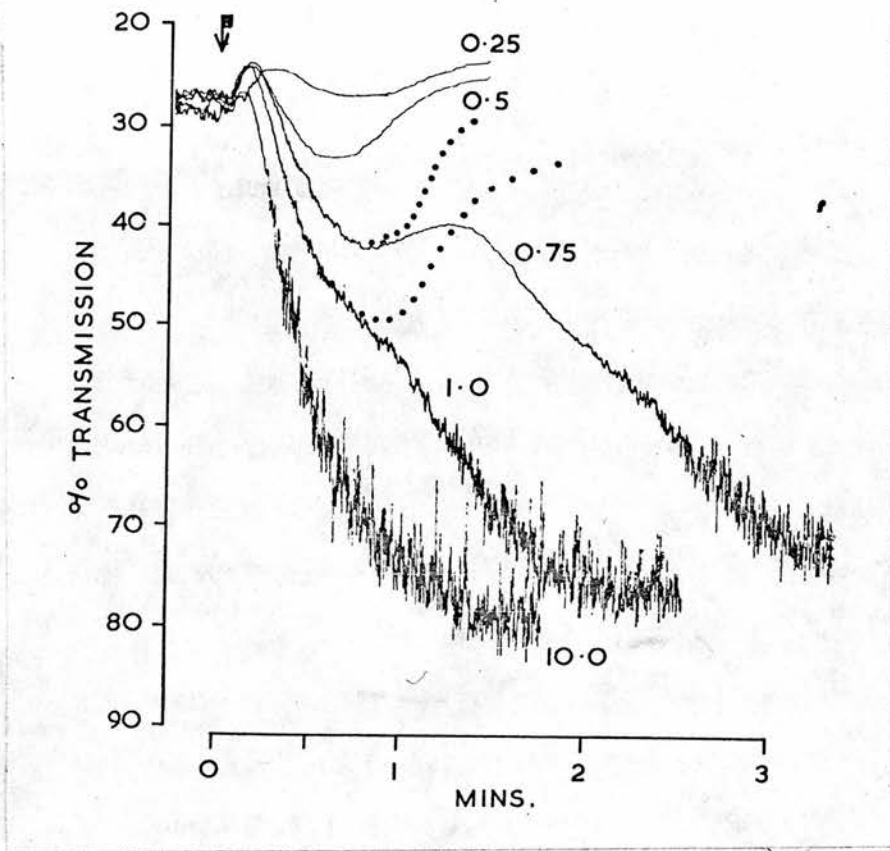
Fig. 4.6.

Dose response curve to ADP showing biphasic S.A. to 0.75μM

The addition of 0.25 to 0.75μM ADP induced P.A. The disaggregation
phase of the 0.75μM tracing was interupted by a secondary aggregation
which continued to a near maximal extent. 1.0μM ADP produced fusion
of P.A. and S.A. 10μM ADP only slightly increased the degree of
aggregation.

.... = expected P.A. response

present after S.A. was markedly the greater of the two. Furthermore, stirring of plasma in which S.A. had occurred for 10 mins did not produce any disaggregation (in marked contrast to even a 10 μM ADP addition in the primary aggregation type of response). A small increase in plasma density occurred when plasma which had undergone S.A. were stirred for 1 hour, but microscopy showed this was due to disruption of aggregates (frayed edges of aggregates and platelet 'debris'), not disaggregation.

The addition of 0.5 μM ADP to the plasma shown in Fig. 4.5 produced a faster onset of S.A., for the S.A. response interrupted the early part of the disaggregation following the P.A. response. 1 μM and 10 μM ADP additions further increased the speed of onset of S.A., and P.A. and S.A. became inseparably fused. But none of these higher ADP additions added significantly to the degree of aggregation.

Fig. 4.6 shows the dose-response curve to ADP of a plasma which gave the S.A. response to a 0.75 μM ADP addition. At this ADP concentration the S.A. process again interrupted the disaggregation following the P.A. response. It had the same very marked aggregation tracing as the plasma depicted by Fig. 4.5.

The ability of the S.A. process to reverse disaggregation resulted in the separation of the two aggregation phases by a disaggregation process. The S.A. in plasma showing this biphasic tracing, of P.A. and S.A. seen separately, will be called 'biphasic' S.A.

Demonstrating the biphasic stage required careful titration of the ADP addition, for as is shown by Figs. 4.5 and 4.6. a small further increase in the ADP addition produced fusion of P.A. and S.A. However, the abrupt increase to near maximal aggregation which S.A. produced was so striking, when compared to the small P.A. response to low (ADP) and the 'step by step' nature of P.A., that, even when P.A. and S.A. were fused, the underlying presence of an S.A. reaction was obvious.

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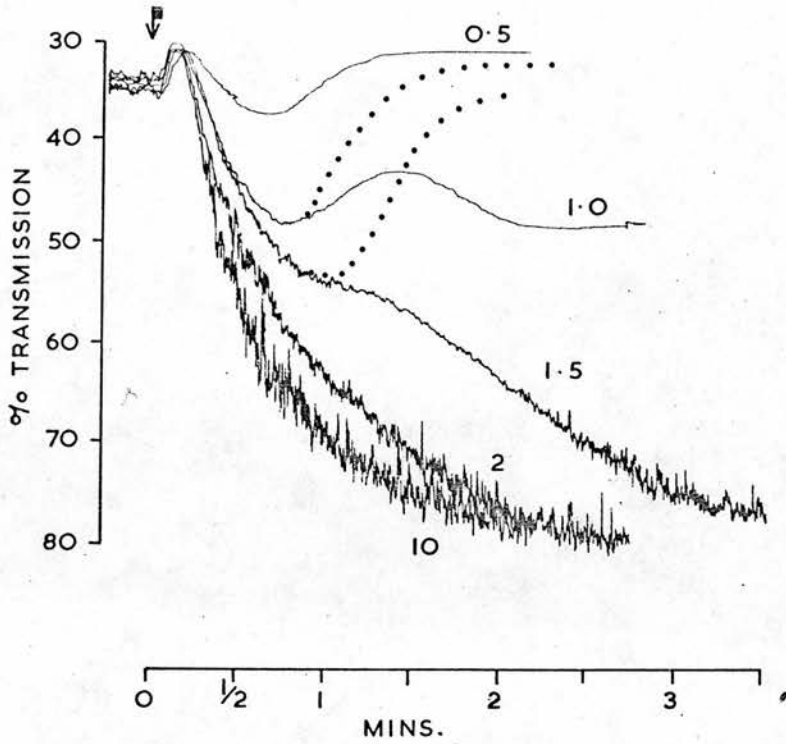
Fig. 4.7.

Dose-response curve to ADP showing biphasic S.A. to 1.5μM ADP

P.A. occurred at 0.5 μM addition. At 1μM ADP addition the disaggregation
phase of P.A. was interupted by a further aggregation process (giving
a biphasic tracing) but this did not develop to a marked extent i.e.
it was partial S.A. The next ADP dose (1.5μM) induced a fully developed
S.A. process; P.A. and S.A. can still be separated. At the next dose
level of 2μM, aggregation of P.A. and S.A. were indistinguishable.
10μM ADP did not increase the aggregation response. (.......... = expected
response from P.A. only).
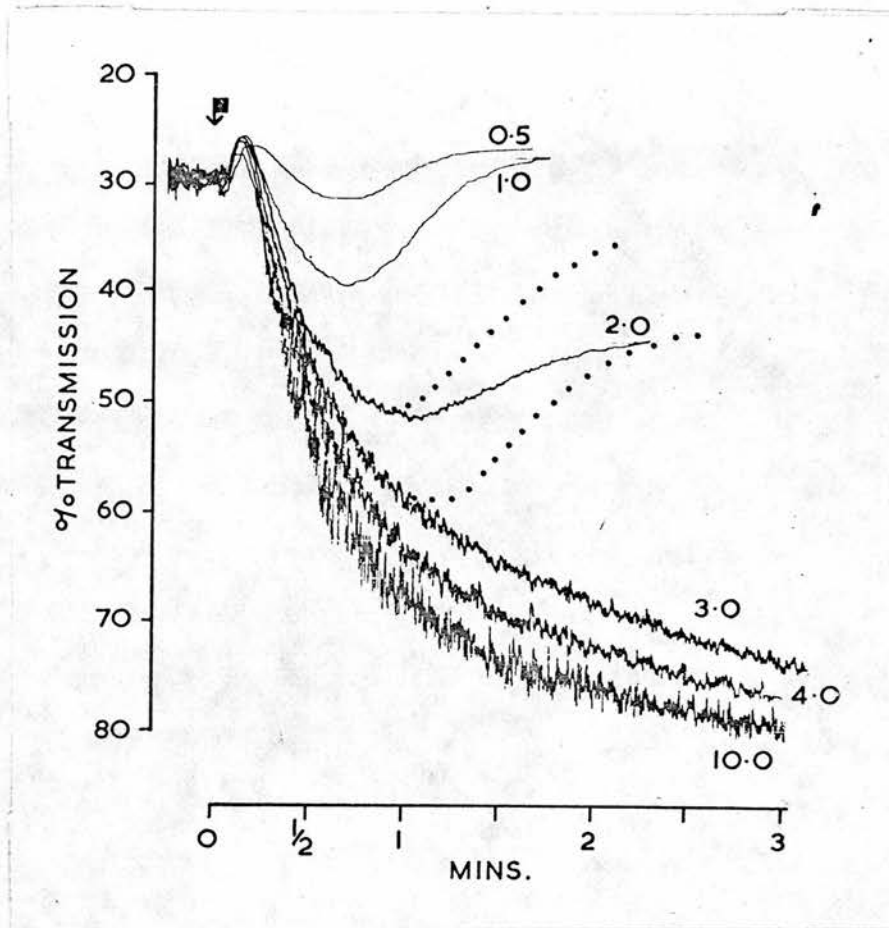
Fig. 4.8.

Dose response curve to ADP of a PRP which showed non-phasic S.A. to

3μM ADP

The response was of P.A. type until the disaggregation phase of the
2μM addition. This was impaired (for expected see dotted line). The
3μM addition was followed by a continuing aggregation process with
P.A. and S.A. fused, which continued to a near maximal response for
the plasma (which was less marked than for biphasic S.A.). The 4μM and
10μM additions showed the same pattern with a small increase at each
dose in the extent of the aggregation.

A further example of 'biphasic' S.A. is given in Fig. 4.7.
This plasma showed a 'pre-S.A.' response, for a second aggregation
interrupted the disaggregation phase which followed the P.A. response
to 1 µM ADP.   But this further aggregation faded away without reaching
a near maximal extent (S.A. was produced by the 1.5 µM ADP addition).
Thus the S.A. process sometimes affects the aggregation in a plasma
at the ADP addition before S.A. occurs.

Fig. 4.8 shows the S.A. process in a plasma where the higher
dose of 3 µM ADP was required to induce secondary aggregation.   The
process was less marked in this plasma, but still produced more
aggregation than 10 µM ADP added to a plasma showing only the P.A.
response.   Again the aggregates persisted for 10 mins without
disaggregating.   This less active S.A. reaction was not strong enough
to reverse an established disaggregation process, though there was a
small 'bump' in the tracings of some plasma where P.A. joined S.A.
This type of S.A. tracing will be called 'non-phasic'.

The ADP addition required to induce S.A. in twenty plasma was
correlated with the type of S.A. tracing which resulted.   Eleven plasma
showed a 'biphasic' response and in these the ADP addition inducing S.A.
was 1.5 µM or less.   Nine plasma showed the 'non-phasic' response, in
eight 2 µM ADP or more was required to induce S.A. and in one 1.5 µM ADP
was sufficient.   The correlation between biphasic S.A. and an inducing
ADP addition of 1.5 µM or less, and non-phasic S.A. and an inducing ADP
dose of 2 µM or more, was significant at the 1% level (Fisher's d test).

There was a sub-division of non-phasic S.A. which became very
interesting when the mechanism of S.A. was investigated (next chapter).
In a few plasma the increased aggregation response of S.A. levelled out
(Fig. 4.9) instead of continuing, as it had in the other plasma.   No
disaggregation occurred in 5 mins but had occurred slightly by 10 mins.
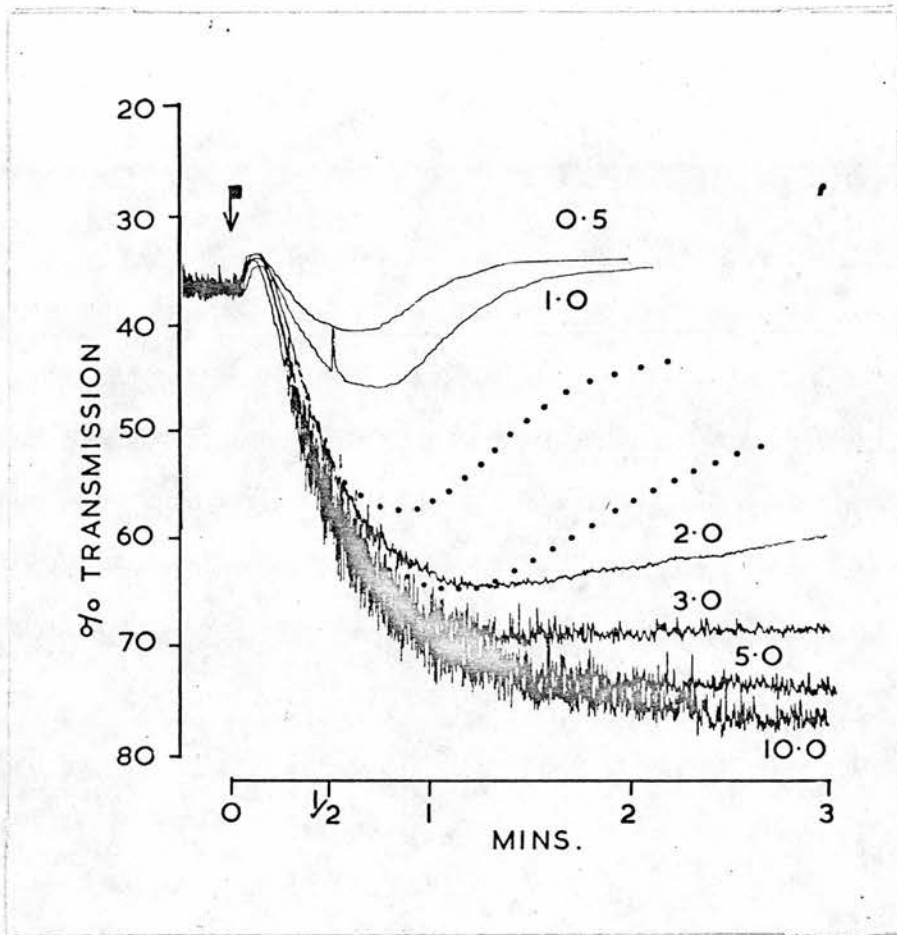
Fig. 4.9.

Dose response curve to ADP of a PRP which showed 'level' non-phasic S.A.
to 3μM ADP.

0.5 and 1.0 μM ADP induced a P.A. response and 2μM ADP induced quite
marked aggregation and there was very little disaggregation. 3μM ADP
was followed by a marked aggregation response which became level and
continued for 3 mins with no disaggregation. 5μM ADP was also followed
by aggregation which became level (cf continuing aggregation of biphasic
and other non-phasic S.A.). 10μM produced a small further increase in
aggregation.

This type of tracing was called 'level' S.A.

<u>Quantifying Secondary Aggregation.</u>

S.A. can be separated on the appearance of the tracings into biphasic, non-phasic, and level.   To increase further the power of discrimination between plasma, the dose of ADP necessary to induce S.A. was used as a measure of S.A. activity.   The fully developed S.A. process was used as the end point, and was defined as a 'continuing aggregation, lasting for at least 3 mins, which reached 80% or more of that occurring with 10 μM ADP'.   This point will be called the 'S.A. point'.

The scale of ADP concentrations used to determine the S.A. point was: 0.25μM, 0.5μM, 0.75μM, 1μM, 1.5μM, 2μM, 3μM, 4μM, 5μM, 7.5μM and 10μM.

<u>Microscopy of specimens after S.A.</u>

Specimens removed from plasma which had undergone S.A. showed the following features:-

1.   Less than 25,000 single platelets/cu.mm. (many of which were deformed).

2.   Very few aggregates in the 2 platelets to $\frac{1}{4}$ HPF range.

3.   Many aggregates sized $\frac{1}{4}$-2 HPF, all of which showed marked adhesion.

After biphasic S.A. the number of single platelets was usually ⟨10th/cu.mm.   Also the aggregate size and the degree of 'marked' adhesion in the aggregates was greater than after non-phasis S.A.   Microscopy of S.A. aggregates which had been stirred for one hour showed no break-up of the aggregates, but their edges had become ragged.   There was a marked increase in the amount of platelet 'debris' (which probably accounts for the small increase which occurred in the density of the plasma).

<u>Summary of secondary aggregation (S.A.)</u>

1.   In most plasma from man a second wave of aggregation occurred during an ADP dose-response curve.   This has been called 'secondary' aggregation (S.A.).

2. On the appearance of the tracings S.A. was divided into:

   (i) Biphasic: S.A. interrupted the disaggregation which followed P.A. (correlated with an inducing ADP concentration of 1.5 μM ADP or less).

   (ii) Non-phasic: S.A. was unable to interrupt established disaggregation (correlated with an ADP addition of 2 μM ADP or more).

   (iii) Level: Non-phasic S.A. where the aggregation reached a steady state of less than maximal degree

3. The presence of the S.A. response in a plasma resulted in:-

   (i) Maximal or near maximal aggregation of the platelets in a plasma, to ADP additions which would otherwise have only a small P.A. response (through biphasic S.A.) or a moderate P.A. response (through non-phasic S.A.).

   (ii) The aggregation of nearly all the platelets into large, very persistent aggregates with marked inter-platelet adhesion (in contrast to the mainly transient aggregates formed during P.A.).

4. The accuracy of the measurement of S.A. was increased beyond the separation of the three types of tracing by determining the lowest ADP addition which induced S.A. (S.A. point (ADP)). Eleven dose levels of ADP in the range 0.25 - 10 μM were used.
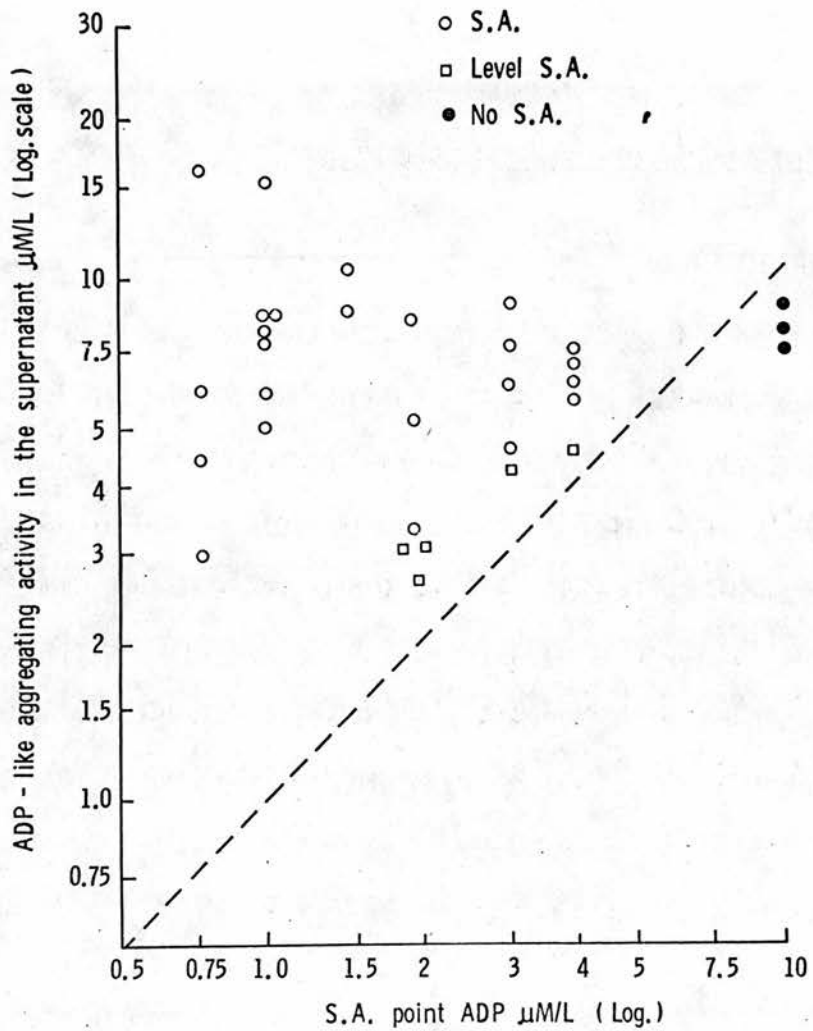
Fig. 5.1.

The ADP-like aggregating activity (µM/L) present in plasma after S.A.

The aggregating activity (log) has been plotted against the S.A. point log [ADP] µM. The diagonal line shows the concentration of the added ADP µM/L.

O = normal S.A.   □ = level S.A.   ● = S.A. absent

THE MECHANISM OF SECONDARY AGGREGATION

INTRODUCTION

In the previous chapter the presence of S.A. in some human plasma was described. The next stage was to try and find out why S.A. occurred. Two primary factors were possible; an increased aggregating response of the platelets, and an increase in the aggregation stimulus in the plasma. As platelets are known to release ADP when stimulated by thrombin and collagen, an increase in aggregating activity in the plasma due to platelets releasing endogenous ADP was the most likely mechanism. Several secondary affects could however be occurring, such as the plasma affecting a platelet's ability to undergo S.A., or there could be a combination of mechanisms which varied in their importance.

EXAMINATION FOR AGGREGATING ACTIVITY IN PLASMA AFTER S.A.

1. ADP-like aggregating activity present after S.A. had occurred.

Plan of study. The supernatant of plasma which had been induced to undergo S.A. was examined for ADP-like aggregating activity (see methods). After addition of the S.A. point ADP concentration the plasma was stirred for 3 mins to allow S.A. to occur, then the supernatant was examined.

Results Fig. 5.1. (32 subjects).

The total ADP-like activity $\mu M/L$ in the supernatant has been plotted against the S.A. point ADP concentration. The diagonal broken line shows the amount of ADP added (this is an over-estimate as some of the added ADP will have been metabolised by the time the supernatant was examined).

All the plasma showing S.A. contained an excess of ADP-like

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Table 5.1. Comparison of the total aggregating activity present after S.A. with the ADP-like activity present.

| Subject | S.A. point of plasma (ADPμM/L) | Aggregating Activity (ADP-equivalent μM/L) | | Excess of (1) over (2) |
		(1) Total	(2) ADP-like	
(1)	0.5	2.8	3.2	None
(2)	1.0	8.1	9.3	None
(3)	2.0	5.1	4.9	0.2
(4)	2.0 (level)	3.2	2.9	0.3
(5)	2.0 (level)	2.5	2.7	None
(6)	4.0	4.9	5.3	None

aggregating activity over the original ADP addition.   The three plasma
which showed only P.A. showed a decrease compared with the ADP addition.
Thus S.A. and an excess of ADP-like activity in the plasma were
correlated.   (Wilcoxon sum of ranks, $p < 0.002$).

The size of this excess of ADP-like activity varied through wide
limits from $< 1 \mu M$ to $> 10 \mu M$.   Plasma with an S.A. point of $1.5 \mu M$ ADP
or less had a higher excess than plasma with an S.A. point of $2 \mu M$ ADP
or more (Wilcoxon, $p < 0.01$).   Five plasma with the 'level' type of
S.A. were tested (marked □ Fig.5.1.).   The release of ADP-like
activity in these plasma was low.

These findings were reported in outline in 1966 (Macmillan, 106)
and in 1968 Mills et al confirmed the presence of an ADP release using
a specific biochemical method (116).

2.   Comparison of total and ADP-like aggregating activity present after S.A.
        Platelets are known to release substances other than adenine
nucleotides and among these are other platelet aggregating agents,
the catechol amines and 5H-T (116, 196).   In addition to producing
aggregation these latter substances are also capable of a synergistic
action with ADP (139, 117).

Present study.   Supernatant from plasma which had undergone S.A. was
tested for both ADP-like activity (i.e. with the inhibitors heparin
and phentolamine present) and for total aggregating activity
(i.e. no inhibitors).

Results.   Table 5.1. (six subjects).

The total aggregating activity was slightly greater than the
ADP-like aggregating activity in two subjects and slightly less in four.

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The group contains biphasic S.A. plasma (two), non-phasic S.A. (two) and level S.A. (two).

Comments. The total aggregating activity released during S.A. was no greater than the ADP-like activity release in six plasma which included all types of S.A.

3. Conditions necessary for the release of ADP-like activity

The release of ADP and S.A. have been associated in the preceding work. To see if the release and S.A. could be separated the following combinations were tested for ADP-like activity.

1. PRP + S.A. point (ADP) - stirred for 3 mins (S.A.).

2. PRP + S.A. point (ADP) - mixed and incubated for 3 mins.

3. PRP + S.A. point (ADP) - stirred for 3 mins.

Six experiments were performed using biphasic S.A. plasma and they gave similar results.

Results. A marked release of aggregating activity occurred only when PRP was stirred after the addition of ADP. ADP and no stirring produced no release or only a small one. In stirred platelet poor plasma a decrease in activity occurred.

Comment. Stirring was necessary for a marked release of ADP at the S.A. point ADP concentration.

4. The timing of the release of ADP-like activity.

The ADP-like activity present in plasma which was undergoing S.A. was measured at four points during it's development; at the top of the rise, and at 30% fall, at 60% fall and at near 100% fall of the tracing.

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Table 5.2. Timing of the release of ADP-like activity during S.A.

| | ADP-like aggregating activity (arbitary units) | | | | | | | |
	(1)	(2)	(3)	(4)	(5)	(6)	Mean	Change from preceeding column
(1) Top of rise	2	6	3	2	0	0	2.2	
(2) Fall of 30%	6	16	20	16	5	4	11.2	Increase*
(3) Fall of 60%	21	38	31	35	14	24	27.2	Increase*
(4) $2\frac{1}{2}$ mins (near 100%)	25	33	33	37	13	27	28.0	N.S.

Results. Table 5.2 (Six subjects). The units are arbitary.

There was only a low ADP-like activity at the top of the rise, probably due to the added ADP. The increase in activity was marked at the 30% fall point (p < 0.05) and showed a further definite increase over this at the 60% fall point (p < 0.05). Little or no further increase in activity occurred between 60% fall and near 100% fall.

Summary. In six plasma the release of ADP-like activity occurred during the first 60% of the fall of the tracing.

5. ADP-like activity in supernatant from the pre-S.A. point specimen.

In some plasma the early stages of S.A. can be detected in the tracing as impaired disaggregation occurring at ADP doses which were less than the S.A. point ADP concentration (see Chapter Four). In these pre-S.A. specimens there was sometimes a small increase in ADP-like aggregating activity in the supernatant above the added ADP concentration.

THE EFFECT OF THE PLASMA ON THE S.A. POINT

The best way to try and answer this question is to separate platelets, wash off as much of their plasma coating as possible, and then resuspend them in another plasma. Unfortunately this is not possible because such a separation can only be performed with EDTA plasma and EDTA prevents the S.A. response (see Chapter Seven, Expt. 3). Only a partial answer therefore is possible.

Present Study. PRP was diluted by 33% with it's own (parent) PPP and the S.A. point was measured. This was compared with the S.A. point of the same PRP similarly diluted with PPP from a separate plasma with a differing S.A. point. Further dilutions would have lowered the S.A. point too much for the test. Both mixtures were incubated at $37^{\circ}C$ for 20 mins before testing, to permit an interchange of constituents between

Table 5.3. Effect on S.A. point of a plasma of incubating it with a foreign platelet poor plasma

| Subject | S.A. point (ADPμM) | | | | | |
	(1)	(2)	(3)	(4)	(5)	(6)
(1) Subject PRP + own PPP	0.5	1.5	2.0	2.5	4.0	7.5
SA point of 'foreign' PRP	7.5	5.0	Nil	1.0	1.0	1.0
(2) Subject PRP + 'foreign' PPP	0.5	1.75	2.5	2.5	4.0	7.5
Difference (1) and (2)	None	0.25	0.5	None	None	None

the platelets and plasma.

Results. Tablet 5.3 (Six subjects)

The difference between the S.A. points of the parent PRP and the 'foreign' PRP was wide in four experiments (Expts. 1,2,3,6). The S.A. point in these four was unaffected by the interchange in two experiments, and minimally affected in the other two (Expts. 2,3). When the S.A. points of the plasma involved was closer (Expts. 4,5), there was no difference.

Comment. 33% dilution of a PRP with PPP from a separate PRP with a differing S.A. point had little or no effect on the S.A. point.
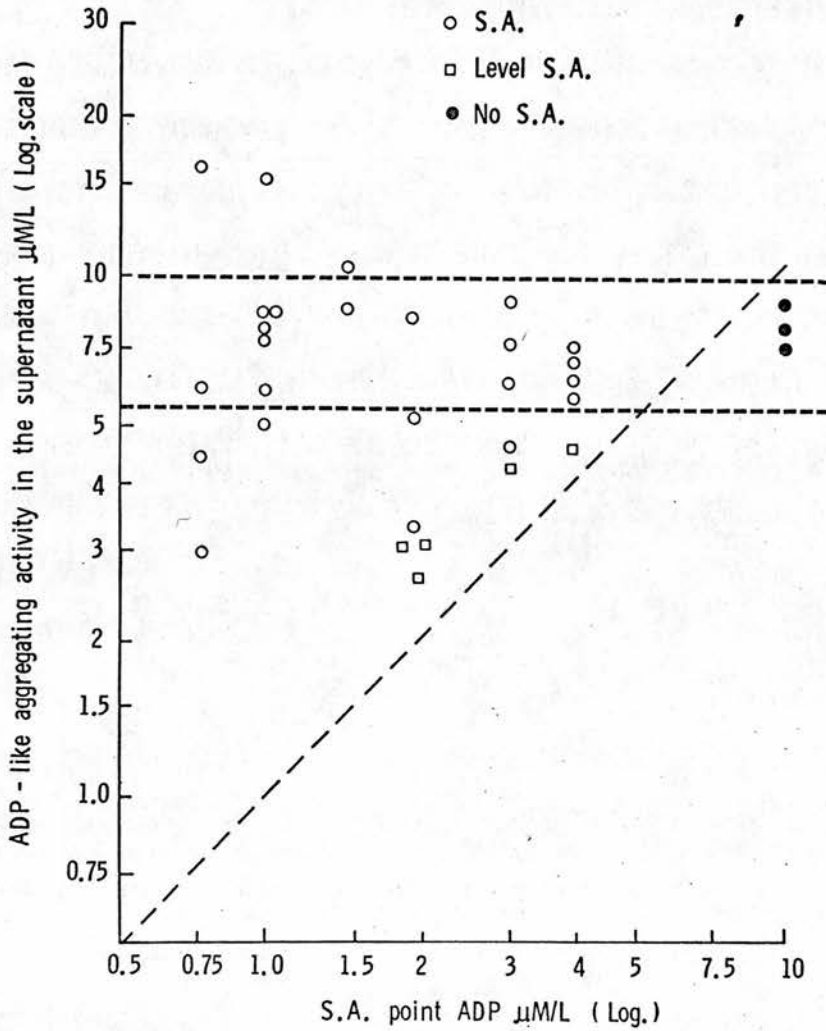
Fig. 5.4.

The ADP-like aggregating activity (μM) present in plasma after S.A.

This figure is a repeat of Fig. 5.1. with horizontal lines at the 10μM and 5.5μM supernatant aggregating activity levels. Of the 29 subjects showing S.A. the ADP-like aggregating activity in the supernatant was $\langle$10μM in 26 and $\langle$5.5μM in 11.

PLATELET RESPONSIVENESS TO ADP AND S.A.

In the introduction to this chapter the theoretical possibilities
of a change in platelet responsiveness or the presence of a release of
aggregating activity were stated.   A release of ADP has been detected,
and it was also found that the total release of aggregating activity and
ADP-like activity were equal.   The results of Chapter Four showed that
S.A. produced a greater aggregation response and failure to disaggregate
(even when non-phasic) than 10µM ADP added to P.A.-only plasma.   Yet a
closer look at the amount of aggregating activity released (Fig. 5.4.)
shows that the activity present in the supernatant of twenty-six of the
twenty-nine plasma showing S.A. was $<$10µM and it was $<$5.5µM in eleven
of the twenty-seven.

Thus it must be assumed that the platelets are more reactive to
the available aggregating activity during S.A. than they are during P.A.
This is particularly so in the plasma showing level S.A., in whom the
release of aggregating activity was particularly small.

An increased aggregation response can be due to either an absolute
increase in aggregation, or to a decrease in disaggregation.   The relative
importance of these two mechanisms cannot be deduced from the present
series of experiments.   However it is worthy of note that the tracings
and microscopy show a marked failure to disaggregate.

The only small increase in aggregating activity in some of the
plasma undergoing full S.A. suggests that possibly only a part of the
aggregating activity released into the plasma is essential for S.A.   If
this is so then an increase in platelet responsiveness would be a major
factor in S.A., with it's importance masked by the large size of the
release of ADP in most S.A. plasma.
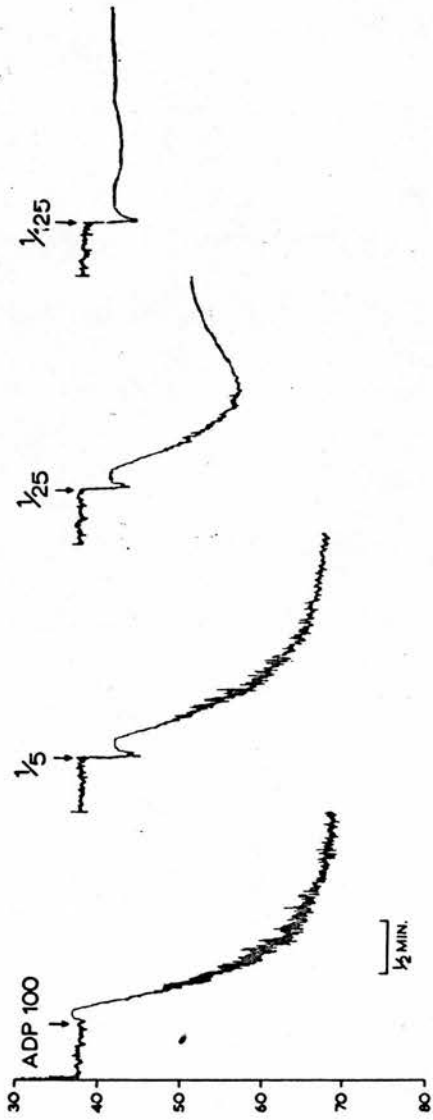
Fig. 5.2.

Effect of transfering supernatant from aggregated P.A.-only
plasma to more P.A.-only plasma

P.A.-only plasma was aggregated by a 100μM ADP addition. (left
specimen); 0.25 mls of supernatant was transferred (1:5 dilution)
to a further aliquot of the plasma. After this plasma had
aggregated the transfer was repeated, (1:25 dilution of original
100μM addition) and repeated again (1:125 dilution). The
aggregation response decreased rapidly.
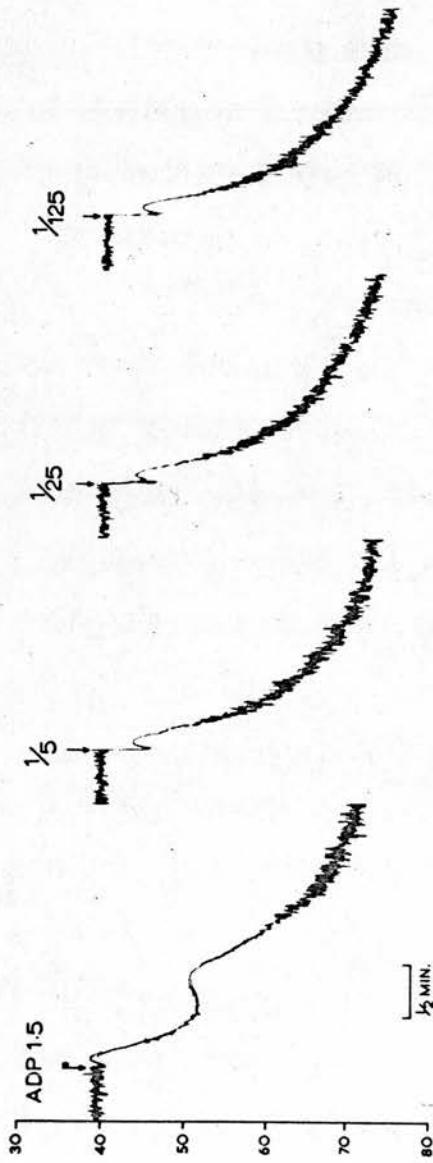
Fig. 5.3.

Effect of transferring supernatant from aggregated biphasic S.A. plasma to more biphasic S.A. plasma

S.A. plasma was induced to undergo S.A. by its S.A. point ADP concentration (1.5µM) and a biphasic tracing occurred (left specimen); 0.25 mls of supernatant was transferred (1:5 dilution) to a further aliquot of the plasma, where it induced an S.A. response with P.A. and S.A. fused.   After this S.A. had occurred further transfers (1:25, 1:125 of original 1.5µM addition) were made.   These again produced S.A.   A chain reaction of maximal aggregation and ADP release was occurring.

AGGREGATION CHAIN REACTION INDUCED BY S.A.

There is available after S.A. endogenous platelet ADP free in
the plasma which theoretically could induce further PRP to undergo S.A.,
with yet further release of endogenous ADP. If such a continuing
reaction occurred it could initiate a chain reaction of aggregation.
This possibility was tested experimentally.

Present study. Plasma showing the P.A.-only response, and other plasma
showing biphasic S.A. were used. The pharmacological dose of 100 µM ADP
was added to the P.A.-only plasma, and the much lower S.A. point (ADP) to
the S.A. plasma. After aggregation had occurred one quarter of the
supernatant (0.25 mls) was added to a further 1 ml. of the same plasma
(this is equivalent to 20% of the released and added ADP). After the
aggregation this produced in the second plasma specimen had occurred,
the transfer, and so the dilution, was repeated, by adding 0.25 mls. of
the supernatant to a further fresh sample of the same plasma.

Results;

1. P.A.-only plasma. Fig. 5.2. 100µM ADP was added to the plasma,
and after aggregation had occurred the transfer described above (20% of
original addition) was performed. The plasma sample showed a slightly
reduced aggregation response. The next transfer (4% of the original 100µM
addition) was followed by a marked reduction in the aggregation. The
third transfer (< 1% of the original addition) was followed by only a
minimal aggregation response. This loss of aggregation response was
that which would be expected from the dilution produced by the transfer.

2. S.A. plasma. Fig. 5.3. shows the result using a plasma with
an S.A. point of 1.5µM ADP. The addition of 1.5µM (i.e. only 1.5 percent
of the dose used in the P.A.-only plasma experiment above) was followed by
a biphasic S.A. tracing. The same transfers, and so dilutions of the

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added dose, were made. In contrast to the P.A.-only plasma the S.A. plasma showed an S.A. response with each transfer. Thus S.A. was producing a release of more than four times the ADP needed to initiate S.A. in a further aliquot of the same plasma. Thus an aggregation chain-reaction was occurring.

The transfers could be continued until the store of plasma was exhausted, and eight to ten transfers were performed on other biphasic S.A. plasma without any break in the chain-reaction. Also, using plasma showing very active S.A., transfer of as little as 0.1 ml. (i.e. only 10% of supernatant) from a post-S.A. specimen was sufficient to initiate, and support, the aggregation chain reaction.

In contrast to biphasic S.A. plasma, non-phasic S.A. plasma supernatant failed to initiate or support an aggregation chain reaction, with the unusual exception of some of the non-phasic S.A. plasma with an S.A. point of 1.5µM.

Quantitation of the aggregation chain-reaction potential of S.A.

The preceding experiment showed that the theoretical possibility that the S.A. process could result in an aggregation chain reaction was correct. The biphasic S.A. plasma nearly always gave the chain-reaction when only 20% of the aggregating activity after S.A. was transferred (in some biphasic S.A. plasma it occurred with the transfer of <10% of the aggregating activity), whereas only a few of the plasma which showed a non-phasic S.A. tracing gave an aggregation chain-reaction with the 20% transfer.

The aggregation chain-reaction potential of the S.A. in a particular plasma, can be considered from the alternative viewpoint of how many further units of plasma could be induced to undergo S.A. by the released aggregating activity. If 20% of the aggregating activity present after S.A. induces one further specimen of plasma to undergo S.A., then the total aggregating activity present after S.A. would be capable

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Table 5.4. Aggregation Chain-Reaction Potential of S.A.

	1	3	6	2	3	5	4
(1) No. of plasma							
(2) ADP-like activity	9.25	8.6	7.3	7.3	3.0	3.4	2.0
(3) S.A. point (ADPµM/L)	0.25	0.75	1.0	1.5	2.0	3.0	4.0
Chain reaction potential of one unit ((2)/(3))							
First cycle =	37.4	11.4	7.3	4.9	1.9	1.5	0.1
Second cycle =	1,370	130	53	24	2	1	1
Third cycle =	1,000,000	16,900	2,800	577	6	1	0

of inducing at least _five_ further units of the same plasma to undergo S.A. (i.e. 100%/20%); the aggregating activity released by S.A. in each of these five units could each induce five more units of plasma to undergo S.A. (i.e. 5^2 or 25) and after the next cycle of S.A. there would be enough aggregating activity present to produce S.A. in 25^2 i.e. 625 units.

The chain-reaction potential of an S.A. plasma can be _exactly_ calculated, by dividing the aggregating activity release released during S.A. by the S.A. point of the plasma. The results of this calculation, using the figures for release of aggregating activity and S.A. point reported at the beginning of this chapter (Fig. 5.1), are given in Table 5.4. To emphasise the effects of the _geometric_ scale of increase which occurs during a chain-reaction, the effects of the second and third cycles of such a reaction are given.

A plasma with an S.A. point of 0.25µM/L ADP and a release producing 9.25µM/L ADP-like activity had the aggregation chain-reaction potential to induce S.A. in 37 further units of plasma in the first cycle, 1370 units in the second cycle, and greater than one million units in the third. In this plasma the potential is obviously enormous. The chain-reaction potential of three cycles of reaction reduces rapidly as the S.A. point ADP concentration rises, being 16,900 for 0.75 S.A. point, 2,800 for 1.0, 577 for 1.5, and then falling to the low level of 6 for the S.A. point of 2µM ADP.

Although the level of released ADP-like activity falls as the S.A. activity of the plasma decreases, it is still, at the 2µM/L ADP S.A. point, 30% of the release occurring at the 0.25µM/L ADP S.A. point. But during the same period, the aggregation chain-reaction potential has fallen to 0.01% of it's value at the 0.25µM/L ADP S.A. point. Thus the major determinant factor of the chain-reaction potential is the S.A. point ADP concentration.

These calculations have shown the crucial importance of relatively small changes in the S.A. point ADP concentration. In the S.A. point range 0.25μM-1.5μM/L ADP a fast reducing, but important, chain-reaction potential was present, whereas S.A. points of 2μM ADP/L and above were associated with minimal potential for a chain-reaction. This point is underlined when one calculates that the increase in S.A. point activity from 2μM to 0.25μM ADP was associated with a one hundred thousand times increase in aggregation chain-reaction potential.

To emphasise this rapid increase in chain-reaction potential, for only a small decrease in the ADP concentration required for S.A. two new terms for describing the S.A. process will be used in the remaining parts of the thesis. The S.A. in a plasma with an S.A. point of 0.25 to 1.5μM ADP/L will be called Chain-Reactive S.A., to stress it's marked ability to induce S.A. in a further sample of the same population of platelets. In plasma with an S.A. point of 2μM or more, the S.A. will be termed Self-limiting S.A., to stress it's relative inability to induce further platelets to undergo S.A. This division corresponds very closely with the previous division into 'biphasic' and 'non-phasic' S.A.

MECHANISM OF SECONDARY AGGREGATION

Added ADP

Becomes
self-perpetuating

(1) Reversible aggregation (P.A.)

(2) Increased and persisting aggregation

(3) Release of platelet ADP stores

} = S.A.
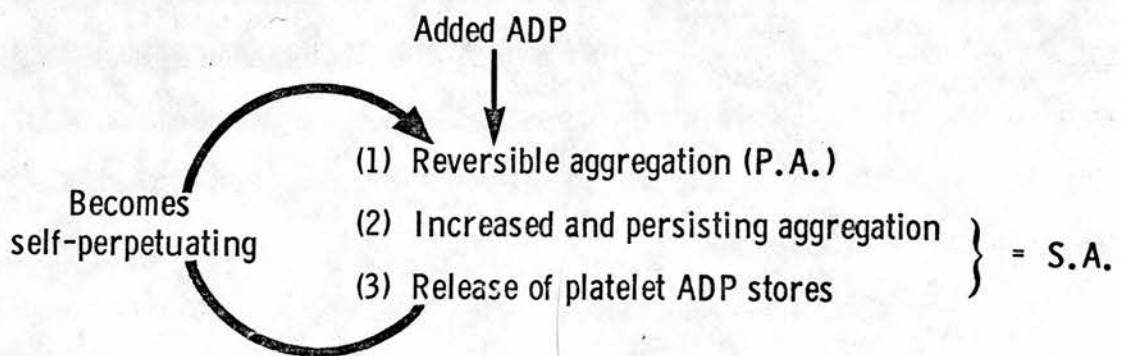
Fig. 5.5.

Diagram to show how the responses of platelets to ADP during
secondary aggregation become self-perpetuating.

SUMMARY OF CHAPTER FIVE

1. During S.A., aggregating activity was released by the platelets into the plasma. This activity behaved as though it was entirely due to ADP.

2. The platelets responsiveness to ADP was increased during S.A. and such a change could be an important part of the S.A. response.

3. The combination of a release of ADP with an increased responsiveness to ADP is sufficient to explain the S.A. process. Thus the ability of ADP to induce S.A. was due to the platelets showing a triple response as compared to the single one (aggregation) which they show during P.A. A self-propagating aggregation process resulted (Fig. 5.5). The added ADP was acting as a trigger, but S.A. soon became self-sufficient.

4. Due to the release of ADP, some S.A. had the ability to initiate a powerful aggregation chain-reaction, an ability which correlated closely with the presence of a biphasic tracing. It correlated even more closely with an S.A. point of 1.5 μM/L ADP or less and such plasma have been called 'chain-reactive' S.A. plasma.

5. The factor which determined the magnitude of the chain-reaction potential was the S.A. point ADP concentration. The potential of three cycles was very large for an S.A. point of 0.25μM and 100,000 times less for an S.A. point of 2 μM ADP. The rapid fall in aggregation chain-reaction potential for only relatively small changes in S.A. point ADP concentration over the range 0.25μM to 2μM ADP has been stressed.

CHAPTER SIX

EFFECT OF S.A. ON THROMBIN, ADRENALINE AND COLLAGEN INDUCED AGGREGATION

INTRODUCTION

The S.A. reaction has been studied so far by adding exogenous ADP. In vivo one would expect the ADP released from injured tissues and damaged red cells to have the same effect as that of exogenous ADP, as it is exogenous to the platelet cell.

A further important source of ADP is the release by the platelets of their rich stores of ADP. This release is induced by thrombin, adrenaline, collagen and 5H.T. (see Chapter 1). Since the platelets will have entered an altered state when they undergo the release reaction, it is important to find out whether or not this endogenous platelet ADP will induce S.A. in these altered platelets.

There are practical difficulties in attempting to show this. Since thrombin and collagen release ADP as part of their direct effect, ADP release is not, as in ADP induced S.A., a marker of S.A. The search for S.A. during the aggregation produced by these substances had therefore to be based on the appearance of the aggregation tracings. A search was made for an abrupt increase to a near maximal aggregation response at a lower than expected dose of aggregating agent. In order to increase the ease of finding any such change, plasma showing the marked S.A. response of biphasic S.A. were compared with plasma which showed only P.A. A direct correlation between S.A. to ADP and the second phase of adrenaline induced aggregation has already been reported (Macmillan 1966) and confirmed (Hardisty et al 1970).

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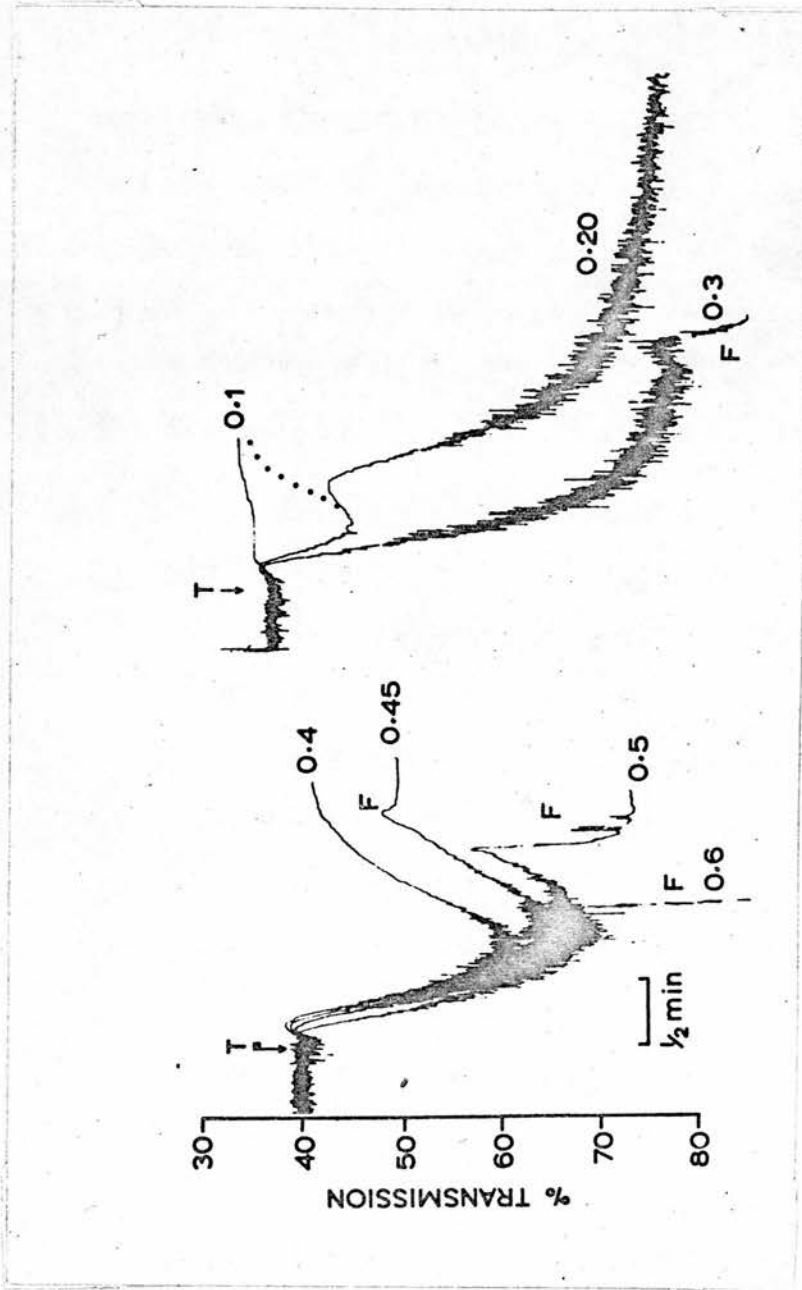
Fig 6.1.

Thrombin induced platelet aggregation

Left Plasma showing only P.A. with ADP. Thrombin (T)

induced aggregation after a short delay. The
aggregation increased in degree with thrombin
concentrations of up to 0.6 units. This was
followed by disaggregation until the point where
fibrin formation (F) prevented further observation.
The response is similar to PA to ADP.

Right Plasma showing biphasic S.A. with ADP. Thrombin (T)

induced a 'P.A.' response at 0.1 units and initially
at 0.2 units. Then a biphasic tracing with secondary
aggregation occurred. 0.3 units induced fusion of
P.A. and S.A. It was interrupted by fibrin formation.
The final extent of aggregation was moderately increased
by the S.A. as compared to that in the P.A.-only plasma.
(....... expected 'primary' thrombin aggregation).

THE SEARCH FOR S.A. TRACINGS DURING THROMBIN, COLLAGEN, AND ADRENALINE
AGGREGATION.

First, plasma which showed P.A. only and biphasic S.A. were
compared, and then, plasma which showed non-phasic S.A. were examined.

Thrombin

Previous work.   Thrombin is an active platelet aggregating agent which
exerts it's full action over a smaller range of concentration than ADP
(139).   At the higher thrombin concentrations fibrin forms and prevents
further observation.   Thomas (177) has demonstrated a biphasic response
to thrombin in some human plasma.

Present study.

1.   Plasma showing P.A.-only to ADP (Fig. 6.1. left).

In these plasma increasing concentrations of thrombin
produced a gradually increasing aggregation response.
This was followed by a decreasing, but always present,
disaggregation.   At higher thrombin concentrations
fibrin formed, initially during the disaggregation phase,
and then during the aggregation phase.
The thrombin concentration required to produce the most
marked aggregation effect was 0.45 to 0.7 units.
The ADP-like aggregating activity released by the stage
of marked aggregation was measured in three plasma.
It was 4.8; 5.2; and 6.0µM.

2.   Plasma showing S.A. to ADP (Fig. 6.1 right).

In biphasic S.A. plasma, a biphasic aggregation tracing
occurred with thrombin (see Fig.6.1 right 0.2 units).
The second aggregation phase had an identical appearance
to the S.A. tracing induced by ADP i.e. it rapidly
resulted in marked aggregation with no subsequent

disaggregation.   Thus an <u>abrupt</u> increase in aggregation had occurred (cf Fig. 6.1 left) with a biphasic tracing. The lowest thrombin dose inducing the S.A. response in this series was 0.01 units i.e only 2.5% of the thrombin concentration producing a lesser aggregation in P.A.-only plasma.   In plasma with non-phasic S.A. an abrupt increase in aggregation occurred but a biphasic tracing was rare.   This aggregation also proceeded to a maximal aggregation with no subsequent disaggregation in 10 mins. Thrombin concentrations of 0.15 to 0.4 u were required.
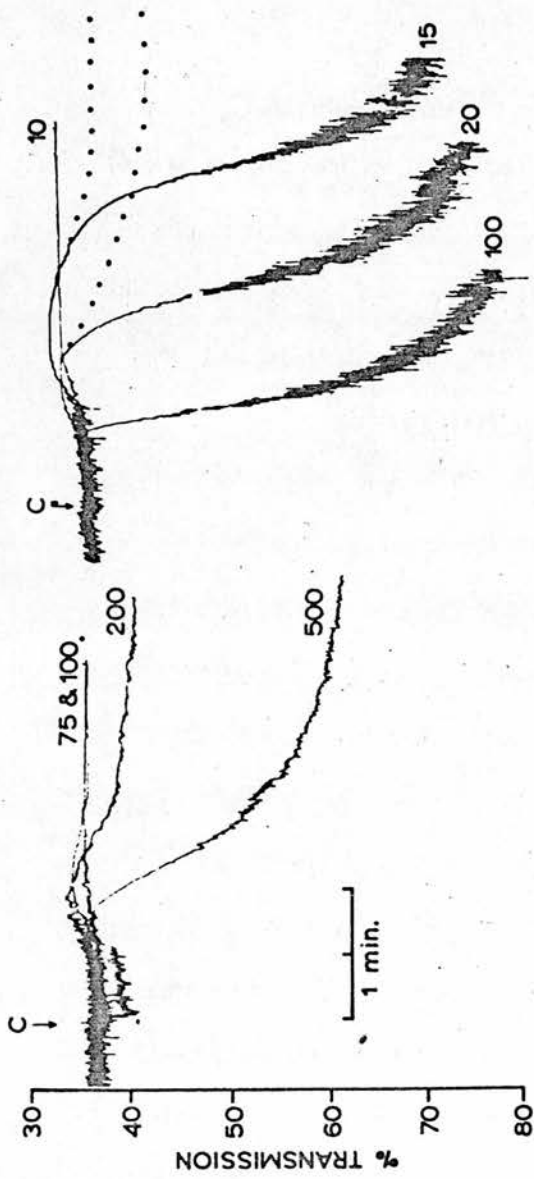
Fig 6.2.

Collagen induced platelet aggregation.

Left   Plasma showing only P.A. to ADP. After a
latent period of 30-90 sec a slow shape change occurred.
Collagen (C) 75 to 200 units produced slight
aggregation, and 500 units produced moderate
aggregation.

Right   Plasma showing biphasic S.A. to ADP. Collagen 10 units
produced a shape change. 15 units was followed by a near
maximal aggregation. This appeared more rapidly, but
increased only by a little in extent with collagen 20 and
100 units. It had an appearance similar to the biphasic
S.A. tracing to ADP; resulted in a marked increase in
aggregation over that of 500 units in the left hand plasma.
(...... expected 'primary' collagen aggregation)

Collagen

Previous work. The aggregation produced by collagen occurs after a latent period, is of slower speed than with other aggregating agents, and is not followed by disaggregation (70, 124).

Present study.

1. **Plasma showing only P.A. to ADP (Fig. 6.2 left).**

   The previous findings were confirmed. There was a latent period of 30-90 secs followed by a slow aggregation process and no disaggregation. The extent of the aggregation was only moderate for 500 units (top of an arbitrary range).

2. **Plasma showing S.A. to ADP (Fig. 6.2. right).**

   In plasma showing a biphasic S.A. response small doses of collagen produced a minimal aggregation (10 units Fig. 6.2. right). A small further increase in collagen dose (15 units) produced an <u>abrupt</u> increase in aggregation to near maximal, which had the S.A. tracing appearance. It thus fulfils the criteria for an S.A. response. S.A. produced a marked increase in the maximum degree of aggregation possible to collagen because 'primary' collagen aggregation is only moderate in extent at it's maximum. The lowest concentration of collagen which was followed by S.A. was half of one unit i.e. only 0.1% of the collagen dose producing considerably less aggregation in 'primary' collagen aggregation.
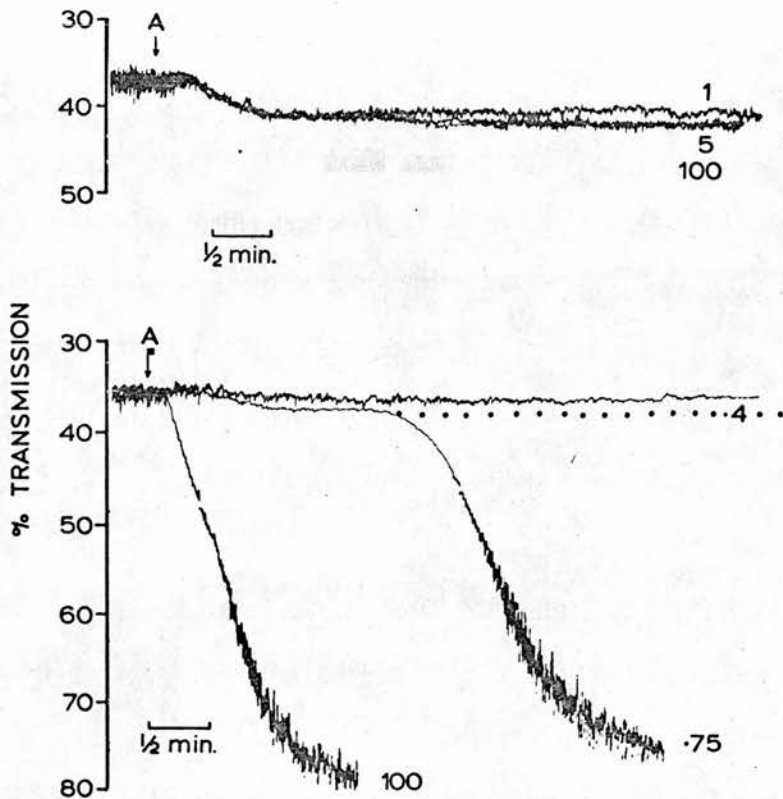
Fig. 6.3.

Adrenaline induced platelet aggregation

Top: Plasma showing only P.A. to ADP: After a latent period of
          about 15 seconds the oscillations decreased and the tracing fell
          slightly. Then aggregation levelled out and remained steady. There
          was only a little difference between the effects of adrenaline
          1µM and 100µM. This is the adrenaline first phase of aggregation.

Bottom: Plasma showing chain-reactive S.A. to ADP: Adrenaline 0.4µM
          induced a first phase. An increase of adrenaline to 0.75µM
          induced, in addition to the first phase, a second aggregation
          which was marked and continued until aggregation was near
          maximal. This is the adrenaline second phase and it is similar
          in appearance to S.A. to ADP. Adrenaline 100µM increased the
          speed of onset but not the extent of the secondary aggregation.
          The second phase allowed a very marked increase in the aggregation
          response over the first phase response (.... Expected 'primary'
          adrenaline aggregation).

Adrenaline.

Previous work.   O'Brien (123) has shown that adrenaline and
noradrenaline induce platelet aggregation in two phases.   The first
is small, and the platelets aggregate without losing their in vivo
disk shape.   ADP is probably not involved (69, 106).   The second
phase produces a maximal response.   O'Brien found that it was absent
in some of the plasma he was examining (123, 124, 125).

Present study.

   1.   Plasma showing P.A.-only to ADP (Fig. 6.3. top).
        These plasma showed only the first phase of adrenaline
        aggregation.   After a short latent period there was a
        small fall in the tracing and oscillations were preserved.
        ADP-like aggregating activity was tested for, but none was
        detected.   There was no spontaneous disaggregation but
        complete disaggregation was produced by the ⟨receptor blocker
        phentolamine (Macmillan 106).

   2.   Plasma showing S.A. to ADP (Fig. 6.3. bottom).
        In these plasma the first phase was interrupted by a rapid
        maximal aggregation (see 0.75 μM), the O'Brien second phase.
        The tracing was identical to the tracing of a biphasic S.A.
        response to ADP.   This maximal response was obtained in all
        plasma showing S.A. with ADP.   The adrenaline concentration
        inducing S.A. varied from 0.005 to 100 μM.
        ADP-like activity was detected at an early stage of the second
        phase and increased in concentration during the phase until it
        was comparable to, or exceeded, the concentration produced by
        S.A. to ADP.

        The increase in aggregation allowed by S.A. was very marked,
        and marked aggregation only occurred when S.A. was present.

Table 6.1. S.A. point dose to ADP, thrombin, adrénaline, and collagen in plasma from 25 subjects.

Subject	Platelet count (10^3/cu mm)	S.A. point concentration			
		ADP μM/L	THR units	Adrenaline μM/L	Collagen units
1*	785	0.25	.15	.005	2
2*	410	0.5	.04	.1	1
3*	340	0.5	.04	.05	1
4*	470	0.5	.01	.5	0.5
5*	280	0.5	.05	.25	1
6*	560	0.75	.125	.05	20
7	210	0.75	.20	.75	15
8*	245	0.75	.225	.5	25
9*	345	1.0	.075	.2	10
10	320	1.0	.15	.1	25
11	455	1.5	.225	1.0	20
12*	195	1.5	.225	3.0	15
13	420	2.5	.15	.5	7.5
14	305	3.0	.25	.5	15
15	240	3.0	.225	.75	20
16*	275	3.0	.225	7.5	75
17	420	4.0	.225	1.5	15
18	275	5.0	.20	100	20
19	360	5.0	.20	5.0	30
20	310	8.0	.45	3.0	60
21	380	10	F 0.5	100	500
22*	530	10	F 0.5	100	500
23	560	10	F 0.6	100	500
24*	260	10	F 0.5	100	250
25	480	10	F 0.5	100	250

F = fibrin formed before S.A. occurred

THR = thrombin   * selected patients

Analysis by Friedmans Test 1937

QUANTATIVE CORRELATION BETWEEN THE S.A. TRACINGS INDUCED BY ADP, ADRENALINE, THROMBIN AND COLLAGEN IN THE SAME SUBJECT.

Previous work.   None.

Present study.

Plan of study.   Each plasma was tested with ADP, thrombin, collagen and adrenaline to determine the S.A. point, if any, to each.   The plasma were taken from patients (selected for high S.A.) and from volunteers, and consisted of 12 with a chain-reactive S.A. response to ADP, 8 with self-limiting S.A. and 5 with only a P.A. response.   The four dose response curves could not always be completed in the 30-90 minute period after venepuncture when the S.A. point to ADP is stable (see Chapter 7), and, to allow for this, the order in which the different aggregating agents were added was randomised.

Results.   Table 6.1.

The results have been tabulated using the S.A. point to ADP as the marker. The platelet counts show no significant difference between the S.A. and P.A.-only plasma.

The results showed an absolute correlation between absence of S.A. to ADP and absence of an S.A. tracing to thrombin, adrenaline and collagen, in the five plasma tested.   Examination of the other results suggested that the S.A. point doses of the four aggregating agents in any one plasma were comparable.   Friedman's correlation test was applied and a very highly significant correlation was found ($p = < 0.001$).

A correlation was also found within the upper ten S.A. plasma group and the lower ten S.A. plasma group.   ($p = < 0.01$ for S.A. points 0.25-1.0μM/L ADP; $p = < 0.01$ for S.A. points 1.5-10μM/L ADP).

Table 6.2 Release of ADP-like activity from the same plasma by four
          aggregating agents.

Aggregating agent	ADP	Thrombin	Adrenaline	Collagen
Subject 1	6.0 (2)	13.8 (1)	5.9 (3)	4.9 (4)
" 2	4.75(3)	6.25(2)	7.25(1)	2.5 (4)
" 3	17.5 (1)	12.0 (2)	9.0 (3)	7.5 (4)
" 4	7.8 (2)	8.1 (1)	7.5 (3)	6.4 (4)

Friedmans correlation test:-

aggregating agent to release $x^2 = 8.4*$

subject to release $x^2 = 6.3$ (N.S.)

The figure in brackets is the ranking order for the release-inducing

power of each aggregating agent.

Release of ADP-like activity during S.A. to different aggregating agents.

No previous work.

Present study. Plasma from four subjects was induced to undergo S.A. by adding ADP, thrombin, adrenaline and collagen. The release of ADP-like activity at the S.A. point of each aggregating agent was measured.

Results. Table 6.2.

There was a wide variation in the degree of release from the four subjects and the four aggregating agents, but there was some order in this. A correlation ($p = < 0.05$) existed between the degree of release induced and the aggregating agent, with thrombin most effective, ADP next, then adrenaline and least effective collagen. The correlation between each subject and the degree of release achieved (on only four subjects) was nearly significant ($p = < 0.10 > 0.05$).

S.A. to thrombin, collagen, adrenaline and the initiation of an aggregation chain-reaction.

In chain-reactive S.A. plasma the supernatant from specimens induced to undergo S.A. by these three substances initiated the aggregation chain-reaction. In self-limiting S.A. plasma the chain-reaction nearly always ceased after the first cycle, presumably because of dilution of the originally added thrombin, collagen and adrenaline, which is, unlike the ADP, not self-generating.

Fig. 6.4. Pathways which can initiate S.A.

SUMMARY OF CHAPTER SIX

1. S.A. was looked for and detected during the aggregation induced by adrenaline, thrombin and collagen.

2. Only those plasma showing S.A. to exogenous ADP showed this response to the endogenous ADP release induced by thrombin and collagen.

3. Through the action of the S.A. process the aggregation response was increased and more persistent aggregates were formed. This increased aggregation occurred, as with the S.A. to ADP, to lower doses of these aggregating agents (often very much lower) than were necessary for a marked 'primary' response. Also, the absolute degree of aggregation was increased in inverse proportion to the size of the 'primary' response, i.e. a little for thrombin, moderately for collagen and markedly for adrenaline.

4. There was a cross-correlation between the doses of ADP, adrenaline, thrombin and collagen required for S.A. in an individual plasma. Thus any of the four may be used for measuring the S.A. activity of a plasma.

5. In chain-reactive S.A. plasma, thrombin, collagen and adrenaline initiated the aggregation chain-reaction. This indicates that the pathways leading to this chain-reaction includes these substances. Fig. 6.4 shows all the pathways.

THE EFFECT ON ADP INDUCED AGGREGATION OF VARYING THE EXPERIMENTAL
CONDITIONS AND THE TIME OF TAKING SAMPLES.

INTRODUCTION.

Platelet function is known to be affected markedly by
differences of experimental techniques, such as anticoagulant and
temperature (Hellem 72 and reviews 100, 61), but in this previous
work total aggregation has been studied, without the separation of
P.A. from S.A.   It was therefore necessary to repeat, with reference
to S.A., much of the work previously performed on total aggregation.
Since these experiments were performed other workers have reported
results relating to some of them and these will be detailed with the
relevant experiments.   In most of the experiments the results
obtained for P.A. are given, as well as those for S.A., to help in
comparisons with previous work.   As well as enabling the construction
of a reliable technique for estimating S.A., many of the experiments
give results of theoretical interest and importance.

Table 7.1.   The effect of storage at 37°C on P.A. to ADP

P. A. percent

S.A. Point (ADPμM/L)	No. 1 3μM			No. 2 4μM			No. 3 2μM			No. 4 3μM			Comparison with baseline
P.A. Dose (ADPμM/L)	0.5	1.0	2.0	1.0	2.0	3.0	1.0	1.5	2.0	0.5	1.0	3.0	
30-40 min	3	13	57	21	44	56	29	51	68	9	23	52	
45-50 min	4	16	59	24	47	60	34	58	69	10	25	54	Increase***
60-70 min	4	15	55	23	46	58	32	49	71	12	25	55	Increase***
75-85 min	4	15	53	23	43	55	30	47	63	10	28	52	N.S.
90-100 min	-	15	-	-	43	54	27	44	58	8	23	53	Decrease*

The highest level in each series is underlined e.g. 55

Analysis by Wilcoxon Signed Ranks Test

Table 7.2. The effect of storage at $37^\circ C$ on S.A. point to ADP

Time after Venepuncture	1	2	3	4	5	6	7	8	Change
30 mins	0.75	1.5	3.0	3.0	3.0	3.0	7.5	Nil	
60 mins	0.75	1.5	4.0	3.0	3.0	4.0	7.5	Nil	
90 mins	1.0	1.0	4.0	3.0	4.0	5.0	7.5	Nil	
120 mins	1.0	3.0	5.0	3.0	7.5	5.0	10	Nil	Decrease*
150 mins	1.0	5.0	10	5.0	10	10	10	Nil	Decrease*
O.D. PRP	0.46	0.33	0.27	0.33	0.45	0.50	0.38	0.41	

Decreases in activity below the baseline value are underlined

Analysis by Wilcoxon Signed Ranks Test

Experiment One.

Effects of storage of citrated plasma at 37°C on P.A. and S.A. to ADP.

Previous work. O'Brien noted that the aggregation response (total) at 37°C decreased with time (137), and other workers agree.

Present study.

1. Primary aggregation. Plasma from four subjects was stored at 37°C. Three dose levels of ADP were added to each plasma in a fixed sequence and the sequences were started at the following times after venepuncture, 30 mins, 45, 60, 75 and 90 mins.
Results. (Table 7.1.). A systematic variation occurred. The P.A. response rose from the baseline level of 30 mins to become significantly higher ($p = <0.002$) at 45 and 60 mins. At 75 mins it was near to baseline levels and at 90 mins had fallen below the baseline levels ($p = <0.05$). The degree of the increase was small (only 1 to 7%).
Comments. P.A. should be measured at a fixed time after venepuncture and between 30 and 75 mins. In survey work 30 to 40 mins was used.

2. Secondary aggregation. Plasma from 8 subjects was stored at 37°C. The S.A. point to ADP was determined 30, 60, 90, 120 and 150 mins after venepuncture.
Results. Table 7.2 shows the results. The baseline values ranged from an S.A. point of 0.75μM/L ADP to S.A. absent. S.A. became less active in two plasma at 60 mins, four at 90 mins and six at 120 mins.
Comments. In survey work the S.A. point should be determined within 90 mins of venepuncture to prevent a storage deterioration of more than one level in the S.A. point. This was done.

Table 7.3. Effect of storage at 4°C, 20-24°C and 37°C on P.A. and S.A. at 37°C

Subject	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	Change from 37°C storage
PRP platelet count or O.D.	349	279	642	237	480	318	0.27	0.36	
P.A. percent (ADP 1μM/L)									
(1) stored 37°C	-	23	27	38	2.5	28	20	26	-
(2) " 20-24°C	-	29	49	52	0	52	29	32	Increase*
(3) " 4°C	-	24	37	16	13	40	29	26	N.S.
(2)-(1) percent (1)	-	+26%	+81%	+37%	0	+86%	+45%	+19%	
S.A. point (ADP μM/L)									
stored 37°C	1	1.5	1.5	1.5	3	Nil	Nil	1.5	-
" 20-24°C	0.5	1	1	1.5	3	Nil	Nil	1	N.S.
" 4°C	1	2	2	2	3	Nil	Nil	1.5	N.S.

The most active result has been underlined

Analysis by Wilcoxon Signed Ranks Test

Experiment Two.

Effect of storing citrated plasma at 4°C, R.T. $(20-24^{\circ}$C) and 37°C on P.A. and S.A. tested at 37°C.

Previous work. Using rabbit plasma, Constantine (26) found total aggregation at 37°C increased from 20 to 90 mins when plasma was stored at R.T. Using the lower test temperature of R.T., Harrison et al (67) reported a stable aggregation response from human plasma stored at R.T.

Present study. Experiment one has shown a very small increase in P.A. when plasma was stored and tested at 37°C. In the present experiment a plasma sample was divided into three parts; one was stored in ice chips, one in water at R.T. $(20-24^{\circ}$C), and one in water at 37.5°C. Testing was started after 20 mins storage. Specimens were incubated at 37.5°C for 2 mins before testing to bring them up to the test temperature.

Results Table 7.3.

1. P.A. Storage at R.T. produced an increased aggregation in six of seven plasma $(p = < 0.05)$. This increase showed a marked variation of from 0 to + 86%. This variation could not be correlated with the initial level of P.A., or with the S.A. activity of the plasma. The P.A. value in plasma stored at 4°C was higher than that in plasma stored at 37°C in five of seven subjects, but the extent of the increase was less than after storage at R.T.

2. S.A. The S.A. point ADP concentration after R.T. storage was equal to that after 37°C storage in two subjects, and one dose level more active in four subjects (N.S.). The S.A. points were equal after 37°C and 4°C storage in three subjects and S.A. was one dose level less active after 4°C storage in the other three subjects (N.S.).

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Conclusion

1. Storage of plasma at R.T. increased the P.A. response relative to storage at 37°C. The extent of this increase varied widely and was unpredictable.

2. S.A. activity was increased by storage at R.T. by one dose level in the majority of plasma.

3. 4°C storage tended to increase P.A. and decrease S.A. activity.

4. Storage of plasma at 37°C is preferable to storage at R.T. or 4°C.
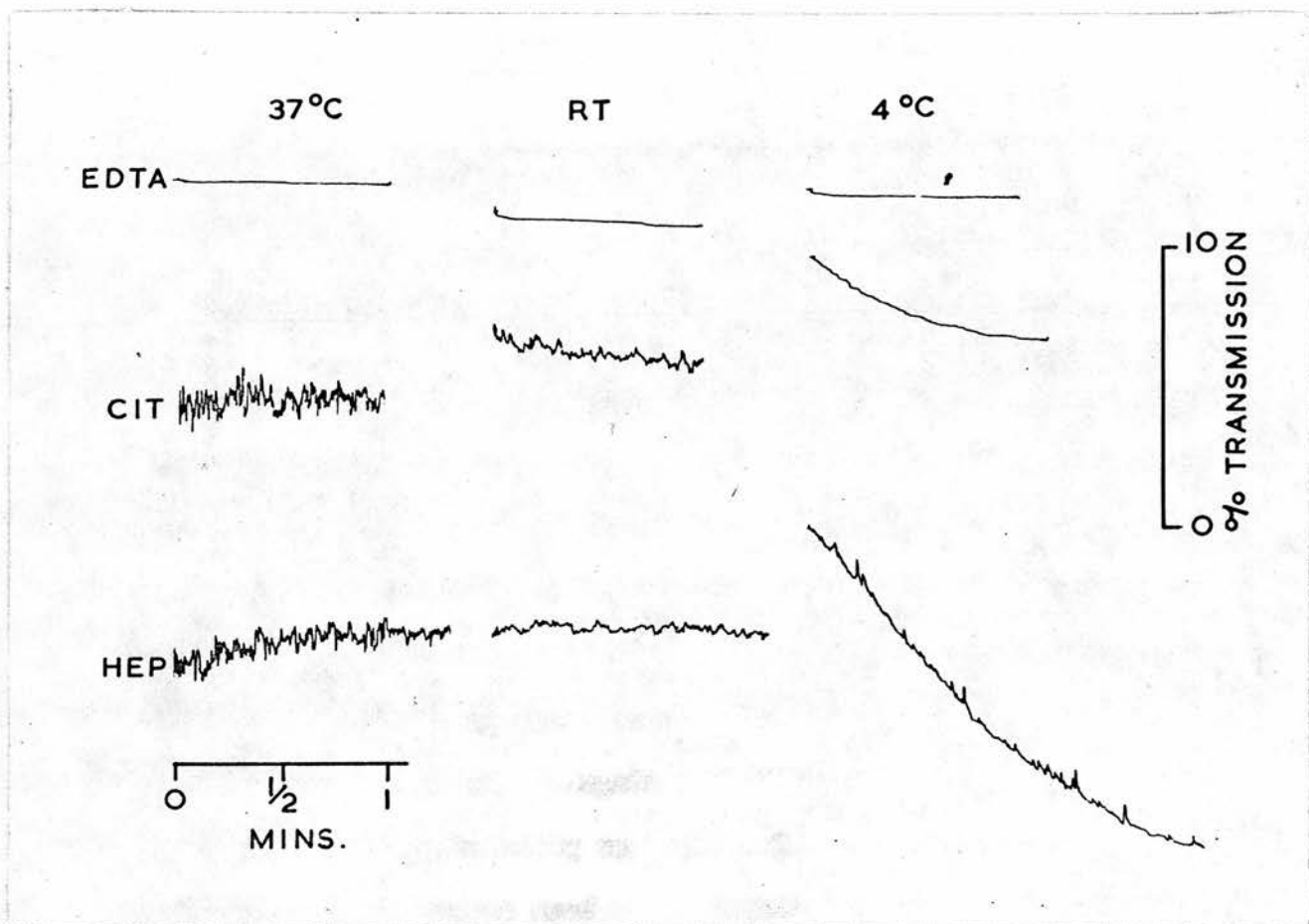
Fig. 7.1.

Effect on platelet shape of three anticoagulants, three plasma
storage temperatures and stirring.

Plasma from the same blood was anticoagulated with 0.38% citrate (CIT),
3 mM EDTA, and 10 u/ml heparin (HEP). After storage at 37°C, 22-24°C
(RT) and 4°C the plasma was warmed to 37°C and stirred. Baseline
oscillations = many disk shaped platelets. No oscillations = mostly
spherical platelets. A fall of the tracing = aggregation.

Experiment Three

Effect of different anticoagulants on platelet shape and stability, and on P.A. and S.A.

1. Shape, storage temperature, and stirring in heparinised, citrated and EDTA plasma.

Previous work. Platelets in vivo are disk shaped. It was shown by Zucker et al (189) that in citrated plasma at 37°C most platelets preserve this disk shape. They found that cooling resulted in sphering of platelets and also produced a few more grotesque shapes. The change from disk to sphere was complete after 20 mins at 4°C. Platelets in heparinised plasma showed the same sequence on cooling, with less preservation of disk shape at all stages. In EDTA, sphering was immediate even at 37°C. The tracings produced, when PRP anticoagulated in these three ways was stirred, have been described (Macmillan and Oliver, 1965).

Plan of study. As has been shown earlier in this thesis (and 141, 144) the stirring of disk shaped platelets produces an oscillating tracing, and of sphered platelets a steady tracing. Citrated (0.38%), heparinised (10u/ml) and EDTA (3mM) PRP were prepared from blood taken at a single venepuncture. Each of these plasma was divided into three. One aliquot was stored at 37°C, and one each at R.T. and 4°C, for 20 to 30 mins. After re-incubation to 37°C. (2 mins in waterbath) the specimen was stirred.

Results. Three experiments which gave similar results. A photograph of one of these is shown in Fig. 7.1. Zucker's findings of shape changes were confirmed and are dmonstrated graphically. The change in citrated plasma from disk shape to sphere on cooling (confirmed by microscopy) was shown by the loss of oscillations in the tracing. The density of

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the plasma rose.   Heparinised plasma showed the same sequence.
Microscopy of 37°C heparinised plasma stirred for 60 secs showed
some sphering and small aggregate formation.   Coinciding with
this was a slight rise in the tracing and a small loss of
oscillations.   Many aggregates, again small, were found on
microscopy of the R.T. Heparin specimens.   Stirring of 4°C citrate
plasma produced a fall in the tracing and aggregates of up to $\frac{1}{2}$ HPF
size were present.   In the 4°C heparinised plasma a much greater
fall occurred and aggregates of 2-5HPF size were present.
In EDTA plasma the platelets were spherical and remained single at
all temperatures.   An unexplained reduction in plasma density
occurred at R.T.

Comments.

1.   Citrated plasma gave a greater preservation of the _in vivo_
disk shape of platelets than the other anticoagulants and
this was optimal at 37°C.

2.   The disk shaped platelets in citrated plasma were more
stable to stirring than those in heparinised plasma.

3. 37°C citrated plasma was optimal from these two viewpoints.

2.   Variation in citrate concentration.

Previous work.   Increasing the citrate concentration reduces platelet
aggregation and adding calcium increases the aggregation (121).   Studies
of S.A. have not been reported.

Present study.   PRP was prepared from blood diluted to 1/20 with 3.8%
citrate.   Immediately before testing 0.9 mls of the PRP was mixed with

(1)   0.1 mls saline = 0.19% citrate;

or   (2)   0.05 mls 3.8% citrate + .05 mls saline = 0.38% citrate;

or   (3)   0.1 mls 3.8% citrate = 0.47% citrate

Table 7.4. Effect of varying citrate concentration on P.A.

[Citrate]	ADPμM producing 20% P.A.						Change from 0.19% citrate
	(1)	(2)	(3)	(4)	(5)	(6)	
0.19%	0.29	0.24	0.57	0.37	0.27	0.37	
0.38%	0.68	0.57	1.2	1.5	0.80	1.1	Increase*
0.47%	1.1	0.68	1.9	1.8	2.3	3.0	Increase*

Table 7.5. Effect of varying the citrate concentration on S.A.

[Citrate]	S.A. point [ADPμM/L]						Change from 0.19% citrate
	(1)	(2)	(3)	(4)	(5)	(6)	
0.19%	1	0.5	5	3	0.75	0.5	
0.38%	3	1.5	10	Nil	3	1.5	Increase*
0.47%	5	1.5	Nil	Nil	Nil	5	N.S.

Dose response curves were carried out on all these concentrations, running the tests of each concentration in parallel.

Results.

 1. Primary Aggregation. Table 7.4.

  Because of the wide range of responses which occurred, there was no ADP dose level which induced aggregation at all levels of citrate concentration, without inducing S.A. in some of the 0.19% citrate plasma. An alternative standard, of the ADP concentration producing 20% P.A., was used. It was calculated from graphs of aggregation percent versus ADP concentration. The results showed that P.A., like total aggregation, was inhibited by citrate.

 2. Secondary Aggregation. Table 7.5.

  The S.A. activity decreased in all six plasma when the citrate concentration was increased from 0.19% to 0.38% ($p = <0.05$). The increase in concentration to 0.47% produced a further decrease in S.A. in four of the five plasma still showing S.A.

  In three subjects (3), (4), (5), there was no S.A. activity detectable at 0.47 citrate concentration. S.A. activity was detected in two of these at 0.38% and appeared in the third at 0.19% citrate concentration. Thus S.A. was uncovered in these subjects by lowering the citrate concentration.

Comments.

 1. The inhibiting effect of citrate on aggregation was confirmed.

 2. This inhibition affected both P.A. and S.A.

 3. Lowering the citrate concentration uncovered a previously undetected S.A. in one plasma.

 4. It may be that S.A. would occur to some extent in all plasma at a citrate concentration of 0.19%.

 3. Heparinised Plasma.

Previous studies. All workers agree that ADP aggregates platelets in

Table 7.6. Comparison of citrate (0.38%) and heparin (10u/ml) as anticoagulants in paired plasma

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	Change
S.A. point of Cit. PRP [ADPμM/L]	0.75	2.0	2.0	3.0	5.0	6.0	7.5	Nil	
Test Dose ADPμM	0.25	1.0	1.0	1.0	1.0	1.0	1.0	1.0	
P.A. percent (1) Cit. PRP	12%	17%	24%	19%	08%	0.5%	29%	18%	
(2) Hep. PRP	52%	68%	52%	63%	70%	57%	63%	60%	Increase**

The highest values are underlined

Analysis by Wilcoxon Signed Ranks Test

Table 7.7. Correlation between disaggregation in heparinised plasma and the S.A. point of a paired citrated specimen.

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
S.A. point [μMADP/L] cit. PRP	0.5	0.5	0.5	0.5	0.75	1.0	1.0	2.0	3	5	7.5	7.5	Nil	Nil
Disaggretion hep. PRP mm	10	5.3	3	5.5	2	1	4	10.5	8	8	14	27	14	33

Specimens (1) to (7) showed less disaggregation than specimens (8) to (14) *** (Wilcoxon Sum of Ranks)

heparinised plasma, but there is disagreement about the comparative effectiveness of the ADP. Some report an equal effect of ADP in citrated and heparinised plasma (14), others that ADP has more effect on platelets in heparinised plasma (118, 145, 174). S.A. has been demonstrated on rare occasions (118) with Born's technique, but using ADP removing enzymes Haslam (69) has uncovered a biphasic tracing more frequently.

Present study. Citrated (0.38%) and heparinised (10 u/ml.) specimens of blood were removed from the same venepuncture.

Plan of study. It was found that after centrifugation at 150g less platelets remained in a heparinised than in a paired citrated plasma, and so the heparinised plasma was centrifuged at 140 g. Even so the platelet counts in nearly all heparinised plasma were less than in a paired citrated plasma (0 to 20%). Specimens from the citrated and heparinised plasma pools were tested alternatively.

Results.

1. Primary aggregation. Table 7.6.

A markedly higher aggregation was present in the heparinised plasma in all eight pairs $(p = < 0.01)$. This increased P.A. activity occurred inspite of the lower platelet counts in the heparinised plasma.

2. Secondary aggregation.

No S.A. tracing was obtained in any of the eight paired specimens, and was only found once in the thirty specimens examined during the work. In an attempt to uncover any S.A. reaction not shown by the aggregation side of the process, disaggregation was studied (Table 7.7).

Table 7.8. ADP-like aggregating activity released from paired citrated and heparinised plasma, by the S.A. point ADP concentration, and during the second phase of adrenaline aggregation.

(1) S.A. to ADP				
Subject no	(1)	(2)	(3)	(4)
S.A. point ADPµM/L	1.0	1.0	3.0	4.0
Release ADP-like activity [µM/L]				
(a) Cit. plasma	5.7	4.0	4.0	2.5
(b) Hep. plasma	Nil (-0.55)	Nil (-0.5)	Nil (-2.25)	Nil (-2.5)
(2) Adrenaline second phase				
Subject no	(5)	(6)	(7)	
Adrenaline addition [µM/L]	8.0	8.0	3.0	
Release ADP-like activity [µM/L]				
(a) Cit. plasma	12.0	7.5	3.6	
(b) Hep. plasma	0.22	0.65	0.45	

The disaggregation occurring, after 10 mins stirring of the sample of heparinised plasma which contained the S.A. point ADP concentration of the paired citrate plasma, was measured i.e. if the citrated plasma S.A. point was 0.5 μM ADP then the disaggregation in heparinised plasma after 0.5μM ADP was measured, and for an S.A. point of 5μM the effect of 5μM ADP on disaggregation in the heparinised pair (Table 7.7).

In spite of the higher ADP concentrations of 2 to 10μM in the specimen of heparinised plasma whose citrate pair showed weak S.A., these samples showed more disaggregation than that occurring after the addition of 0.5 - 1.0μM ADP to heparinised samples paired with chain-reactive S.A. citrate plasma ($p = <0.002$).

Comments.

1. Aggregation induced by ADP was much greater in heparinised compared to citrated plasma.

2. Although an S.A. tracing was not demonstrated there was a marked correlation between chain-reactive S.A. in a citrated plasma and reduced disaggregation in the paired heparinised plasma.

Further studies using heparinised plasma.

Haslam's work, and the preceding section, suggested that a second process is affecting aggregation and disaggregation in heparinised plasma, but to a lesser extent than in citrated plasma. It was possible that a release-reaction was occurring but not showing on the tracing. However no excess of ADP-like aggregating activity over added ADP was found in four aggregated specimens of heparinised plasma (Table 7.8). There was a definite release of aggregating activity in the paired

citrated plasma. In the one of thirty heparinised plasma in which a second aggregation was detected only a minimal release of ADP-like activity was detected (0.2μM).

In citrated plasma there is a strong correlation between S.A. to ADP and the presence of the adrenaline second aggregation phase (see Chapter Six). Only suggestive evidence to show that S.A. occurs in heparinised plasma to ADP has been found so far, yet adrenaline is known to induce a second aggregation phase in heparinised plasma (O'Brien 139), although of a lesser extent than that occurring in citrated plasma (O'Brien et al 145).

The release of ADP-like activity during the second adrenaline aggregation phase was measured in paired citrated and heparinised plasma (Table 7.8 bottom). There was a marked release of aggregating activity into citrated plasma but only a very small one into the heparinised paired plasma (from 2-12% of that in the citrated plasma). Varying the heparin concentration through the range 10 to 400 units (6 experiments). The extent of aggregation was comparable for the same ADP concentrations at each of the heparin concentrations and the small variations which occurred were not systematic.

Summary of results in heparinised plasma.

1.   A second aggregation process could only be detected in the dose-response curve tracing of one of thirty heparinised plasma. Any release of ADP-like activity from platelets in heparinised induced by ADP was too small to produce an excess over the added ADP dose, with one exception.

2.   Any secondary aggregation occurring in heparinised plasma can only be inducing the release of a very small proportion of the ADP available for release reactions.

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3. Disaggregation was significantly depressed in heparinised
    plasma when the citrated paired plasma showed chain-reactive
    S.A.   This suggests that the increased aggregation
    responsiveness/impaired disaggregation part of the S.A. process
    may be occurring.

4. The adrenaline induced second phase of aggregation which occurs
    in heparinised plasma produced only a very small release of
    ADP-like activity, an order or more smaller than that occurring
    in a paired citrated plasma.

5. Heparin has an inhibiting action on the S.A. process and so was
    an unsuitable anticoagulant for S.A. studies

4. EDTA

Previous studies.   EDTA is a powerful chelating agent and when used to
chelate calcium is much more effective than citrate; so much so that
only when more calcium has been added to the plasma can ADP produce
platelet aggregation (68).   It is the only anticoagulant which, after
the separation of platelets from plasma, allows their re-suspension in
further plasma or in saline without large aggregates forming (68), and
so it has an important place in platelet research.   Incubation of
platelets in EDTA plasma at $37^{\circ}C$ makes them incapable of aggregation (58).
No studies into the effect of EDTA on P.A. and S.A. have been reported.
Present study.

Methods.   The syringe containing EDTA was cooled before venepuncture
and the blood-EDTA (3mM/ml) mixture was immediately transferred to glass
centrifuge tubes in ice chips $(4^{\circ}C)$.   Centrifugation was at $4^{\circ}C$ (10 mins).
The plasma was stored at $4^{\circ}C$ and aliquots were warmed to $37^{\circ}C$ just before
testing.

The calcium addition was titrated in each plasma to find the

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Table 7.9.   Comparison of P.A. in paired EDTA (3mM) and citrate (0.38%) paired plasma

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	Change
S.A. point citrated (ADPµM)	1.0	1.0	1.5	2	3	7.5	Nil	Nil	
P.A. percent									
Test Dose [ADPuM/L]	0.5 Cit ED	0.75 Cit Ed 0.5	1.0 0.5	1.0	1.0	1.0	1.0	1.0	
Citrated plasma	14%	19%	12%	22%	08%	05%	13%	43%	
EDTA plasma	30%	43%	30%	59%	40%	49%	52%	80%	Increase**

Analysis by Wilcoxon Signed Ranks Test

Table 7.10.   ADP-like activity in paired citrated and EDTA plasma at the S.A. (citrated) point ADP concentration

	(1)	(2)	(3)
ADP added (µM/L)	0.75	1.0	1.0
ADP-like activity (µM/L)			
(1) citrated PRP	4.8	8.2	15.0
(2) EDTA PRP	0.5	0.25	0.1

minimum addition which supported a maximal aggregation to 100µM ADP, and this addition was used in further specimens of that plasma. It varied from 2.6 to 3.4 mM in different plasma.

Results.

1. The aggregation response decreased rapidly when the plasma was incubated at 37°C. After 15-30 mins incubation at 37°C no aggregation response could be obtained, even with massive calcium and ADP additions. The aggregation response sometimes showed a small increase and sometimes a small decrease during storage at 4°C.

2. Primary aggregation. Table 7.9. Eight subjects.

P.A. was markedly greater in EDTA plasma compared with a paired citrated plasma. ($p = < 0.01$).

3. Secondary aggregation. 18 subjects (paired EDTA and citrated specimens) were studied. In only one was an S.A. tracing obtained. Unfortunately an ADP-like activity assay could not be performed on this occasion.

The eighteen pairs included eight in which citrated specimens showed chain-reactive S.A. The degree of disaggregation in 10 mins in the EDTA plasma paired with these eight, was compared with the disaggregation in the EDTA plasma paired with citrated plasma showing less active or absent S.A. There was a significant depression of disaggregation (Wilcoxon sum of ranks $p = < 0.002$) in the EDTA plasma paired with chain-reactive S.A. citrated plasma.

4. The release of ADP-like activity after aggregation by ADP was measured in three pairs of EDTA and citrated plasma (Table 7.10). Citrated plasma with chain-reactive (S.A. points 0.75, 1.0 and 1.0µM/L ADP) were chosen and these showed a marked release of ADP-like activity. The EDTA plasma of the pairs showed a fall in ADP-like activity from the ADP addition level.

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Summary.

1. Platelet aggregation could only be studied in EDTA plasma which was stored at 4°C.

2. The P.A. response to ADP was much higher in EDTA plasma than in citrated plasma.

3. Only one S.A. tracing occurred in eighteen plasma. No ADP-like activity release was detected in EDTA plasma paired with chain-reactive S.A. citrated plasma.

4. However there was a correlation between a failure of platelets to disaggregate in EDTA plasma and chain-reactive S.A. in the paired citrated plasma.

5. In S.A. studies citrated plasma is preferable to EDTA plasma.

Table 7.11. Effect of fast and steady withdrawl of blood on P.A. and S.A.

	(1)	(2)	(3)	(4)	(5)	(6)
P.A. percent (1μM/LADP)						
Fast removal	22	21	34	20	25	28
Slow removal	23	28	42	25	28	28
S.A. point (ADPμM/L)						
Fast removal	5	1.5	2	2	1.5	4
Slow removal	7.5	1.5	1.5	2	1.0	4

The more active response in each pair has been underlined.

Experiment Four.

The effect of fast and steady withdrawal of blood on P.A. and S.A.

No previous work.   The release of ADP from red blood cells is
sufficient for it to be the stimulus to platelet aggregation in
platelet stickiness tests.   It is theoretically possible for such
a release to occur during a venepuncture and so affect the aggregation
of platelets in the subsequently separated platelet rich plasma.

Present study.   Blood was withdrawn from a venepuncture into two
syringes; one as fast as vein collapse would allow ('fast'), and
the other at a steady unhurried rate ('steady').   The order of fast
and steady withdrawal was randomised.   Six subjects were studied.
Dose response curves to ADP were performed, testing aliquots from
the 'fast' and 'steady' plasma samples alternately.

Results.   Table 7.11.

1.   Primary aggregation was higher in plasma separated from
blood withdrawn steadily compared with the paired 'fast' withdrawal
sample in five subjects, and equivalent in one subject.

2.   Secondary aggregation was more active in two 'steady'
withdrawal plasma, less active in one, and equal in the other three
pairs.   When a difference was present it was only of one S.A. point
level.

Conclusion.   P.A. was reduced by a fast withdrawal of blood.
No effect was detected on the S.A. point.

Table 7.12. Comparison of P.A. and S.A. in the top and bottom portions of the plasma column

	(1)	(2)	(3)	(4)	(5)	(6)	Change
P.A. percent (µM/LADP)							
Top	12	24	23	15	14	-	
Bottom	05	26	24	18	11	-	N.S.
S.A. point (ADPµM/L)							
Top	10	1.5	4	Nil	3	1	
Bottom	10	1.5	3	Nil	3	0.75	N.S.

The more active response in each pair has been underlined

Experiment Five

Comparison of P.A. and S.A. in plasma from the top and the bottom of the plasma column.

Previous work.   Aggregation percent has been found to be increased in plasma taken from the top, over plasma from the bottom, of a plasma column in a centrifuge tube, at a test temperature of R.T. (Harrison et al 66).   This change was ascribed to degradation of ADP by the white blood cells in the bottom portion of the plasma.   The experiment has not been performed in plasma stored and tested at 37°C.

Present study.   Two citrated blood samples of 20 mls were taken from the same venepuncture.   After centrifugation the top halves of the plasma columns were withdrawn and pooled and the bottom halves were treated similarly.   Dose-response curves to ADP were performed, testing specimens from the two pools alternately.

Results.   Six subjects.   Tablet 7.12.

1.   Primary aggregation.   The plasma from the top of the column gave a higher aggregation percent in two subjects and a lower reading in three subjects.   The differences were small.

2.   Secondary aggregation.   The S.A. point was equal in four plasma pairs and one dose level more active in two of the 'bottom' plasma.

Conclusion.   No significant difference was detected between the aggregation of platelets in the top and bottom portions of a plasma column.   The aggregation process occurs at a faster rate at 37°C and so may be complete before any white cell effect on ADP can affect the result.
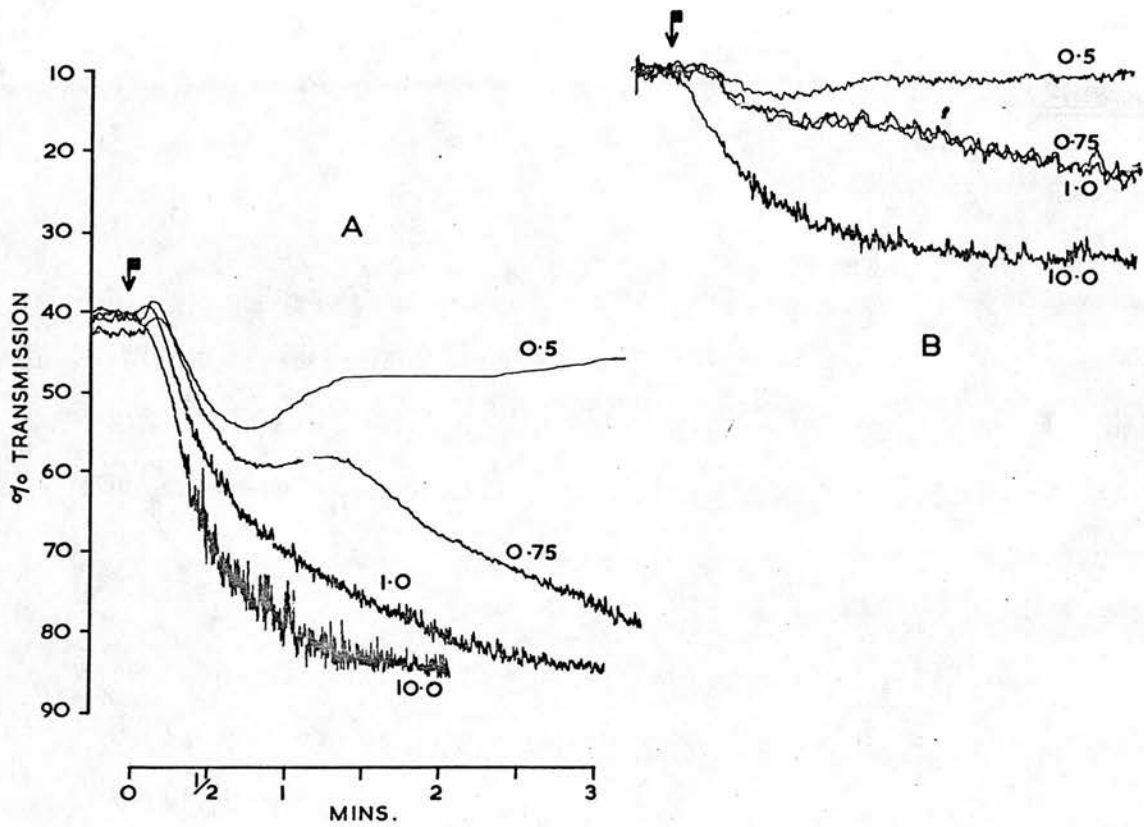
Fig. 7.2.

Effect of R.B.C.'s on the dose-response curve of plasma to ADP

Plasma A shows S.A. point (with biphasic tracing) of 0.75μM ADP.
Plasma B is plasma A plus 32th. R.B.C.'s and has a shorter range of
response. The S.A. point is also 0.75μM ADP.

Experiment Six.

Effect of red cells on S.A.

No previous work.

Present study.   Plasma from three subjects were each divided into two
equal aliquots.   To one of these a small drop of centrifuged blood
was added.   This produced a marked reddenning of the plasma and the
red cell count was 20 to 45th/cm.mm.   The S.A. point of each specimen
was measured.

Results.   Fig. 7.2.

Samples from the aliquot containing red cells had an
elevated density.   The red cells produced a visible swirling effect
and this produced oscillations in the tracing which persisted after
the addition of ADP.

The form of the tracing was the same in both aliquots but
with much smaller changes in plasma density during aggregation in the
red cell specimen.   Microscopy of the $0.75\mu M$ tracing specimens showed
maximal aggregation in the platelet aggregates from both.   The S.A.
points were equal in the three subjects ($0.75\mu M$ and $0.75\mu M$; $2.5 \mu M$ and
$2.5\mu M$; and $4\mu M$ and $4\mu M$ ADP).

Summary.   Intentional contamination with red cells did not affect the
S.A. point in three subjects.

Table 7.13. Comparison of P.A. and S.A. in duplicate specimens from the same venepuncture

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	Mean
P.A. percent (1μMADP)																	
1st Specimen	22	6	8	10	16	16	5	13	12	16	16	39	37	11	25	–	12.1
2nd Specimen	20	7	11	11	14	16	13	13	4	13	24	33	48	11	28	–	13.7
S.A. point (ADPμM)																	
1st Specimen	2	3	2	3	3	3	Nil	4	3	2.5	2	2.5	3	5	1.5	0.75	2.51
2nd Specimen	2	3	2	3.5	3	3	Nil	4.5	2.5	3	1.5	2.5	3	5	1	0.5	2.50

Experiment Seven.

Reproducibility of P.A. and S.A. in duplicate samples.

Previous work. Harrison et al (67) who tested aggregation at room temperature, have found that the mean values of paired specimens removed from the same venepuncture were equivalent.

Present study. Two blood samples were withdrawn from the same venepuncture in sixteen subjects. The blood samples were processed separately and samples from the resulting plasma were tested alternately.

Results. Table 7.13.

1. Primary aggregation. The P.A. response was very similar in eleven specimens and less equivalent in four (7), (9), (11), (13). Higher aggregation percent was present in five 'first' specimens and 'seven' second specimens.

2. Secondary aggregation. The S.A. point was identical in nine pairs of plasma. In the remaining seven pairs there was a variation of one dose level or less.

Conclusion.

1. In duplicate specimens, P.A. was reproducible in most.

2. S.A. was reproducible to within one dose level in all specimens.

<u>Table 7.15.</u> <u>The degree of extraction of platelets from whole blood to the</u>
<u>supernatant plasma during centrifugation</u>

	Whole Blood platelet-count (10^3/cu mm)	Plasma platelet-count (10^3/cu mm)	Plasma volume (mls)	Plasma platelets 18 (10^3/cu mm)	Extraction of platelets to plasma (percent)
1)	243	331	8.7	161	66
2)	181	332	10.3	196	108
3)	181	305	8.3	142	78
4)	130	180	7.9	79	61
5)	200	281	7.0	110	55
6)	184	334	9.0	166	90
7)	121	224	8.2	101	84
8)	234	300	8.7	145	62
9)	135	304	6.0	102	75
10)	199	255	9.8	139	70
11)	134	221	10.6	133	99

<u>Range</u> 55-108%

<u>Mean</u> 77%

<u>Experiment Eight.</u>

<u>Effect of platelet count and platelet population on P.A. and S.A.</u>

1. <u>Percentage extraction of platelets from whole blood to platelet rich plasma.</u>

<u>Present study.</u>   The percentage extraction from whole blood to platelet rich plasma depends on the centrifugation procedure.   The platelet extraction percent of the centrifugation procedure used in this thesis was measured, using citrated blood.

<u>Results.</u>   Table 7.15.   Eleven subjects.

The mean extraction of platelets from whole blood into the supernatant plasma showed wide variation.   There was a mean extraction of 77% and a range of 55-108%.

<u>Summary.</u>   The degree of extraction of platelets from whole blood into PRP varied widely.

Table 7.14. Effect on P.A. of diluting plasma with platelet poor plasma

	(1)		(2)		(3)		(4)		(5)		Mean
O.D. plasma	0.44	0.44	0.49	0.49	0.44	0.44	0.49	0.49	0.58	0.58	
S.A. point (ADPμM)	1.5		5.0		2.0		4.0		3.0		
P.A. dose ADP (μM/L)	1.0		1.0		1.0		1.0		1.0		
P.A. of Initial 100% plasma	28(100)	29(100)	11(100)	18(100)	23(100)	20(100)	17(100)	20(100)	24(100)	23(100)	23.7(100)
P.A. of Final 100% plasma	29	29	18	22	20	20	20	24	23	24	23.4
Change Initial to final (percent initial)	+4%	0	+64%	+18%	-13%	0	+18%	+20%	-4%	+4%	
P.A. of dilutions of PRP											
1) 80%	24(84)[1]	25(86)	11(100)	17(85)	19(89)	19(95)	19(102)	20(90)	27(115)	23(98)	21.4(90%)
2) 60%	17(60)	18(62)	11(100)	14(70)	15(70)	16(80)	15(81)	17(77)	26(110)	25(106)	17.0(72%)
3) 40%	14(49)	10(36)	7(64)	11(55)	11(52)	11(55)	11(60)	15(68)	20(85)	20(85)	13.7(54%)
4) 20%	6(21)	5(17)	-	7(35)	9(42)	8(40)	11(60)	9(41)	16(68)	20(85)	10.1(42%)

1. Percentage of initial P.A. of 100% plasma

2.   The effect of platelet count on P.A. and S.A.

A.   Primary Aggregation.

Previous work.   There is general agreement that aggregation decreases with the platelet count.   O'Brien has attempted to quantify this decrease for total aggregation, to enable inter-plasma and intra-plasma comparison. No work has been reported on S.A.

Present study.   Plasma was diluted with it's own platelet poor plasma (PPP) to concentrations of 80, 60, 40 and 20%, immediately before testing. The P.A. response of the plasma and it's dilutions, to 1µM ADP, was measured.   The response of the parent (100%) plasma was then rechecked. There were two series of measurements on each plasma.

Results.   (Table 7.14) 5 plasma giving 10 results.

The results were comparable in four pairs.   They were dissimilar in pair (2) where the control value increased by 64% during experiment (A), and this result was not analysed.   In the remainder, a gradual reduction in response occurred with dilution, except for pair (5) where there was a rise in P.A. at 80% and 60% dilutions above the P.A. in 100% plasma.   However the degree of reduction was far from uniform.   The inter-plasma pair differences were larger than the intra-plasma pair differences, suggesting that the variations were biological rather than technical.

Comment.   P.A. decreased with the platelet count, but not sufficiently uniformly for an accurate prediction of the change to be made.

B.   Secondary Aggregation.

No previous work.

Present study.   Plasma was diluted with it's own PPP to 75% and 50%, or to 60% and 30%.   The S.A. points of whole plasma and the two dilutions were measured.   Samples from the three plasma were tested alternately.

EFFECT of DILUTION of PLASMA on S.A. POINT
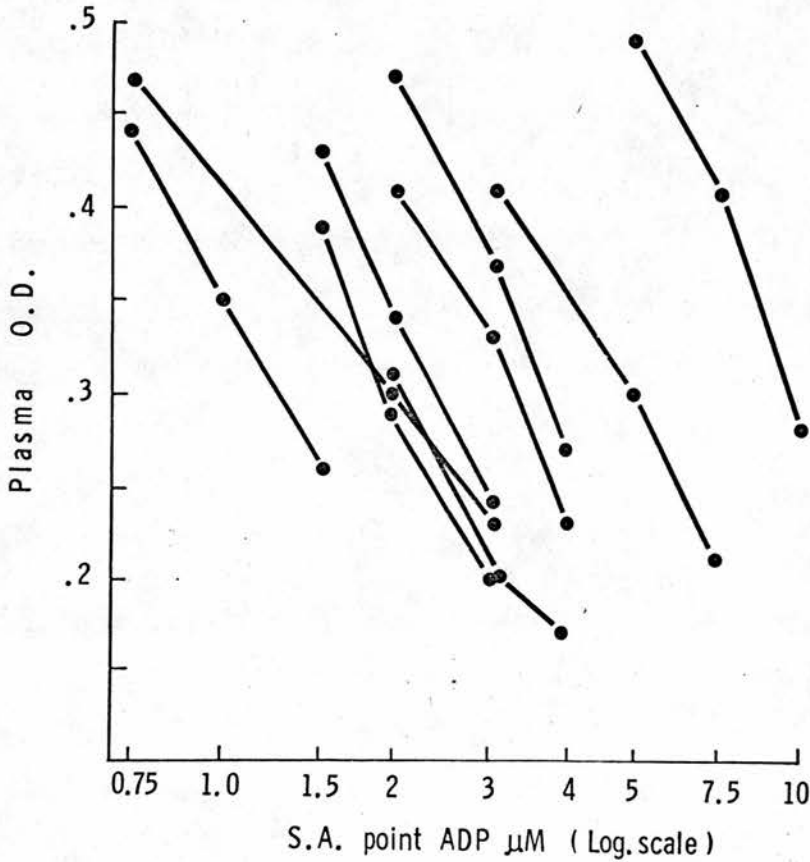
Fig. 7.3.

Effect of dilution of PRP with PPP on the S.A. point (ADPµM)

Plasma was diluted to either 60 and 30 percent or 50 and 25 percent
with its own PPP. The S.A. points of the three resulting specimens
have been joined by a line. Nine subjects.

<u>Results</u>. Fig. 7.3. Nine subjects.

The three points for each subject have been joined. Plasma specimens covered the S.A. range. Using a **logarithmic scale for the** ADP concentration of the S.A. point, a linear relationship to O.D. and comparable slopes between the plasma are suggested. Analysis of the first and second slopes showed no significant divergence from a straight line, and the slopes of the S.A. points of the 10 subjects did not differ significantly from each other (Mr.~W~ Lutz, ~~Public Health~~ Social Medicine Department, Edinburgh). The mean slope was 62.1°.

<u>Summary</u>. The changes in S.A. point produced by dilution of a plasma with it's own PPP were linearly related. In nine experiments the slope of this change was **similar**. The mean slope was 62.1°.

3. <u>Effect of reducing platelet count by repeated centrifugation on</u>
<u>S.A. point</u>.

<u>Introduction</u>. A theoretical criticism which has been levelled against methods using platelet rich plasma, is that the population of platelets in PRP may not be a true sample of those in whole blood. The relationship between platelet size and platelet aggregation potential is uncertain. Some workers have shown a relationship between large size and youth (105) and others between large size and metabolic activity (95) and platelet release reactions (95a). Both these groups of workers altered the <u>in vivo</u> disk shape of the platelet to that of a sphere by using EDTA in their experiments. These workers may therefore be showing that resistance to the sphering effect of EDTA, youth, and high activity are inter-related. There is no work relating the size of the disk shaped platelets of citrated plasma (which predominate <u>in vivo</u> and in this work) to their aggregating ability.

EFFECT of CENTRIFUGATION of PLASMA on S.A. POINT
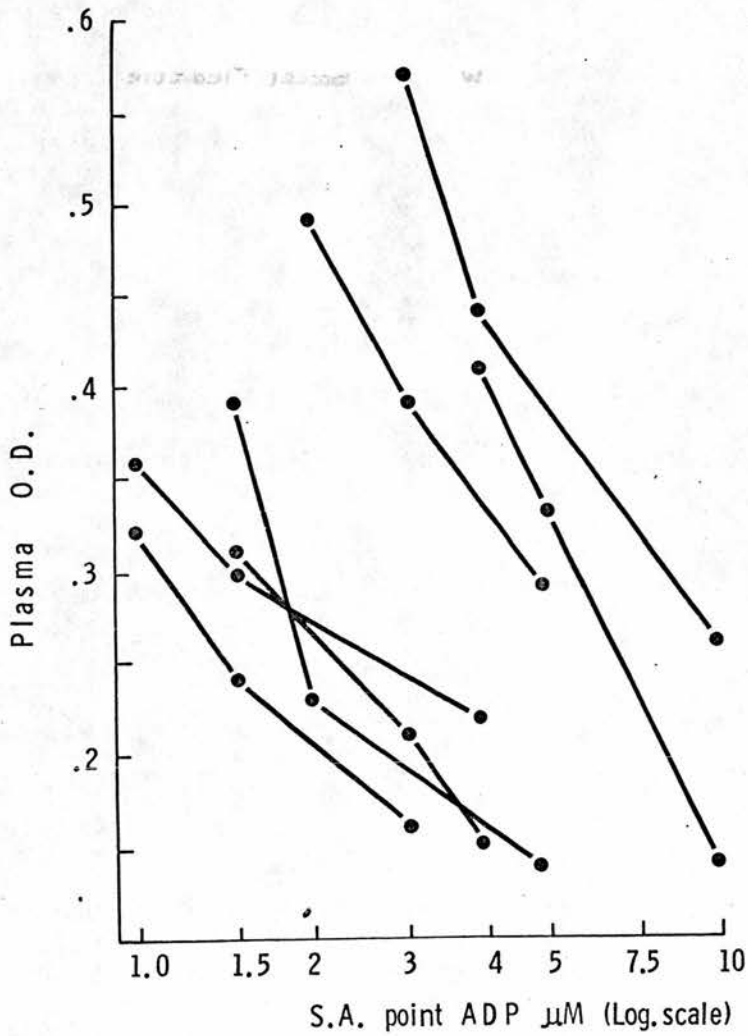
Fig. 7.4.

Effect of centrifugation of PRP on the S.A. point (ADPuM)

Plasma was recentrifuged twice at 210g for 5 mins. The S.A. point
of the three resulting specimens have been joined by a line. Seven
subjects.

<u>Present study</u>.   In order to allow the larger platelets to sediment
plasma was recentrifuged in the following way:- 15 mls of plasma from
the same subject were mixed and 4 mls removed.   The remainder was
recentrifuged for 5 mins at 210g.   The supernatant plasma was removed,
leaving the bottom one cm and the sedimented platelets.   4 mls were
removed from the plasma and the remainder was similarly recentrifuged
and a 4 ml aliquot was again set aside.   The S.A. points of the three
samples (original plasma and first and second recentrifugations) were
measured.   Specimens from the three samples were tested alternately.
<u>Results</u>. Fig. 7.4. Seven subjects.

The initial O.D. was comparable to those in the dilution
experiment (Fig. 7.3) in three plasma and the same angle of slope
was present in both experiments.   The initial O.D. was lower than that
in the dilution experiment plasma in the other four and the slope angle
was also lower in these four plasma.   A mean slope of 54.1° was present;
it may however be in part lower because of the lower initial O.D.'s.

The difference between the slopes obtained by dilution
(62.1° mean) and by recentrifugation (54.1° mean) was significant
($p = < 0.05$).   To find out the practical effect of this difference in
slope, the S.A. point which would occur when a plasma of O.D. 0.4 and
S.A. point 1µM ADP was diluted or recentrifuged to halve the platelet
number (about 400,000 to 200,000 platelets per cu.mm.), was calculated.
The S.A. point by dilution would be 2.5µM ADP and by recentrifugation
3.5µM, a small difference only.   This suggests that changes of platelet
population produced by centrifugation are of minor importance only, and
that <u>citrated</u> PRP gives a good measure of the S.A. activity of the total
platelet population.

Summary.

1.   In seven subjects the effect on S.A. point of reducing the number of platelets by centrifugation (thus allowing differential loss of the more sedimentable platelets), was measured.

2.   The effect of this manoeuvre on S.A. was to reduce S.A. activity a little more quickly than dilution by PPP did. The difference was small in practical terms.

3.   It is likely that the method of plasma preparation used in this work, produces a PRP population whose S.A. activity gives a good measure of S.A. activity of the whole blood platelet population.

Summary of Experiment Eight.

1.   The degree of extraction of platelets from centrifuged whole blood into the plasma supernatant was a mean of 77% with a range of 55-108%.

2.   Reducing the platelet count by dilution reduced P.A. percent and S.A. activity.

3.   The degree of reduction in P.A. was too variable for accurate corrections to be made for differences in platelet count.

4.   The reduction in S.A. was uniform. Thus accurate corrections can be made for differences in platelet count.

5.   S.A. activity was reduced slightly more quickly by recentrifugation than by dilution. The difference was small and may be in part due to differences in plasma density, suggesting that the S.A. response of citrated PRP is a good measure of that of the platelets in whole blood.

Table 7.16. The effect on P.A. and S.A. of testing aggregation at R.T. and 37°C

Subject	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	Change
Initial test temperature	R.T.	R.T.	37°C	37°C	R.T.	37°C	R.T.	37°C	37°C	R.T.	
(1) P.A. dose ADP(µM/L)											
(a) 37°C	0.5	1.0	1.0	1.0	0.75	0.75	0.25	0.5	1.0	–	
(b) R.T.	0.25	0.25	0.5	1.0	0.25	0.25	0.25	0.5	1.0	–	
P.A. percent (a) 37°C	15	18	39	32	22	25	11	22	54	–	Increase***
(b) R.T.	36	48	62	34	40	29	50	57	60	–	
Difference percent	–	–	–	+6%	–	–	+445%	+160%	+9%	–	
(2) S.A. point (ADPµM)											
(a) 37°C	1.0	2.0	4.0	5.0	1.0	1.0	0.5	0.75	2.0	1.0	Increase*
(b) R.T.	0.5	0.75	2.0	5.0	0.5	0.75	0.5	0.75	1.0	0.5	
Difference (S.A. levels)	-2	-3	-2	0	-2	-1	0	0	-2	-2	

The highest ADP doses in a P.A. pair and the most active responses (P.A. and S.A.) have been underlined

Analysis by Wilcoxon Signed Ranks Test

Experiment Nine.

Effect of testing at RT and $37^{\circ}C$ on P.A. and S.A.

1. The dose-response curve.

Previous work. There is general agreement that the degree of aggregation is increased by testing a PRP at R.T. as against $37^{\circ}C$ and that subsequent disaggregation is decreased (for review see Ref.111).

Present study. Plasma was stored at R.T. for 20 mins and then tested at R.T. and $37^{\circ}C$. The same apparatus was used for both test temperatures, and so the complete dose-response curve at a test temperature had to be performed before the alternative temperature was used. The order of testing was randomised. A temperature equilibration period for the apparatus of 20 mins was used between the two dose-response curves.

Results. Table 7.16. Ten plasma.

    1. Primary Aggregation. It was confirmed that P.A. is greater at R.T. compared with $37^{\circ}C$ ($p = <0.002$). The degree of the increase at R.T. varied widely and was in no way predictable from the result at $37^{\circ}C$.

    2. Secondary Aggregation. Non-phasic S.A. tracings were present at R.T. but a biphasic tracing was not detected. The S.A. point ADP was lower at R.T. ($p = <0.05$) with the degree of the increase in S.A. activity varying. It was zero (3 plasma), one level (1 plasma), two (5 plasma) and three (1 plasma). The degree of variability was not related to the level of the S.A. point.

2. Correlations between S.A. tracings and release of ADP-like activity.

Plan of study. The increased aggregation and decreased disaggregation occurring at R.T. may have produced an S.A. tracing appearance in the absence of ADP release or the presence of only a small release, and so

Table 7.17. Release of ADP-like activity during S.A. at R.T.

R.T.	Spec 1		Spec 2		Spec 3	
	Dose	ADP-like activity (µM/L)	Dose	ADP-like activity (µM/L)	Dose	ADP-like activity (µM/L)
P.A. tracing	0.5	not detected	0.25	not detected	1.0	1.0
1st S.A. tracing	1.0	not detected	0.5	1.25	2.0	5.5
2nd S.A. tracing	2.0	5.0	1.0	2.5	3.0	5.5
3rd S.A. tracing	3.0	5.0	2.0	6.0	-	-
37°C S.A. point (ADPµM/L)	2		1.5		3	

the tracings were correlated with the ADP-like activity in the supernatant, in three plasma. The tracings at $37^{\circ}C$ testing have been found to correlate with the release of ADP-like activity (see Chapter Five).

Results.   Table 7.17.

In contrast to S.A. at $37^{\circ}C$, the correlation between the tracing and the release of ADP-like activity was variable at R.T. (Table 7.17).   In specimen (1) an S.A. tracing occurred before a release and in specimen (2) an S.A. tracing occurred in association with a small release which subsequently increased when the dose of ADP was raised.   Specimen (3) showed complete correlation between tracing and release.

Summary.

1.   R.T. testing increased P.A.   The degree of the increase varied widely and was not predictable.

2.   A non-phasic S.A. tracing can be detected at R.T. and occurs at a lower ADP dose.   But it does not have always the same meaning as the S.A. tracings occurring at $37^{\circ}C$, as it can occur without an ADP release or only a small release.

Table 7.18. Variation in P.A. and S.A. through a day

Primary Aggregation (%)					
Time	(1)	(2)	(3)	(4)	(5)
9 AM	26(1)	28(1)	29(1)	37(1)	08(4)
12 MD	16(4)	18(4)	18(4)	36(2)	09(3)
3 PM	18(3)	26(2)	26(2)	35(3)	20(1)
6 PM	23(2)	20(3)	21(3)	34(4)	13(2)

The ranking of each level is shown in brackets

Secondary Aggregation (S.A. point ADPµM/L)												
Time	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
9 AM	2	5	3	1	2	2	1	1.5	2	3	4	Nil
12 MD	2	7.5	4	1	2	3	1	2	1.5	3	4	Nil
3 PM	3	4	4	1.5	1.5	2	0.75	2	2	4	3	Nil
6 PM	2	4	3	1	1.5	1.5	0.75	2	1.5	3	4	Nil
Range of variation	1	2	1	1	1	2	1	1	1	1	1	0

Experiment Ten.

Intra-person and inter-person variations in S.A.

Previous work. Variations with time in S.A. to ADP have not been reported but the changes in the related second phase of adrenaline aggregation were studied in ten subjects over a month by O'Brien (140). O'Brien used a single dose of adrenaline (5μM/L) and detected the second phase on all occasions in four subjects, on two-thirds or more occasions in four, and on less than half the occasions in two subjects. He suggested that considerable variations occur in S.A. activity from day to day

Present study. This differed in two respects from O'Brien's study. First ADP was used to stimulate S.A. Second the dose of ADP was varied to detect the S.A. point exactly, thus obtaining a more accurate measure of S.A. activity.

1. Variation through a day.

Plan of study. The blood was taken from volunteers at 9 am., 12md., 3 pm. and 6 pm. They were fasting for the first specimen, had a light breakfast at 9.30 am., a fatty meal at 1 pm. and a light tea at 3.30 pm.

Results. Table 7.18.

1. Primary Aggregation was followed in five subjects. In four subjects the peak of aggregation was 9 am., with a mid-morning fall and a rise to near fasting (9 am.) levels in the afternoon. In the fifth subject low morning values were followed by a peak at 3 pm. With the exception of this latter person the values varied up to ± 25% of the mean.

2. Secondary Aggregation was followed in twelve subjects. Eleven subjects showed S.A., in the other (No. 12) there was no S.A. throughout the day. Of the eleven, nine showed a variation of only

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Table 7.19. Variation in S.A. Day to Day and Week to Week

| | S.A. point ADP (µM/L) | | | | | | |
| | Group one | | | | Group two | | |
Subject	(1)	(2)	(3)	(4)	(5)	(6)	(7)
Day 1	1.5	-	-	-	5	-	4
2	1.5	-	-	-	7.5	-	4
3	1	-	-	-	5	-	5
4	1.5	-	-	-	5	-	3
5	1.5	-	-	-	10	-	4
8	1.5	2	3	4	7.5	7.5	4
9	1.5	1.5	4	4	-	-	3
10	2	2	3	2	4	5	3
11	3	1.5	3	4	4	7.5	3
12	-	2	4	7.5	-	-	4
Week 3	2	-	2	7.5	4	5	2
4	1.5	2	3	4	7.5	5	3
5	-	2	(nil)	4	-	-	4
6	-	1.5	(nil)	(7.5)	-	-	4
7	-	1.5	2	(nil)	-	-	-
8	-	1.5	2	5	-	-	-
Mean	1.7	1.8	2.6	4.6	6.4	6.0	3.6
Variation in S.A. point level							
1) day to day	3	1	1	4	3	1	3
2) Total time	3	1	2(6)	4(6)	3	1	3

one S.A. point level and in the other two it was 2 levels.   A
systematic pattern could not be detected in the through a day
variation in S.A. point.

Comment.

1.   The S.A. point was steady through the day.

2.   P.A. showed, as in other experiments, more variation.
      The results also suggest the possibility of a mid-morning
      fall in P.A.

2.   Variation from day to day and week to week.

Plan of study.   Fasting volunteers (males aged 18 to 48) were bled
at 9 am. on various days over two eight-week periods.   The subjects
were tested in groups, one of four followed by one of three.   This
allowed a check to be made for any systematic change across a group.

Results.   Table 7.19.   Seven subjects.

The top section of the table shows the day to day variation
in five-day periods (10 periods in all).   The variation was one S.A.
point dose level (three subjects), three (three subjects) and four
(one subject).   S.A. was present on all occasions.

The variation during the three to eight weeks period
(bottom section Table 7.19) was only slightly greater, and was
remarkably similar to the individual subjects day to day variation.
In group one, two volunteers, who worked together, showed a marked
fall in S.A. activity (subject (3) weeks 5,6 and subject (4) weeks
6,7) when they had an upper respiratory tract infection.   The effect
of aspirin-like drugs was not known at the time of this survey and so
were not inquired into at the time.   These two pairs of results are
in brackets and were not included in the analysis.   The other
volunteer in group one maintained a steady response over this period.

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The mean S.A. point value varied widely, from 1.7 to 6.4µM ADP/L. The variation in S.A. point level (bottom table 7.19) was low (1-2 levels) in three subjects and moderate (3-4 levels) in the other four. The variation in the day to day and in the total period were markedly similar in a particular subject. The presence of low or moderate variation did not correlate with either low or high S.A. activity.

Summary.

1.   The variation in S.A. activity, tested at four times during a day of normal eating and activity, varied by one dose level or less in ten of twelve subjects. This is little different from the variation found in duplicate estimations (Exp. Seven this Chapter). In the other two the variation was one dose level higher.

2.   The variation in S.A. activity day to day, and week to week was low (1-2 levels) in three of seven volunteers and was moderate (3-4 dose levels) in the other three. Two pairs of aberrant results were present.

3.   The mean S.A. point ADP concentration varied widely (1.7 - 6.4µM).

4.   The degree of variation was similar in the day to day period and the week to week period in any one individual.

5.   These results suggest that S.A. activity may remain constant in an individual although varying widely in it's level between individuals. The degree of variation about the mean level may also be constant in any individual.

GENERAL SUMMARY OF CHAPTER SEVEN.

1. Citrated PRP stored at $37^{\circ}C$ showed a relatively stable P.A. percent response between 30 and 75 minutes of venepuncture; and an S.A. response which was stable for 30 to nearly 90 minutes from venepuncture.

2. Any variation in storage or test temperature produced unsystematic and sometimes large changes in the aggregation response. Because these changes were unsystematic corrections could not be made for them.

3. Heparin and EDTA inhibited S.A., though evidence of slight S.A. activity could be detected when the S.A. process was very active. Heparin and EDTA are not considered to be suitable for studies of S.A.

4. The withdrawal of blood at a fast rate slightly affected P.A. but did not affect S.A. Contamination of plasma with RBC's and WBC's (bottom of plasma column) did not affect the S.A. point level.

5. Reduction of platelet count by dilution with PPP produced a fall of widely varying extent in P.A., and a remarkably constant degree of fall in S.A. point level. The changes in S.A. point level were sufficiently constant for valid corrections to be possible for changes in platelet count.

6. There was only a small difference between the effects on S.A. point level of diluting a plasma (i.e. same plasma population) and recentrifuging (i.e. loss of larger platelets). It is therefore likely that S.A. activity measurements in citrated plasma are a good estimate of S.A. activity in the citrated whole blood platelet population.

7. Through a day and over an eight-week period the level of S.A. activity in a given healthy volunteer remained constant within a variation of four dose levels (in most it was less than four levels). The mean level of S.A. activity varied widely.

CHAPTER EIGHT

PRIMARY AND SECONDARY AGGREGATION IN HEALTHY BLOOD DONORS

Previous work. In the course of basic studies on small groups of
volunteers and patients plus volunteers S.A. has been found in about
80% of plasma by several workers (Macmillan 106, Mills and Roberts 116,
Zucker and Peterson 197). There has been some variation in the
interpretation of the tracings in these reports which will be
discussed at the end of the thesis. Recently Hardisty et al (1970
ref 65) reported an incidence of S.A. of 100% in 60 laboratory workers.
He used the Born principle, but had included a major modification in
technique.

The study reported in this chapter was on healthy blood
donors, and the intention was to survey both sexes and each decade
from 21 to 60 yrs. as evenly as possible.

Present study. This was made possible by the co-operation of
Dr. R. A. Cummings, Director of the Scottish South Eastern Regional
Board Transfusion Centre. The blood donation centre was situated
very close to the laboratory and so quick transport of blood specimens
was possible.

The blood donors. Blood donors were asked to give a further specimen
of blood for research during a routine blood donation. The donors
asked were Caucasian. Initially all donors were asked who were aged
21 years to 60 years, both male and female. When 10 donors of the
one sex in one of the four decades had been surveyed no more in this
age/sex group were approached. In this way an almost even spread of
sex and age was achieved.

All volunteers were asked the following questions and if a
positive answer was given they were excluded:-

1. Have you had any illness, however minor, in the past ten days?

Table 8.1. P.A. and S.A. in 74 blood donors

Subject	S.A. Point (ADPμM)				P.A. (%) (1μMADP)			
	Male		Female		Male		Female	
	21-40	41-60	21-40	41-60	21-40	41-60	21-40	41-60
1	4 3	2 2	1.5 1	1.5 5	10 27	33 53	38 +	49 40
2	1 3	4 3	2 3	2 2	+ 25	29 36	30 20	44 31
3	7.5 10	10 7.5	3 3	1.5 1.5	6 34	3 20	21 25	51 33
4	3 Nil	1.5 Nil	3 1	3 3	14 5	27 26	33 +	38 36
5	2 7.5	1.5 Nil	2 5	7.5 3	23 0	36 9	26 30	41 50
6	2 10	5 2	1.5 4	7.5 10	8 30	36 40	51 38	25 10
7	2 3	3 2	3 2	3 2	26 18	27 20	35 35	18 31
8	5 3	3 2	2 1	3 0.75	29 14	43 35	43 +	31 +
9	3 3	4 5	(0.75) 3	- 1	19 12	27 61	(+) 12	- -
10	1.5 7.5	2 4	- (1)	- -	41 22	26 7	- (+)	- -
Average	4.7	4.2	2.2	3.4	19.1	29.7	31.3	33.9

The readings in brackets were from donors who were rejected (one for low Hb and the other for high ESR)
+ indicates that an early S.A. point prevented a P.A. (%) reading
The P.A. and S.A. results of a donor are in the corresponding place in the table
The results of those females who were pre-menopausal are underlined
Analysis by Wilcoxon Sum of Ranks Test

2. Are you taking any tablets, or medicines, or the birth (contraceptive) pill?

3. Have you taken any of the following tablets in the past week:-

   (a) Aspirin, aspro, codis, phenacetin, or other pain killing tablets.

   (b) Butazolidin, Tanderil, or other anti-rheumatic tablets.

Seven donors were rejected for minor illness, or aspirin ingestion, or contraception with hormone tablets.

All the donors who were approached volunteered to take part.

The following estimations were made:-

1. P.A. percent to 1µM ADP.

2. S.A. point dose ADPµM/L.

3. Plasma platelet count.

4. Hb., W.C.C., E.S.R. (1st hour Westergren).

   Those with Hb $< 75\%$ and/or E.S.R. > 20 mms. were excluded.

   On this basis two donors were excluded, one with an Hb. of 64% and one with an E.S.R. of 35 mms 1st hour.

The results of the survey of the remaining 74 donors are given below.

Results.

The full results for the 74 donors, arranged by sex and age by decade, are given in Table 8.1. For analysis the results were sub-grouped into young adults (21-40) and middle aged adults (41-60) and further sub-divided by sex. Each of these groupings contained approximately twenty donors.

1. Primary aggregation. The average value for P.A. was highest in the sub-group of middle aged females (33.9%), next were young females (31.3%), then middle aged males (29.7%) and, distinctly lower, were young males (19.1%). The young males showed a significantly lower

COMPARISON of P.A. with the S.A. POINT (ADP μM)
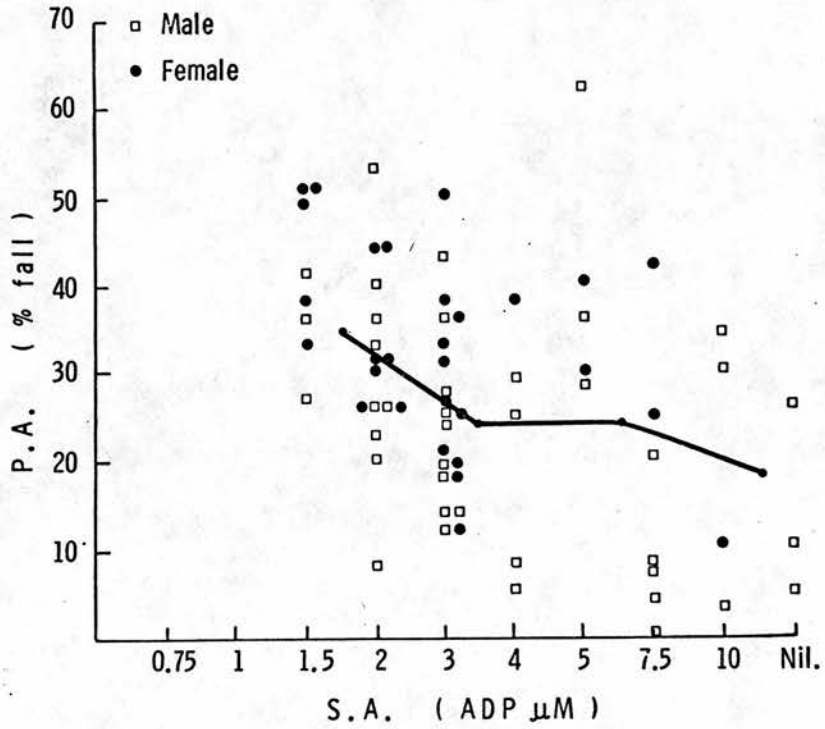
Fig. 8.1.

A comparison of P.A. (to 1μM ADP/L) with S.A. point

The P.A. results of the blood donors with an S.A. point of 1.5μM ADP plus are shown. The line joins the mean P.A. values for paired S.A. points (1.5 and 2μM; 3 and 4μM etc.)

□ male   ● female

SECONDARY AGGREGATION in BLOOD DONORS
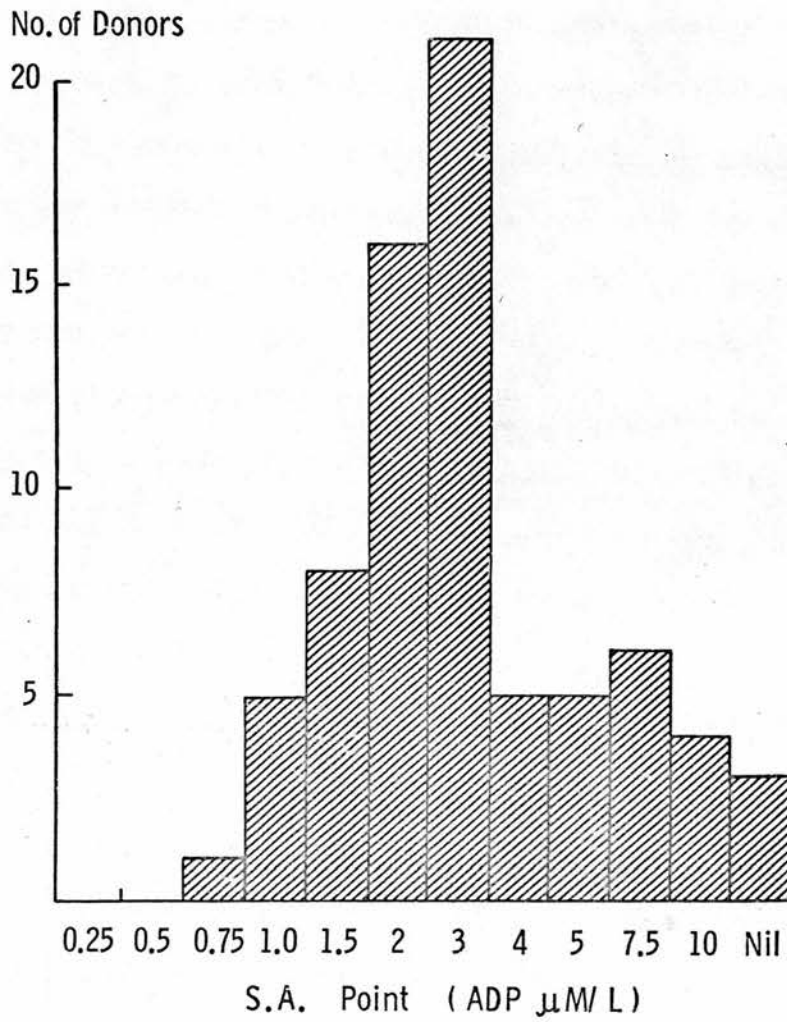
Fig 8.2.

Histogram of the S.A. point ADP concentration of 74 Blood donors

P.A. response than each of the other three groupings (middle aged males, both age groupings of females $p = <0.05$). There was no significant difference between the other three groups.

2. **Relationship of P.A. to S.A.** The pattern of the P.A./S.A. relationship in males and females was the same and so the results have been combined, Fig. 8.1. P.A. decreased as S.A. became less active, from 41% at 1.5μM ADP to 16% for P.A.-only. The pattern of this decrease was a rapid fall from 1.5μM to 3μM followed by a plateau which had an aberrant peak at 5μM. The differences in P.A. between pairs of S.A. points were analysed. The fall of P.A. percent from the S.A. points 1.5 and 2μM ADP to the S.A. points 3 and 4μM ADP was significant ($p = < 0.002$). The differences in P.A. percent between the 3μM + 4μM S.A. points and the other pairs of S.A. points were not significant.

This relationship between S.A. and P.A., which occurred when the S.A. point was near to the P.A. test dose, could be due to either process influencing the other. However, as the S.A. process is acting before the fully developed form used in the definition of the S.A. point (see Chapters Four, Five), it is more likely that S.A. is increasing the P.A. response than the reverse.

3. **Secondary Aggregation.**

(1) **Total results.** (fig. 8.2)

The S.A. points in the blood donors ranged from 0.75μM ADP (one) to Nil S.A. i.e. P.A.-only (three). The mode value was 3μM and the average was 3.8μM. The histogram shows a regular rise and prolonged tail indicating a slight skew to the left. The percentage of donors showing the different degrees of S.A. activity were:-

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BLOOD DONORS

Sex and Age comparison of S.A. point
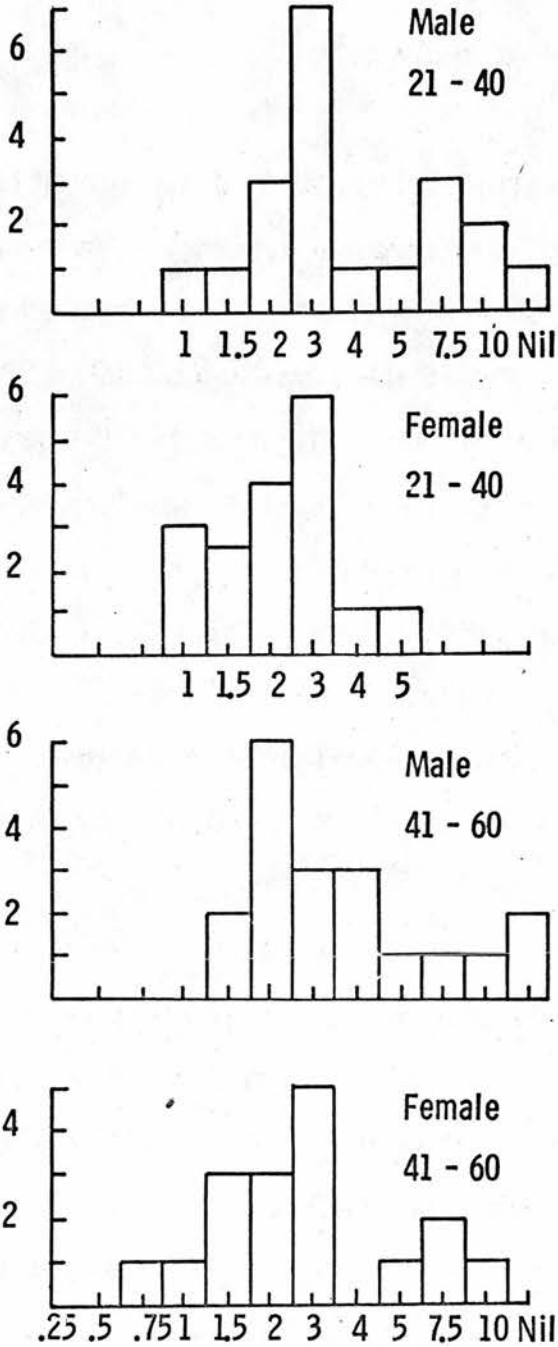
Fig. 8.3.

Histogram of the S.A. points (ADPµM) of 74 blood donors divided into age groups (21 to 40 yrs, and 41 to 60 yrs) and subdivided by sex.

(1) chain-reactive S.A. - 12 (16%).

(2) self-limiting S.A. - 59 (80%)
      non-phasic (73%)
      level (7%)

(3) P.A.-only - 3 (4%).

(2) Sex and age sub-groups.

The mean values for S.A. point (ADPμM/L) in these groups (Table 8.1)
show that the most active S.A. response occurred in young adult
females (2.2), next in middle aged females (3.4), then in middle
aged men (4.2) and least active were young adult men (4.7).

A histogram of these sub-groups (Fig. 8.3) showed that three of the
groups had a mode value of 3μM ADP and middle aged males had the
slightly more active mode value of 2μM ADP. The high average S.A.
activity in the young females is seen to be due to 'front loading'
of the values with the absence of a 'tail', rather than to higher
absolute values. In the remaining three groups a 'tail' was
present, and in F 41-60 and M 21-40 there was the suggestion of
a second 'hump' at 7.5 μM, i.e. of two populations.

Analysis of these results showed a significant difference ($p = < 0.05$)
between the young adult females and the young adult males groups and
a nearly significant ($p = 0.06$) difference between the young females
and the middle aged males. Other differences were not significant.

(3) Intra-group differences in females.

To see if the menstrual cycle was affecting the S.A. point, the
twenty-four menstruating women were analysed further, for differences
between the first half (haemorrhage effect? hormonal effect?), and the
second half (hormonal effect?) of the cycle. Only seven out of the
twenty-four were in the first half of their cycle, indicating self-
selection. The average S.A. points were, first half 3.3μM and

second half 2.8µM (N.S.).   So any intra-cycle differences was not
detectable in these small groups.   Next the twenty-four
pre-menopausal women were compared with the thirteen post-menopausal
women.   The average S.A. points were 2.9 and 3.1µM respectively (N.S.).

(4) Differences in Hb. (and citrate concentration).

The higher citrate concentration in plasma derived from whole blood
with a high Hb, could have influenced the calcium dependent platelet
aggregation in the male plasma.   The mean Hb (%) and mean S.A.
point (ADP) for the groups were;   young adult men, 98.8% and 4.7µM,
and women 88.8% and 2.2µM;   middle aged men, 96.1% and 4.2µM, and
women 84.4% and 3.2µM.   The highest values of Hb and S.A. point
were associated, but the lowest values were not.

Spearmans correlation test was applied to the values for Hb and S.A.
point ADP concentration.   For the total female group $z = 1.81$, and
for males plus females $z = 1.86$ ($p = <0.10>0.05$).   This suggests
that some association is present but that it is a weak one.

The histogram (Fig. 8.3.) does not suggest that the values for S.A.
point are moving because of a change in citrate concentration.   The
shape of the histogram varies in the different groups of age and sex
as opposed to being similar in shape but in different positions;
also the most active S.A. point and S.A. mode value are not in the
group with the most active mean S.A. point value (F21-40).   The main
difference between F21-40 and the other groups is absence of a 'tail' -
and a 'tail' is present in the group with the lowest mean Hb
concentration (F41-60).

SUMMARY OF CHAPTER EIGHT.

1. A survey was conducted of P.A. and S.A. in specimens from 74 healthy donors. The age group 21 to 60 years was studied and both sexes included. The donors were questioned about minor illnesses and drugs before being admitted to the survey.

2. P.A. appeared to be influenced by an active S.A. process (1.5, 2 μM ADP S.A. point), probably due to the early stages of the S.A. process. P.A. percent was lower in males aged 21-40 than in the other groups of blood donors.

3. The range of S.A. point was 0.75μM ADP to absent; the mode value was 3μM and the values were skewed slightly to the left. S.A. was chain-reactive in 16%, was self-limiting in 80%, and was absent in 4% of the donors.

4. Analysis of the results by age and sex showed S.A. to be most active in young females and least active in young males. The difference between these groups was significant ($p = < 0.05$). Menstruating women did not show a higher S.A. activity than post-menopausal women, and there was no difference between women who were examined in the first 14 days of the menstrual cycle compared with those examined at a later stage of the cycle. Differences in Hb level did not account for more than a small part of the difference.

CHAPTER NINE

ACUTE ILLNESS AND S.A.

INTRODUCTION.

Following acute illness humans become highly susceptible to venous thrombosis. The three components of this hyperthrombotic state are the vein wall, changes in blood flow, and changes in the blood. There is firm pathological evidence that the initial event in a venous thrombosis is a white (i.e. platelet rich) thrombus developing in the turbulent blood flowing around venous valves (see review Chapter One). Subsequent red (coagulation) thrombus develops downstream to the block caused by the white thrombus.

While white thrombus formation is the initiating event, it is the red thrombus which produces disease, either by blocking the vein and so preventing the return of venous blood to the heart (a deep vein thrombosis), or by becoming detached and passing to the lungs (a pulmonary embolus).

Deep vein thrombosis and it's sequelae are important in both medical (169) and surgical patients (106). Clinical signs are notoriously unreliable for detecting deep vein thrombosis, because many thrombi do not cause local symptoms, and also because the clinical features which suggest thrombosis are not always due to thrombosis (161). So venography and radio-active fibrinogen are used to detect subclinical venous thrombi, (venography correlates well with necropsy findings (103) and such a study is not yet available for radio-active fibrinogen). Reliable estimates of the incidence of venous thrombosis depend on results from these special investigations and on post-mortem studies

Table 9.1.   Previous work on platelet function during acute illness

	Control group	Medical illness 0-48 hrs	Medical illness 5-10 days	Surgical illness 0-48 hrs	Surgical illness 5-10 days	Postpartum
Platelet count	-	-	-	Fall(5,9)	Rise(all)	Rise(2,11)
Platelet stickiness		-	-	Fall(9)	No change(9,11)	Rise(2)
(1)Long contact time (rotating bulb)	-	-	-	No change (10)	Increase(2,10)	No change (11)
(2)Short contact (glass bead column)	-	-	-	Rise(10)	Fall to (10) initial increase(7)	-
Platelet aggregation	-	-	-	-	Increased(6)	-
Electrophoretic mobility	-	Increased (8)	Falling (8)	Increase (8)	Falling (8)	-

1. Dawborn, 1928   (35)
2. Wright, 1942   (186)
3. Pepper, 1960   (150)
4. Egeberg, 1962   (41)
5. Innes, 1964   (83)

6. Emmons, 1965   (42)
7. Bygdeman, 166   (24)
8. Hampton, 1966   (60)
9. Ham, 1967   (59)
10. Shaper, 1968   (164)

In post-mortem studies the incidence of deep vein thrombosis varies from about 20 to 60 percent depending on the patients age and the severity, site, and duration of the preceding illness (162, 103). In prospective studies on surgical patients an incidence of 40 percent was present in elderly trauma patients (venography, 47) and of 21 percent in routine surgical cases (radio-active fibrinogen, 22).

It can therefore be stated that deep venous thrombosis is a common occurence after acute illness. The incidence of small platelet-fibrin thrombi on venous valves is not known.

Previous work. Table 9.1.

The first study of platelet behaviour in vitro in acute illness was undertaken in 1942 by Payling Wright (186), using her rotating bulb method of measuring 'platelet stickiness'. She found a slow increase in platelet count after operations and also an increase in platelet stickiness. Since 1942 several investigations of post-operative and post-partum patients have been carried out using platelet stickiness tests and one investigation using platelet aggregation.

All investigations have found a post-operative rise in platelet count occurring after several days, and in one investigation a fall was detected during the first 48 hours (59). Platelet stickiness has been found unchanged by some workers and increased by others (see Table 9.1).

In the one investigation of platelet aggregation Emmons et al (42) studied, in eighteen post-operative patients, the total aggregation response (i.e. P.A. and S.A. not separated) at room temperature, to fixed doses of ADP and adrenaline. Samples were taken post-operatively, and at nine to ten days, and thirty plus days post-operatively. A significant increase in the mean aggregation percent

was present at the nine to ten days observation point, with a return towards pre-operative levels at thirty plus days. The authors noted that there was wide variation in the changes in individual patients and, also, in one woman examined from day to day. They were unable to correlate the aggregation increase with age, sex, and the magnitude of the change in platelet count, nor was aggregation greater after 'major' compared with 'minor' operations. The purpose of the present study was to separate P.A. and S.A. changes. The gaps in the Table 9.1. indicate the desirability of also studying a control group and acute medical illness, as well as the post-operative state, and this was done.

Plan of study.

1. **Pilot study.** Experiment Ten Chapter Seven investigated the changes in S.A. point through time in healthy subjects, but in illness this might differ so four patients were studied day to day for about two and a half weeks and less frequently for a further two weeks.

2. **Main study.** On the basis of the results of the pilot study the remainder of the patients were studied at admission (or pre-operatively) and at three further times of 3-4 days, 7-8 days and 10-12 days after admission or operation.

Observations and measurements.

(a) **Diagnostic and prognostic measurements** were recorded on the patients illness and it's severity. Especially useful was the E.S.R.

(b) **Therapeutic measures.** Many drugs are now known to affect S.A. (142, 143, 62), particularly aspirin. No patient under study received aspirin or related anti-inflammatory drugs in hospital. Other drugs given are noted in the results. Some acute admissions

may have received aspirin before admission, as only the later patients were questioned about aspirin ingestion because during the earlier stages of the survey the anti-S.A. action of this drug was not known.

(c) Laboratory measurements.

Platelet count (blood and plasma).

Hb., W.C.C., E.S.R.,

P.A. percent (1µM ADP).

S.A. point ADPµM.

The specimens of blood were removed on admission, and thereafter at 8-30 to 9-30 a.m. after breakfast.

The Patients.

(1) Pilot study. Four patients who were in bed for at least two weeks, one without an acute illness, one with a minor illness, and two with a major illness.

(2) Control group. Seven patients with chronic, but not acute, illness (hypertension and ischaemic heart disease) who were in hospital for investigations were kept in bed for seven days, only being allowed up for toilet purposes. Six ambulant volunteers were also studied because some patients in the study were mobilised early.

(3) Acute medical illness. Only patients who became acutely ill in the 48 hours before admission were studied. Within this restriction, as wide a group of conditions as was possible were included. There were twenty patients with conditions ranging from acute myocardial infarction and cerebrovascular accident to haematemesis and urinary infection, and both mild and severe illness.

(4) <u>Acute surgical illness</u>.   Both patients undergoing cold

surgery and emergency admissions (within 48 hours of onset of

illness) were studied.   Again several conditions and differing

degrees of illness were included.   There were twenty patients

in this group also.

DAY to DAY CHANGES in AGGREGATION in ACUTE ILLNESS
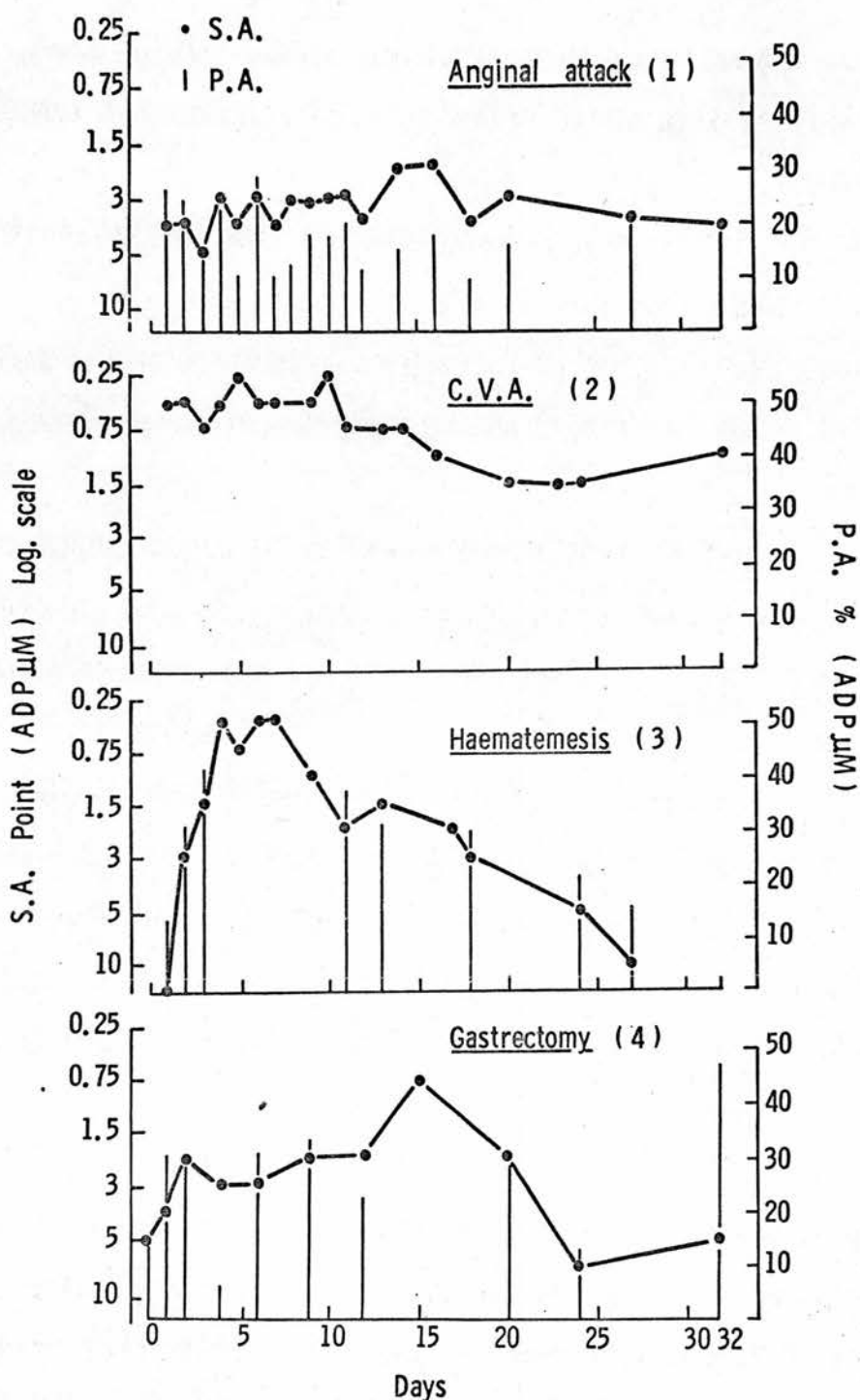
Fig. 9.1.

Graphs of P.A. and S.A. in four patients followed for three to four weeks.

⬤ = S.A.   Vertical lines = P.A.

THE PILOT STUDY

Four patients were studied in detail and the results are shown in
Fig. 9.1.

(1) This patient had 5 mins of severe cardiac pain at rest,
and special investigations failed to detect evidence of myocardial
infarction.

Nonspecific tests of tissue damage such as temperature, white cell
count and E.S.R. were normal.

S.A. remained steady throughout the observation period.   P.A.
tended to mirror the S.A. changes but with a wider variation than
for S.A.

(2) Patient two had a mild hemiplegia on the day of admission from
which he made a complete clinical recovery.   S.A. was abnormally
active on admission and remained so for 10 days before falling
slightly.   S.A. was too active for P.A. measurements to be made.

(3) Patient three had a two pint haematemesis from a duodenal
ulcer on the day of admission, and continued to bleed intermittently
for the next three days.   S.A. was absent on admission and rose
to abnormally active levels in four days.   It remained elevated
for four days and then slowly returned to the pre-admission level
over three weeks.

P.A. mirrored the S.A. changes.

(4) This patient had a partial gastrectomy for chronic duodenal
ulceration.   After operation S.A. activity increased quickly by
a small amount, then remained on a plateau for 10 days, finally
reaching a peak at fifteen days.   Over the next two weeks S.A.
activity returned to the patient's normal.   P.A. showed more
variation than S.A.   It tended to be higher when S.A. was active.

Comments on the pilot study.

(1) In the absence of acute illness (patient 1) S.A. activity showed a variation of three S.A. point dose levels. This degree of steadiness of response is within the limits found in normals (Chapter Seven, Experiment 10).

(2) In the two patients (Nos. 3 and 4) showing a rise in S.A. activity the day to day readings showed remarkably little variation about the mean trend.

(3) The peak S.A. activity was 4 to 7 days after the haematemesis, with a later peak after the partial gastrectomy. The high level of S.A. activity after the C.V.A. (patient 2) was maintained for 14 days.

(4) P.A. tended to follow S.A. In the patient with the greatest number of S.A. points of 1.5 and 2 μM (No.3) there was a positive correlation between the levels of S.A. and P.A. ($p = < 0.01$); in the others the correlation was not significant.

(5) It was concluded that variations in S.A. about a trend line day to day were small enough to allow less frequent readings to characterise a trend. This allowed a larger number of patients to be examined. In order to detect both the timing and height of any increase in S.A. activity, measurements were taken at 3-4 days, 7-8 days, and 10-12 days. This schedule seems likely to detect most of the changes in S.A. activity.

Table 9.2. Mean values of platelet counts (10^3/cu mm)

		n	Day 0-1	Day 3-4	Day 7-8	Day 10-12
Control (Bed rest only)	Blood	7	180	200	193	240*
	PRP		401	396	450	460
Medical	Blood	20	180	185	231*	210*
	PRP		375	372	398	458**
Surgical	Blood	20	219	239*	214*	278***
	PRP		377	371	402	482*

Analysis by Wilcoxon Signed Ranks Test

Table 9.3. Mean values for P.A. in acute illness

	PA percent 1µMADP			
Day	0,1	3,4	7,8	10-12
Acute Medical	22.8	23.1	22.2	24.2
Acute Surgical	23.5	23.6	22.7	21.1

RESULTS OF MAIN STUDY

Platelet count.

       The individual results are given in Table 9.10 (at end
of chapter).   There was wide variation from the initial value in
both directions.   The individual results were analysed by intra-
individual comparison and table 9.2 gives the mean values and the
results of the analysis.

       The blood platelet count rose in all groups to become
significantly elevated by 3-4 days in the surgical group, by 7-8 days
in the medical group and by 10-12 days in the control group.   The
PRP count remained steady in all groups at 3-4 days.   It then rose
and this change was significant by 10-12 days in the acute illness
groups.

Primary aggregation.

       The full results are given in detail in Table 9.11 at the
end of the chapter.   As the S.A. activity (see next section) rose
to $1\mu M$ and above in many patients, there were several occasions on
which P.A. could not be measured.   On the available results no
significant changes occurred (Table 9.3).

Secondary aggregation.

(1) Control group. Table 9.4.

       During the period of observation the seven patients in bed
and six ambulant normals showed no change or only a small one (rise
of two dose levels patients (3) and (4).   These changes were not
significant.

(2) Acute medical illness. Table 9.5.

       The group contained eight patients with myocardial infarctions,
four with a haematemesis, two with cerebrovascular accidents (C.V.A.)

Table 9.4 Changes in S.A. point and ESR in control group

Patient	Age	Sex	S.A. point (ADPμM/L)				ESRrange	Drugs
			Day 0,1	3,4	7,8	10to12		
1) Hypertension	46	M	10	7.5	10	7.5	4-19	Methyl dopa
2) "	50	M	1.5	1	2	1	7-15	Seconal,methyl dopa
3) "	54	M	4	5	2	2	1	CPIB
4) "	50	M	10	7.5	10	7.5	1	Bethanidine + D
5) Angina pectoris	53	M	3	4	4	3	5-6	Sod.amytal
6) Arteriosclerosis	54	F	1.5	2	2	1.5	3-9	Librium,sulph.
7) Hypertension	35	F	1	0.75	0.75	0.5	3-14	Chlopromazine
8) Normal	19	M	1.5	1.5	1.5	2	-	None
9) "	22	M	2	1.5	2	1.5	-	"
10) "	43	M	3	3	4	2	-	"
11) "	58	M	5	5	7.5	4	-	"
12) "	31	M	7.5	5	7.5	5	-	"
13) "	27	M	4	3	3	4	-	"

Drugs
D = chlrothiazide diuretic
Sulph = sulphadimidine

Table 9.5   Changes in S.A. point during Acute Medical Illness

	Patients	Age	Sex	S.A. point (ADPμM/L) Day 0,1	3,4	7,8	10-12	ESR range	Drugs and therapy
1	Haematemesis	55	M	Nil	3	0.75	1.5	21-57*	Blood (1pt); 1;B
2	"	44	M	Nil	0.5	0.5	2	18-33	Blood (4pt); M;I; Cycl;B.
3	M.I.	61	M	3	3	1.5	0.75	3-46	M;D;C.
4	C.V.A.	65	M	5	2	1.5	2	45-60*	Beth; MD; Chp; DF118
5	Biliary colic	50	F	Nil	4	2	4	-	M; Cycl; B.
6	M.I.	73	M	4	1.5	1	0.75	29-41	M; Dig; Chp; B.
7	Pericarditis	58	F	7.5	0.75	0.75	1	42-89*	-
8	M.I.	57	F	Nil	2	4	1.5	19-31	D; A; B.
9	'Allergic' purpura	66	F	5	7.5	2	2	46-79*	Pen; DF118
10	M.I.	65	M	0.75	0.75	1.5	1.5	21-32	M; D; A; Chpd.
11	C.V.A.	71?	M	0.5	0.5	0.5	0.75	24-56*	-
12	M.I.	61	M	7.5	4	1.5	1	-	M; Dig; D; Tri; B.
13	Haemoptysis	57	M	3	3	1	1	2-13	B.
14	M.I.	58	M	Nil	Nil	7.5	4	10	-
15	M.I.	56	M	Nil	2	2	2	15	M; D; Anticoag; B.
16	Haematemesis	50	M	7.5	3	1	1.5	2-18	B; I.
17	M.I.	61	M	2	1.5	0.75	0.5	21-110*	M; Cycl; D; Anticoag; Mx.
18	Renal colic	54	M	3	2	1.5	1.5	-	M; Atrop; Cycl; Prop; B.
19	Urinary infection	65	M	Nil	2	2	1	7-10	Atrop; DF118; Mx.
20	Haematemesis	67	M	3	1	1	2	7-26	Lib; Mog.
	Mean(at Nil = 10)			6.1	2.7	1.7	1.6		

M.I. = myocardial infarction   C.V.A. = cerebro-vascular accident   * = 'major' illness

Analysis by Wilcoxon Signed Ranks Test.   The peak activity is underlined

Abreviations

A= aminophilline
Anticoag= dividevan or warfarin
Atrop= atropine
B= Barbiturate
Beth= Bethanidine
C= chloral
Chp= chlorpromazine
Chpd= chlorpropamide
D= chlorothiazide duretics
Dig= digoxin
I= iron
M= morphine , pethadine
MD= methyl dopa
Mog= mogadon
Mx= mandrax
Pen= penicillin
Prop= propantheline

and one each with six other conditions ranging from urinary infection and pericarditis to allergic purpura. The drugs administered during hospitalisation are noted in the table.

The initial level of S.A. activity was low, as eight out of twenty patients had no S.A. activity. This low level was near to significantly lower ($p < 0.01 > 0.05$) than the level in age and sex matched blood donors taken from the normal population described in Chapter Eight. In one patient (No.11) the initial S.A. point dose was above the normal range at 0.5 µM ADP, and another (No.10) was at the highest normal level (of 0.75 µM ADP).

A rise in S.A. activity occurred in all the other eighteen patients. The S.A. activity rose above the initial level in fifteen of the eighteen by 3 to 4 days and in all eighteen by 7-8 days. The peak value for S.A. activity was reached by four patients in 3-4 days, by seven in 7-8 days and in the remaining seven at 10-12 days. At the peak values 80 percent of the patients were showing chain-reactive S.A. (compared with 16 percent of the blood donor population). Analysis of these results will be given with those of the surgical group.

(3) Acute surgical illness. Table 9.6.

Eight patients in this group were undergoing cold surgery (three a cholecystectomy, two a partial gastrectomy, two a hernia repair, and one a prostatectomy). The acute surgery group consisted of one patient with perforation of a duodenal ulcer, seven with acute appendicitis, and four with Collés fracture.

Post-operatively the S.A. activity rose in seventeen of the twenty patients; in ten by 3-4 days, thirteen by 7-8 days and the full

Table 9.6   Changes in S.A. point after Acute Surgical Illness

Patient		Age	Sex	S.A. point ADPμM/L				ESR range
				Day 0,1	3,4	7,8	10-12	
1	Cholecystectomy	70	M	2	1.5	1.5	0.5	-
2	Gastrectomy	52	M	5	1.5	2	1.5	10-51[*]
3	Cholecystectomy	57	F	1.5	2	0.75	0.5	3-61[*]
4	Hernia	59	M	Nil	3	3	3	5-30
5	Cholecystectomy	67	F	1.5	1.5	1	0.75	30-62[*]
6	Hernia	44	F	3	1	4	0.5	3-46
7	Prostatectomy	64	M	1.5	1.5	0.75	0.5	15-60[*]
8	Gastrectomy	44	M	10	2	3	2	12-22
9	Perforated D.U.	40	M	2	1.5	2	0.75	-50[*]
10	Appendicectomy	31	F	3	1.5	0.75	1.5	-
11	"	53	M	2	2	2	2	35
12	"	26	F	Nil	Nil	3	2	14-21
13	"	56	M	Nil	1.5	2	DVT	-99[*]
14	"	44	F	4	7.5	2	2	-53[*]
15	"	43	F	Nil	2	1	1	10-30
16	"	53	F	4	3	1.5	3	-22
17	Collés fracture	69	F	5	-	0.75	0.75	7-14
18	"	56	F	3	-	10	3	6-10
19	"	52	F	3	-	3	3	19-32
20	"	62	F	3	-	0.5	0.25	15-10
Mean (at Nil = 10)				4.8	-	2.2	1.5	

The peak value is underlined   *='major' illness

Analysis by Wilcoxon   Signed Ranks Test

seventeen by 10-12 days.   In three patients, one after an appendicectomy and two after Collés fracture, no rise occurred.   The peak values were reached by four in 3-4 days, by five at 7-8 days and the remaining eight at 10-12 days.

The increase affected some patients with all the conditions examined and this included two of the patients with the relatively minor incident of a Collés fracture.

Table 9.7. Analysis of the increase in S.A. following acute medical and surgical illness

Day	0,1	3,4	7,8	10-12
Acute Medical incident:-				
(1) Initial result (Day 0,1)	–	***	****	****
(2) Day 3,4	–	–	*	*
(3) Day 7,8	–	–	–	N.S.
(4) Matched blood donors	N.S.	*	****	****
Acute Surgical incident:-				
(1) Initial result (Day 0,1)	–	N.S.	**	****
(2) Day 3,4	–	–	N.S.	**
(3) Day 7,8	–	–	–	*
(4) Matched blood donors	N.S.	N.S.	*	***

Intragroup analysis by Wilcoxon Signed Ranks Test
Intergroup analysis by Wilcoxon Sum of Ranks Test

$$* = p < 0.05$$
$$** = p < 0.01$$
$$*** = p < 0.002$$
$$**** = p < 0.001$$

ANALYSIS OF S.A. CHANGES FOLLOWING ACUTE ILLNESS.

(1) Changes in S.A. activity in medical and surgical acute illness.

The changes in S.A. were analysed by intra-individual analysis, and were also compared by group with an age and sex matched sample of the blood donors.

In both the medical and surgical groups there was a highly or very highly significant rise in S.A. activity. This significance was achieved by 3-4 days ($p = < 0.002$) in the medical group which also showed a significant ($p = < 0.05$) further rise between 3 to 4 days and the later times. In the surgical group the rise occurred more slowly and only became significant at 7 to 8 days. It reached a peak of significance at 10-12 days. The 10-12 day values were significantly ($p = < 0.05$) higher than the 7-8 days values.

The comparisons with age and sex matched donors produced the same pattern of significance, although because the donors had higher baseline S.A. point values, some of the levels of significance were lower (Table 8.7). This comparison with normals shows that the rise in S.A. activity after acute illness was an absolute one.

The peak S.A. point values occurring during acute illness and those of the blood donor control group are shown in Table 9.8. They are arranged into No-S.A. plus self-limiting S.A., and chain-reactive S.A. (Table 9.8 (1)). The chain-reactive S.A. was then sub-divided into two levels of chain-reaction potential (Table 9.8, (2)).

The acute illness patients had 29 (72%) of peak values showing chain- reactive S.A. as against 6 (15%) for blood donors, a very highly significant increase ($p = < 0.001$). Within the chain-reactive group itself, the values reached by the patients were significantly higher than the levels of chain-reactive S.A. shown by the donors ($p = < 0.05$).

Table 9.8.

(1) Comparison of Chain-reactive S.A. in donors and peak level during acute illness

	No S.A. and Self-limiting S.A.	Chain-reactive S.A.
Donors	34	6
Acute illness peak level	11	29***

50% probability test (1713)

(2) Comparison of degree of chain-reactive S.A.

	Chain reactive S.A.	
	1.5 and 1.0μMADP	0.75 and 0.5μMADP
Donors	6	0
Acute illness peak level	11	12*

Binomial Test (1713)

This absolute increase in S.A. will be associated with a very marked increase in the aggregation chain-reaction potential (the very large increase$_\wedge^s$ in potential, inherent in it's geometric progression, for small changes in the chain-reactive S.A. point ADP concentration, were described in Chapter Five).

(2) <u>Effect of platelet count changes on the rise in S.A. activity.</u>

The mean rise in plasma platelet count of about 85th in the medical group and about 100th in the surgical group would only elevate the S.A. point by one to two levels (see Chapter Seven, Experiment 8). Also the peak values of S.A. point in the medical group occurred earlier than the peak values of platelet count (coincident in three, divergent in fourteen). The peak values for S.A. occurred later in the surgical than in the medical group, but even so only coincided with the highest platelet count in seven patients, being divergent in eight patients. Thus the increase in S.A. activity was not directly relatable to the rise in platelet count. When the S.A. activity results were adjusted to a constant platelet count the pattern of change was not altered and the significance levels were little affected.

(3) <u>Effect of the severity of the acute illness.</u>

Estimates of the severity of the illness, based on the clinical course and investigations, correlated well with the peak ESR value. The illnesses in which the peak ESR was $<$ 50 (twenty-three) were compared with those with a peak ESR of 50 plus (thirteen). The patients constituting the severer illness group were two with haematemeses, the viral pericarditis and allergic purpura patients, two patients with C.V.A's., two who had cholecystectomies, two post-appendicectomy patients, one after gastrectomy and the man who had a prostatectomy.

The peak S.A. activity in the thirteen patients who had the severer illness was higher than that in the twenty-three patients with

Table 9.9. Comparison by age of peak values of S.A. point

Age	Peak S.A. point (µMADP/L)								Total	Major Illnesses
	0.25	0.5	0.75	1	1.5	2	3	4		
61+	1	4	6	3	1	1	-	-	16	6
51-60	-	1	2	1	5	3	2	1	15	5
41-50	-	2	-	2	-	3	-	-	7	1
31-40	-	-	2	-	-	-	-	-	2	1
Total	1	7	10	6	6	7	2	1	40	13

a less severe illness $(p = <0.02)$.

The rises in S.A. activity in the twenty-three patients with the less severe illness were analysed alone. The rise in S.A. activity in this less severely ill group was still significant $(p = <0.01)$.

(4) Age and Sex.

The peak values for the ages by decades are shown in Table 9.9. The over 60 age group (sixteen) had peak values which were highly significantly $(p = <0.002)$ greater than those in the 51-60 age group (fifteen). The number of severe illnesses were comparable in the two groups (six for age 61+ and five for ages 51-60).

(5) Drugs.

Many anti-inflammatory agents markedly depress S.A. and none of these were given to the patients in this survey. Self-medication before admission cannot be excluded and was suspected in the medical group. Some other drugs have a smaller effect on platelets, but usually only in high concentration. These include drugs used for heart disease (62). No suppressive effect was apparent from digoxin (Medical patients No.6 and 12). Nor did any other of the drugs show a consistent inhibitory or enhancing action on the S.A. point.

Interestingly the two patients on oral anticoagulants (medical patients Nos.15 and 17) showed an increase in S.A. activity comparable to that in other patients with a similar severity of illness.

(6) Nature of illness.

Only myocardial infarction patients (eight) and appendicectomy patients (seven) were sufficiently numerous for comparisons. Within each of these two groups there was a wide variation in the initial level, the degree of increase, and the peak value of the S.A. point. Severity of illness appeared to be most important determining factor of this variation.

SUMMARY OF CHAPTER NINE

1. The changes in S.A. activity occurring in the two weeks following
    the onset of the acute medical (20 patients) and surgical illness
    (20 patients) have been studied.

2. In a control group of seven non-acutely ill patients in bed, and
    of six ambulant normals, there was no significant change in S.A.
    activity or P.A. percent.

3. After acute medical and surgical illness the S.A. activity rose
    to a highly significant degree.   This rise occurred earlier in
    the medical group.   P.A. did not change significantly.

4. Chain-reactive S.A. was very highly significantly more prevalent
    in the acute illness group than in a normal group (blood donors).
    Also the _level_ of chain-reactive S.A. was higher in the acute
    illness group than in the normals, which means an even greater
    elevation of the aggregation chain-reaction potential (see
    Chapter Five).

5. The increase in S.A. activity was only slightly due to the rise
    in platelet count.   Thus the major part of the change in S.A.
    was due to changes in the platelets or the plasma.

6. The rise in S.A. activity was significantly greater in those
    patients with severer illnesses, and in those patients aged over
    sixty years.   The rise did not correlate with the patient's type
    of illness or with their sex, and no drug effects were detected
    after admission.

7. Thus the 'hyperthrombotic' state which follows acute illness was
    associated with a marked increase in S.A. activity, and in the
    majority of patients (73%) chain-reactive S.A. was present.

	Day	BLOOD 0,1	3,4	7,8	10 to 12	Day 0,1	PLASMA 3,4	7,8	10 to 12
Control Patients	(1)	174	184	181	211	345	420	420	436
	(2)	162	184	150	170	302	418	218	210
	(3)	188	172	179	191	286	296	364	392
	(4)	136	143	186	284	285	302	375	568
	(5)	165	170	244	262	542	472	444	424
	(6)	295	300	270	340	615	445	520	660
	(7)	160	245	140	225	415	490	480	525
	Mean	180	200	193	240	401	396	460	460
Acute Medical Illness	(1)	217	230	325	325	421	286	610	610
	(2)	240	303	377	301	371	502	570	719
	(3)	152	195	260	235	344	293	235	320
	(4)	124	103	175	130	-	-	-	-
	(5)	175	104	133	86	-	-	-	-
	(6)	166	162	172	137	380	421	330	428
	(7)	275	200	216	212	-	-	-	-
	(8)	165	242	235	264	421	-	480	435
	(9)	113	116	384	256	420	335	617	566
	(10)	164	185	193	219	312	295	415	432
	(11)	152	157	251	242	385	330	504	614
	(12)	210	220	165	279	620	580	469	490
	(13)	110	88	94	120	179	161	121	163
	(14)	224	247	200	222	249	413	460	510
	(15)	260	113	334	324	467	367	429	467
	(16)	140	220	400	324	467	367	429	467
	(17)	215	230	220	340	540	490	390	680
	(18)	225	175	220	230	416	396	-	400
	(19)	295	168	110	187	361	318	318	337
	(20)	140	210	195	165	310	395	465	460
	Mean	180	185	231	210	375	372	398	458

Table 9.10 (contd)

Acute Surgical Illness		BLOOD				PLASMA			
	Day	0,1 -	3,4 -	7,8 -	10 to 12	Day 0,1 -	3,4 -	7,8 -	10 to 12
	(1)	130	137	123	84	286	282	300	-
	(2)	222	153	151	320	473	325	219	711
	(3)	92	85	207	204	160	187	280	452
	(4)	125	234	275	194	458	531	539	436
	(5)	145	140	250	220	375	300	450	315
	(6)	150	199	340	280	410	255	460	360
	(7)	140	134	220	335	365	221	435	450
	(8)	148	154	206	418	301	233	631	611
	(9)	207	-	-	282	267	-	-	349
	(10)	201	250	230	252	595	472	450	552
	(11)	188	235	212	179	692	417	426	367
	(12)	267	235	182	D.V.T.	396	467	405	D.V.T.
	(13)	186	300	193	314	359	728	403	564
	(14)	206	220	247	242	337	435	585	539
	(15)	126	193	247	312	327	391	571	487
	(16)	206	247	171	257	345	322	280	568
	(17)	94	177	227	241	326	-	395	418
	(18)	200	-	231	-	327	-	360	-
	(19)	136	-	162	190	217	-	238	241
	(20)	149	-	178	165	328	-	243	313
	Mean	219	239	214	278	377	371	402	482

Table 9.11. The degree of P.A. percent during Acute Illness

Patient Number	MEDICAL P.A. percent 1μMADP				SURGICAL P.A. percent 1μMADP			
	Day 0,1	3,4	7,8	10-12	Day 0,1	3,4	7,8	10-12
(1)	-	22	=	18	31	26	9	=
(2)	18	=	=	23	-	36	10	18
(3)	26	12	32	=	22	14	=	=
(4)	0	14	26	15	9	23	24	26
(5)	8	25	25	16	45	35	=	=
(6)	33	24	=	=	34	=	52	=
(7)	44	=	=	=	37	53	=	=
(8)	17	25	13	20	16	30	5	31
(9)	38	38	36	18	17	22	=	16
(10)	=	=	16	25	15	19	4	14
(11)	=	=	=	=	22	21	14	11
(12)	28	38	17	30	28	17	18	D.V.T.
(13)	27	17	=	=	0	3	19	7
(14)	10	0	4	16	23	8	=	=
(15)	17	12	15	20	21	15	30	25
(16)	8	28	=	35	32	32	42	=
(17)	31	35	=	=	48	-	-	-
(18)	39	19	38	54	18	-	29	20
(19)	10	37	22	=	58	-	50	41
(20)	57	=	=	24	60	-	=	=
Mean	22.8	23.1	22.2	24.2	23.5	23.6	22.7	21.1

The patient numbers correspond to those in other tables. The mean values of surgical cases exclude the cases of Colles fracture (17-20)

= indicates S.A. too active for a P.A. measurement
- indicates no measurement made

CHAPTER TEN

SECONDARY AGGREGATION IN ANIMAL PLASMA.

INTRODUCTION.

The detection of a powerful aggregation chain-reaction process
in man makes one wonder if this could be playing a part atherosclerotic
disease in man, especially as pathologists are continually reminding us
that this is a thrombo-atherosclerosis (29, 30, 51, 122).   Since the
main initial component of the thrombus on an atherosclerotic plaque is
a platelet mass (51, 122, 151), could it be that S.A., particularly
chain-reactive S.A., allows thrombi to form more easily in man than in
animals?   It has proved difficult to induce marked fibrotic (as opposed
to fat laden) lesions in animals (Constantinides 28, Hartroft et al 70)
and even more difficult to induce the thrombotic element.   Constantinides
(28) was only able to produce thrombosis in rabbits when, in addition to
hyperlipidaemia, he induced both hypertension and hypercoagulability
(Russells Viper Venom).   Even then only a limited thrombosis occurred.

Therefore it was considered worthwhile to investigate
different species of animals for S.A. activity.   Within the limits of
availability species were selected for two reasons - first, similarity
to man i.e. other primates - and second, that they were commonly used
in the laboratory in investigations of atherosclerotic disease.   Previous
work had already shown that platelet aggregation differs in different
species.   Cat platelets aggregate more vigorously than those of man (15)
and it has recently been shown that the tracing is biphasic (Macmillan and
Sim 110, Tschopp 179);   some guinea-pig platelets give a biphasic tracing
to ADP (27);   and rat platelets possess a very strong ability to
disaggregate (32, 170).   Comparative studies of total aggregation to
one ADP dose have been reported on a group of mammals (Sinakos and Caen

Table 10.1 Details of animals and bleeding procedure

Animal	Variety	Anasethetic	Withdrawal site	No. animals used for 20ml blood sample
Human	Caucasian	None	Ante-cubital fossa	1
Baboon	(1) Papio cynecephalus (2) Papio anubis	Phencyclidine Hydrochloride	Femoral vein or artery	1
Cats	Random bred	None	Cardiac puncture	1
Squirrel monkey	-	Phencyclidine Hydrochloride	Cardiac puncture	1
Rabbits	New Zealand white	None	"	1
Guinea pigs	(1) Albino (2) Rosette (3) Piebald	None	"	2
Pigs	-	Slaughtered	Carotid Artery	1
Rats	Albino	100% N_2	Cardiac puncture	4
Dog	Beagle	None	Ante-brachial cephalic vein	1

171) and on different sub-varieties of baboon (Hawkey and Symons 71).

No one has yet surveyed a group of mammals for S.A. and in particular chain-reactive S.A.

Materials and Methods.

Table 10.1 shows the species studied, and details of specimen collection (anaesthetic, if any; puncture site; and number of animals per plasma specimen, which was usually one).

The sensitivity of S.A. detection was increased by reducing the citrate concentration to 0.19%, except in rabbit blood where 0.38% was required to prevent coagulation. Specimens were maintained at about $37^{\circ}C$ during transfer to the laboratory. The centrifugation was at 140 to 310g and was continued for 4-10 mins, depending on the species, so as to produce the best combination of plasma yield and platelet count. The platelet count varied from $300-1,000 \times 10^{3}$ per cu.mm.

Storage and testing were performed at $37^{\circ}C$ as for human plasma.

Plan of study.

Each plasma was tested with ADP, and a dose response curve using concentrations up to 100 μM ADP was performed. The S.A. point concentration of any S.A. activity present was determined and some of the plasma showing S.A. were tested for a release of ADP-like activity. Each plasma was also tested with 100μM adrenaline, and if a second phase of aggregation (i.e. S.A.) occurred the S.A. point concentration for adrenaline was determined.
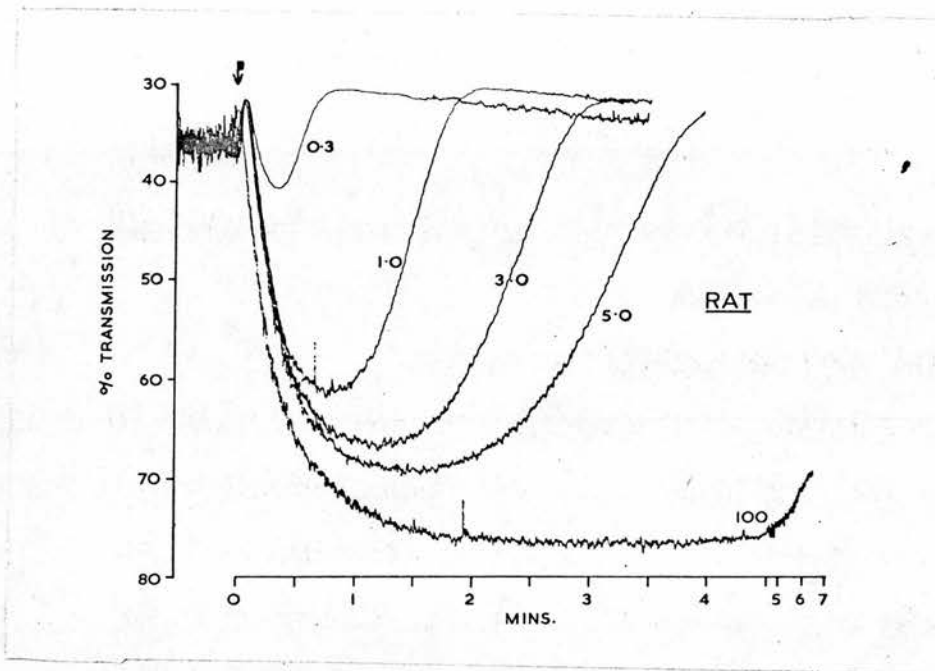
Fig. 10.1.

Rat plasma.   Dose response curve to ADP. Only P.A.

Aggregation increased as the ADP was increased, up to 100μM,
and was followed by a very marked disaggregation.   A P.A.-
only tracing.
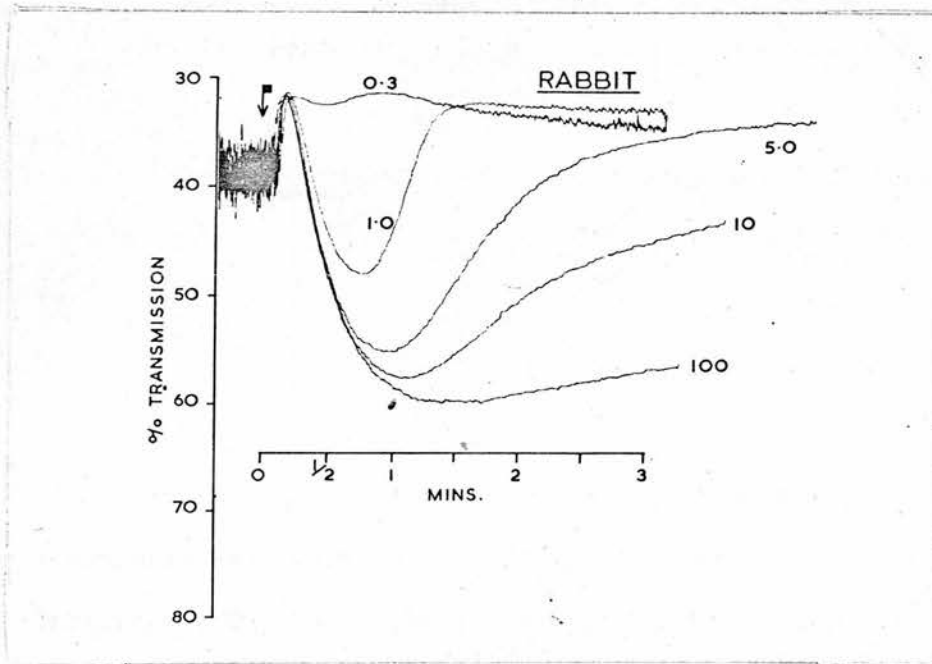
Fig. 10.2.

Rabbit plasma.   Dose response curve to ADP.   Only P.A.

. Aggregation increased as the ADP concentration increased up to
100μM.   Disaggregation was marked but less than in the rat
plasma.   A P.A.-only tracing.

RESULTS

1. Dose response curves showing S.A. and P.A.-only tracings are shown in Figs. 19.1 to 10.8.

   The findings are explained in the legends.

2. No S.A. response to ADP or adrenaline was detected in the following:-

   (1) Papio anubis (baboon)   15 experiments (n = 15)
   (2) Squirrel monkey   3 experiments (n = 3)
   (3) Rabbit   8 experiments (n = 8)
   (4) Rat   6 experiments (n = 24)
   (5) Pig   6 experiments (n = 6)

   In plasma showing only P.A., doses of 10μM ADP (and above) were followed by disaggregation. The extent of the P.A. response was highest in baboons and rabbits, with humans a little less (all with 0.38% citrate, five experiments).

3. S.A. was detected in some or all of the animals studied in the following species:-

   (1) Humans (see previous chapters).
   (2) Papio cynecephalus (baboons).
   (3) Cats.
   (4) Guinea pigs - rosette, piebald and albino.
   (5) Dogs.

   The form of the S.A. tracings in the species showing that response was biphasic in the cats, rosette and piebald guinea-pigs, and the dog (in which it developed very slowly), and was non-phasic in baboons and albino guinea-pigs.

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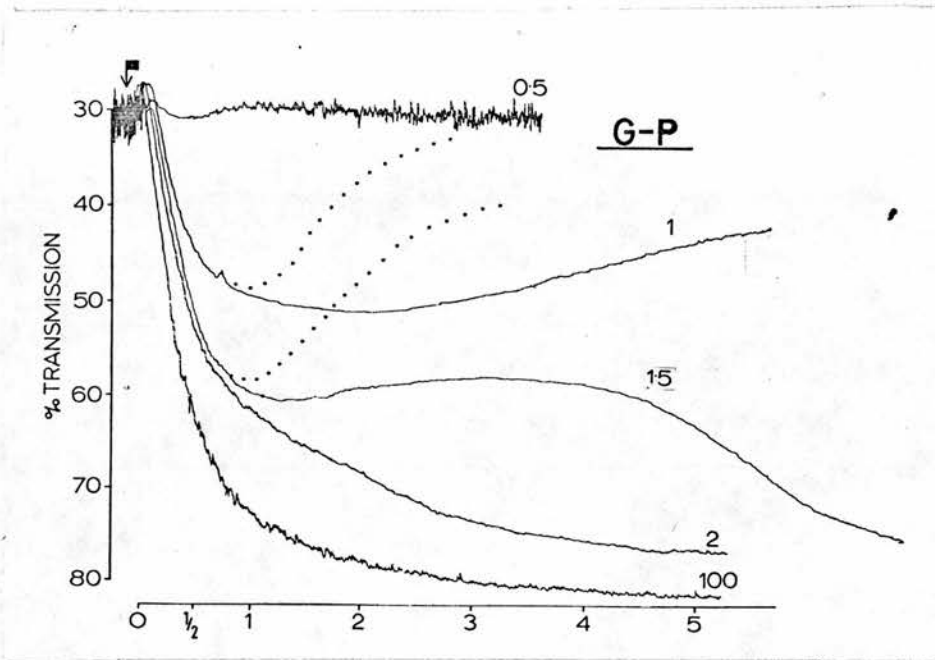
Fig. 10. 3.

Albino guinea-pig. Dose response curve to ADP. Non-phasic S.A.

0.3μM ADP produced a P.A. response with marked disaggregation.
1.0μM ADP was followed by marked aggregation with impaired
disaggregation and 2.0μM ADP by a marked and continuing
aggregation, which was only a little increased by 10μM ADP.
A non-phasic S.A. tracing
(.... = response expected from only P.A.)
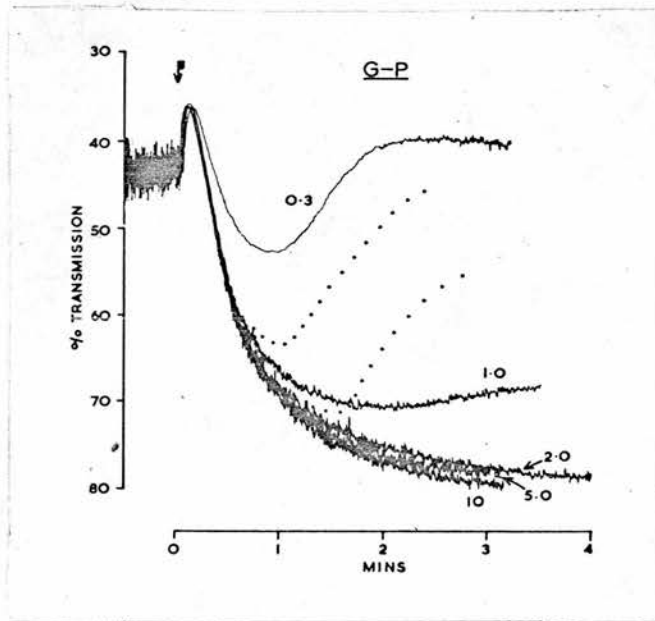
Fig. 10.4.

Rosette guinea-pig. Dose response curve to ADP. Biphasic S.A.

0.5μM ADP produced only a small aggregation and there was a
rapid return of the disk shape. The 1.0μM dose produced a
moderate aggregation followed by impaired disaggregation, and
after 1.5μM ADP a biphaisic aggregation response occurred which
was finally nearly as extensive as that produced by 100μM ADP.
A biphasic S.A. tracing
(.... = expected P.A. response)
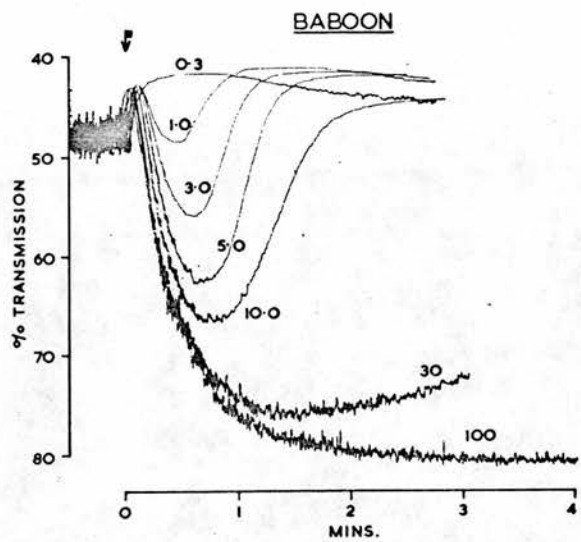
Fig. 10.5.

Baboon (Papio cynecaphalus) plasma. Dose response curve to ADP.

Only P.A.

This plasma showed a P.A. response, with disaggregation still occurring after 30µM ADP addition. A P.A.-only tracing.
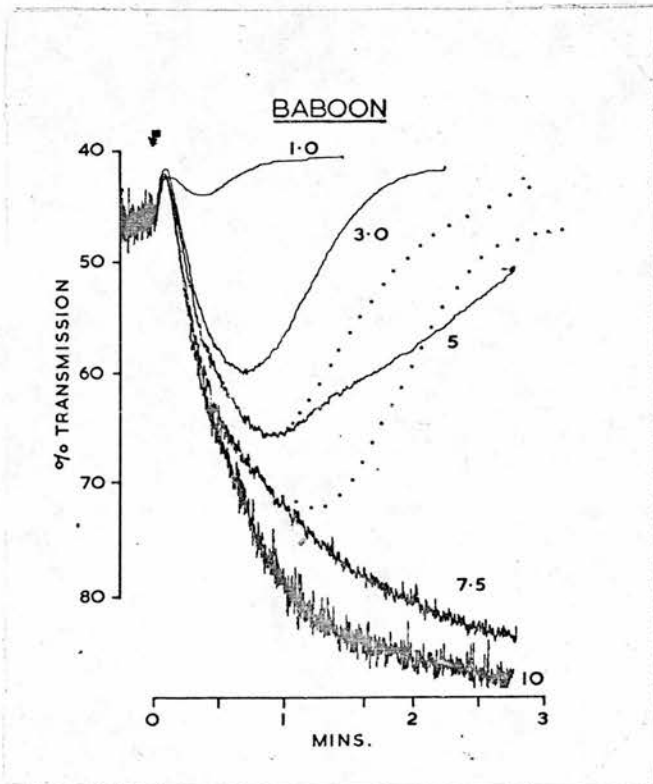
Fig. 10.6.

Baboon (Papio cynecephalus) plasma. Dose response curve to ADP
Non-phasic S.A.

The P.A. response occurred until the impaired disaggregation
following the 5µM addition. The 7.5µM dose was followed by a
fusion of P.A. into S.A. and produced aggregation almost as marked
as that occurring to the 10µM dose. A Non-phasic S.A. tracing
(.... = response expected from only P.A.)
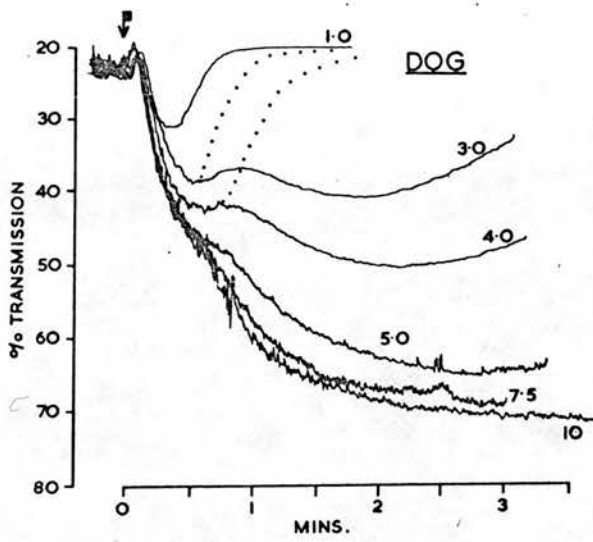
Fig. 10.7.

Dog plasma. Dose-response curve to ADP. Biphasic S.A.

A P.A. response occurred to 1.0µM ADP. After the 3.0µM dose a small
secondary aggregation occurred which was followed by partial disaggregation.
The S.A. became more marked as the ADP dose was increased to 5.0µM, when
it was almost as extensive as that ocurring with 10µM ADP. Biphasic S.A.
present. (....= response expected from only P.A.)
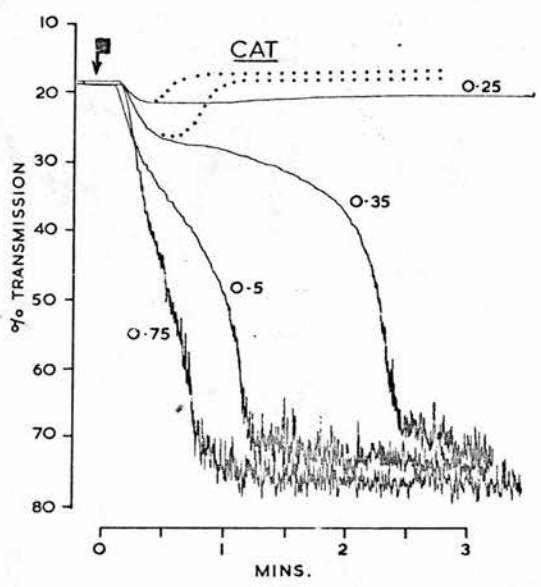
Fig. 10.8.

Cat plasma. Dose-response curve to ADP. Biphasic S.A.

0.25µM ADP produced a small aggregation and there was slight disaggregation.
The 0.35µM dose produced an initial small aggregation which was followed
by a very marked S.A. response, and after 0.75µM ADP the onset of S.A. was
more rapid. Biphasic S.A. present.
(.... = response expected from only P.A.)

Table 10.2. Secondary Aggregation characteristics in some animals

	Humans[1]	Papio cyneceph[2]	Cats[2]	Albino guinea-pigs[2]	Rosette guinea-pig[2]	Piebald guinea-pig[2]	Dog[2]
No. experiments	25	34	6	8	6	8	6
S.A. to ADP							
(1) number (%)	23 (92%)	30 (88%)	6 (100%)	8 (100%)	4 (67%)	5 (62.5%)	3 (50%)
(2) S.A. point (µM/L)	0.25-10	0.015-5	0.25-0.75	0.5-2	0.25-5	0.25-1.5	5-15
S.A. to Adrenaline							
(1) number (%)	23 (92%)	26 (76%)	6 (100%)	Absent	3 (60%)	Not done	1 (17%)
(2) S.A. point (µM/L)	0.005-100	0.002-100	0.01-1	-	0.7-10	-	100

1 = 0.38% citrate

2 = 0.19% citrate

4. The full results for the species showing S.A. are given in
Table 10.2.

The results for man are from the preceding chapters, and it
will be remembered that the citrate concentration in these was 0.38%
rather than the 0.19% present in the animal plasma. (Experiment 3
Chapter Seven showed that the S.A. point ADP concentration in plasma
from man decreased by about three S.A. point dose levels when the
citrate concentration was reduced from 0.38% to 0.19%).

The baboons were a selected group. Experiments soon showed
that S.A. was more frequent and more vigorous in adult females (as in
man, Chapter Eight) and there was a preponderance of these in the
group. Other animal groups were not pre-selected for their S.A.
response but numbers are too small for percentage frequencies to
act as more than a rough guide.

The percentage showing S.A. was high in man, baboons, cats (all)
and albino guinea-pigs; and 50 to 70% of rosette and piebald guinea-
pigs and dogs showed S.A. The S.A. point ADP dose possess two
characteristics, level and range. Low S.A. point dose levels
combined with a small range (i.e. all were low), were present in cats
and the rosette and albino guinea-pigs. While the S.A. dose level
was low in some baboons and humans the range of dose level was wide.
Dogs had a high S.A. point ADP dose. The S.A. response of adrenaline
aggregation (O'Briens' second phase) occurred only in those animal
plasma which had shown S.A. to added ADP, but several ADP-S.A.-
responsive plasma did not show S.A. to adrenaline 100μM (Table 10.2),
and that of albino guinea-pigs never did so. These results differ
from those in man where there was a complete correlation between S.A.
to ADP and to adrenaline. The only animal plasma which showed a
complete correlation was the cat plasma, in which very low adrenaline
concentrations initiated the S.A. reaction.

Table 10.3. The aggregation chain reaction potential of S.A. to ADP in animals.

	Man[1]	Cat[2]	Guinea-Pig[2]	Baboon[2]
n	19	4	7	9
S.A. point (ADPµM/L)	0.25-10	0.25-0.75	0.25-1	0.015 to 5
ADP-like release (µM/L)	12-0.4	2.25-1.2	0.2-2	0.4 to 0
Aggregation chain reaction potential	37 to 0.4	9 to 3.4	0.8 to 0	0.25 to 0
2nd Cycle	1370 to 0.2	80-10	0.6	0.06
3rd Cycle	1,000,000 to 0.04	6,400-100	0.4	-

1.= 0.38% citrate

2.= 0.19% citrate

5. The ADP-like activity present after S.A. was measured in a small
    group of the S.A. responsive animals (Table 10.3).   A definite
    ADP-like aggregating activity release was detected in only the cat.
    In the baboon and guinea-pig there was little or no excess activity
    over the ADP addition.   The total aggregating activity present after
    S.A. was measured in four baboon plasma to see if some aggregating
    agent other than ADP was being released.   None was detected.

    The low or absent release of ADP in baboon and guinea pig
    plasma was not sufficient to initiate an aggregation chain-reaction
    (Table 10.3), and so the S.A. in these animals was self-limiting.
    In contrast, a marked chain-reaction potential was present in all
    the cats tested, i.e. S.A. was chain-reactive.   Chain-reactive S.A.
    was more prevalent in the cat than in the human plasma but it must
    be remembered that the citrate concentration was lower in cat plasma
    (0.19%) than in human plasma (0.38%), and that the concentration of
    ADP-like activity released by platelets from man was higher than
    that for the cat (see Chapter Five).

6. Two intra-variety differences in the presence of S.A. were detected.
    In the baboons, S.A. was present in some cynecephalus animals but
    not in any anubis ones.   The rosette and piebald guinea-pigs
    sometimes showed biphasic S.A. to ADP and adrenaline, whereas the
    albino guinea-pigs all showed non-phasic S.A. to ADP but none showed
    S.A. to adrenaline.

SUMMARY OF CHAPTER TEN

1. Nine varieties of animal species were surveyed for S.A. One sub-variety was examined in six of the animals, two of baboon, and three sub-varieties of guinea pig.

2. S.A. was not detected in rabbits, rats, pigs, and squirrel monkeys.

3. S.A. to ADP was detected in baboons, guinea pigs and dogs. The S.A. was self-limiting with little or no ADP release. The low or absent release suggests that an increased aggregation-responsiveness of the platelets to ADP was the major factor in S.A. in these species (as it probably was in 'level' non-phasic S.A. in man, Chapter Five).

4. Differences were detected between sub-varieties of baboons and guinea-pigs.

5. Chain-reactive S.A. was detected in all of six cat plasma and low adrenaline concentrations initiated S.A. in these plasma.

6. Of the animals tested only the <u>cat</u> showed an S.A. activity comparable to that occurring in S.A. in humans.

COMMENT.

The cat is the only animal so far known to develop disease in it's natural environment from a platelet-fibrin thrombus. Occasional instances of aorta-femoral block (the Leriche syndrome) have been detected in cats, and post-mortem has shown thrombus as the cause of the occlusion (Freak 46, Mitchell 120a). It has been postulated that this thrombus may be the result of embolism from the left auricle (Freak 46).

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DISCUSSION

Chapter by Chapter

In Chapter one the discovery of ADP as the stimulator of the aggregation of blood platelets was described. ADP is the final common pathway for the aggregation-inducing action of all known stimulators of this process, baring the first phase of catecholamine aggregation. The morphological evidence showing that aggregates of platelets form the major and initial mass of haemostatic plugs and white thrombi was also reviewed, as was the increasing evidence that white thrombi are the major factor in the pathogenesis of atherosclerotic plaques, are the main cause of coronary and cerebral artery occlusion, and are the initiating event of deep vein thrombosis.

Platelet aggregation was studied in this thesis by the turbidimetric method of Born (Chapter two) using equipment made in Professor Born's department. This method gives a continuous recording of the aggregation and disaggregation of blood platelets. Chapter two also describes the methodology of the further investigations which the method of Born permits, namely removal of samples which can then be used for microscopical studies or for investigations of changes in the aggregating activity of the plasma. The aggregating activity present in a plasma was measured biologically by recording it's effect on further specimens of platelet rich plasma. Such readings measure the sum of the interaction of the stimulators and inhibitors present in a plasma, and so may be more relevant than chemical estimates to the biological calculations for which they were used in this thesis. The total aggregating activity in a plasma could be measured, or by the use of substances which specifically inhibited other aggregating agents, only the ADP-like aggregating activity. The values obtained in this

biological system are comparable to those obtained by chemical methods
(firefly method and PK-PEP system, Mills et al 116, 120, Hardisty et
al 65).

Chapter three outlines the development of the different methods
for measuring the adhesion and aggregating behaviour of blood platelets and
then the advantages and disadvantages of these methods were described.
In essence, one can either measure both adhesion and aggregation processes
using whole blood, thus obtaining a composite result (platelet 'stickiness')
which is good as a screening test. Or one can separate the platelets from
the red blood cells and examine a single process in detail, either adhesion
or aggregation. Such a separation nearly always necessitates using
anticoagulants. Their use inserts a barrier to the relating of _in vitro_
investigations to the behaviour of blood platelets in the intact animal
which can only really be overcome by doing _in vivo_ confirmatory studies.

In Chapter four, the appearance of platelet aggregation –
disaggregation dose–response curves to exogenous ADP (0.25–10 μM) was
described. All plasma from man showed a response of increasing aggregation
as the (ADP) was raised, and this was followed by a decreasing degree of
disaggregation (the 'primary' aggregation or P.A. response). A study
combining tracings with microscopy, demonstrated that the change of
platelet shape, from a disk to a sphere, produced by ADP (Zucker and
Borelli 195), can be followed in the tracings. A further microscopical
study, this time of the aggregation – disaggregation process, showed a
rapid aggregation of most platelets and that increasing inter-platelet
adhesion occurred in the aggregates as the extent of the aggregation
increased. This finding confirms the work of Born and Hume (19). In
addition, it was found that large aggregates are already present before
the tracing starts to fall, showing that aggregation is faster than the
tracing suggests. Also, the degree of _disaggregation_ which followed the

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aggregation was less microscopically than that suggested by the tracing.

In addition to 'primary' aggregation, most plasma from man showed a further ('secondary') aggregation process (S.A.). This process resulted in a very marked and persistent aggregation response (microscopy showed that nearly all platelets in the plasma were in aggregates showing marked inter-platelet adhesion). When this process was initiated by small doses of ADP (0.25 to about 1.5μM) a markedly biphasic tracing occurred, for the S.A. process was powerful enough to reverse the disaggregation which followed the P.A. response ('biphasic' S.A.). When larger doses of ADP were required to induce S.A. (2μM ADP/or more), the tracing was continuous or showed a slight 'bump' ('non-phasic' S.A.). These results showed that S.A. is a process which converts a small ('biphasic' S.A.) or moderate (non-phasic S.A.) and temporary P.A. aggregation into a very marked persistent aggregation.

Investigation of the mechanism of S.A. (Chapter five) showed that it was accompanied by the release of an aggregating substance by the platelets, and that P.A. was not. The released activity behaved like ADP, and no additional aggregating activity could be detected from the 5H-T which is also known to be released during S.A. (Zucker and Peterson 196). The size of the release of ADP-like aggregating activity was insufficient (especially so in some plasma) to explain either the very marked aggregation of S.A. or it's property of persistence. Presumably during S.A. the platelets, in addition to releasing ADP, became both more aggregation-responsive to the ADP and less able to disaggregate. Further support for this hypothesis was given by the subsequent demonstration that platelets could release an equivalent amount of ADP-like activity to that released during S.A. when they were stimulated to aggregate by thrombin (Chapter six), and yet retain the ability to undergo quite marked disaggregation; and by the finding, still later (Chapter ten), that the

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properties of increased aggregation-responsiveness and aggregate-
persistence occurred in platelets from baboons, to produce an S.A. tracing,
even although there was no increase in aggregating activity in the plasma.
Thus, in the baboon, the only factor which was found to explain S.A. was
the increased ADP-responsiveness of the platelets.

The observation that S.A. was associated with a release of ADP is
probably the most important finding in this work.   Hitherto aggregating
agents have been shown to act through a two tier system (Fig. 1.2 review),
which is an inherently self-limiting process.   Any given dose of
aggregating agent (e.g. thrombin and collagen) produces a dose-dependent
ADP release from the platelets and will then cease to have any further
ADP-release inducing action.   But when ADP itself induces a release
reaction the process becomes a single tier one, for the released ADP, in
addition to causing aggregation, is at the same time increasing the
stimulus for a release of more ADP from more platelets.   A self-
propagating platelet aggregation/ADP release reaction is thus produced,
which has as it's aggregation expression the S.A. process.   The self-
propagation is aided by the increased aggregation responsiveness to ADP
described above.

The discovery that S.A. was due to a self-propagating
aggregation-ADP release reaction suggested that the released ADP may
have the power to induce further specimens of the same plasma to undergo
S.A., and so initiate an aggregation chain reaction.   Experiments
(Chapter five) showed that this occurred, however, this effect was only
marked in plasma showing the active biphasic S.A. response.   The
aggregation chain-reaction potential of a plasma depended on two factors,
the concentration of ADP-like activity released, and the ADP required to
induce S.A. in the plasma , i.e. the S.A. point (ADP).   Calculations of

the chain reaction potential for different levels of the S.A. point showed (Chapter five) that the potential was very high for an S.A. point of 0.25μM. It then fell precipitously to reach small levels for S.A. points of 2μM ADP or more. The S.A. in those plasma with a high potential (S.A. points 0.25-1.5μM) was called 'chain-reactive' S.A. and that in the remainder 'self-limiting' S.A.

The calculations of the aggregation chain-reaction potential of S.A. at different S.A. point levels demonstrated a basic fact of chain-reactions, namely that when they are initiated by a wide range of concentrations of a substance, the variations in the power of the chain-reaction are related not to the linear scale of the initiating range of concentration, but to the geometric one of chain-reactions. The corollary of this relationship is that small falls in the initiating concentration produce very large increases in the size of the chain-reaction.

The values used to calculate the chain-reaction potential are the ADP-like activity release and the S.A. point (ADP). The levels of ADP-like activity release found in Chapter five are comparable to those of ADP found by Mills et al (116). The levels of S.A. point (ADP) recorded in this work are close to those of Mills (115) (who used the same technique) and, when differences in the interpretation of tracings are allowed for, to those of Zucker and Peterson (197). Using a very vigorous stirring technique and a storage procedure for the plasma that increases S.A. activity, Hardisty et al (65) found an S.A. point range of 0.2-1.2μM ADP in a normal population. Presumably Hardisty's technique would give a range for pathological states starting at 0.05-0.1μM ADP. When the S.A. point (ADP) range found by Hardisty et al is used in calculations of the aggregation chain-reaction potential of S.A. a higher potential is obtained. However, since the width of the range found in Chapter four and in the work of Hardisty et al is the same, changes in S.A. point on either scale produce

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comparable changes in the chain-reaction potential i.e. the potentials
for the two ranges are at different absolute levels but run parallel to
each other.

The question of how relevant the S.A. process is to platelet-
fibrin mass formation **in vivo** will only be answered with certainty by
in vivo investigations.   The reader will realise that the platelet
concentration is higher in plasma than in whole blood, and that the
turbulent maximal stirring in the titrator will produce a higher number
of platelet to platelet 'hits' than occurs during laminar flow in a blood
vessel.   This enhancement of aggregation in the titrator is in part
counter-balanced by the marked depression of aggregation produced by the
complexing calcium ions by the citrate anticoagulant (Mitchell and
Sharp 121, Chapter Seven, Experiment 3).   In laminar flow situations in
blood vessels, any ADP released by platelets would probably be rapidly
dispersed and so soon reach an inactive concentration.   On the other
hand in the turbulent flow areas surrounding a projecting thrombus, where
there are slower changes in the layer of plasma immediately adjacent to
the platelet aggregates, the circumstances are not all that far removed
from those occurring in the titrator.   Also, a build up of ADP probably
occurs during haemostasis, as the flow of blood out of a damaged vessel
is slowed by vessel contraction and by the formation of blood platelet
masses.   In summary, it would seem likely that S.A. would have little
effect in a laminar flow situation, but could be exerting a marked effect
at sites of haemostasis and thrombosis where turbulent flow tends to
develop.

Up to this stage in the work, the S.A. process was studied by
using an ADP stimulus which was exogenous to the platelets.   In Chapter six
the effect of the S.A. process on the aggregation response of a plasma to
thrombin, collagen and adrenaline was studied i.e. a situation where the
ADP was derived from platelets themselves.   In P.A.-only plasma these

substances produced an aggregation which was dose dependent i.e. 'primary' thrombin, collagen, and adrenaline aggregation. The primary thrombin aggregation was marked but was followed by partial disaggregation, that induced by collagen was moderate, and that by adrenaline, small. Plasma showing S.A. to exogenous ADP also showed S.A. to the endogenous ADP release induced by thrombin and collagen, and to adrenaline. Furthermore, the degree of S.A. activity which a plasma showed to ADP was mirrored by a comparable degree of S.A. activity to testing with other aggregation agents.

The presence of S.A. activity in a plasma enhanced it's aggregation response to thrombin, collagen and adrenaline in three ways. Firstly, it produced persistent aggregates. Secondly, it increased the degree of aggregation achieved to maximal (this meant a small increase for thrombin, a moderate increase for collagen, and a very marked increase for adrenaline). And thirdly, this enhancement of aggregation occurred with doses of these substances which would have produced only a small aggregation in the absence of S.A.

A corollary of the demonstration of a cross correlation between the concentrations of the four aggregating agents required to initiate S.A. in any one specimen of plasma, is that the level of S.A. activity (and changes in this level) could be monitored by any of the four substances.

These conclusions on S.A. during the aggregation induced by aggregating agents other than ADP, were of necessity based on the appearance of the dose-response curve tracings. They are supported by the two other findings. First, patients with a congenital absence of S.A. to ADP (Hardisty and Hulton 63, Weis et al 182), and second, patients in whom S.A. has been inhibited by aspirin (O'Brien, 142, 143, Zucker and Peterson 197, Macmillan 107), showed aggregation tracings to thrombin, collagen and adrenaline which were identical to those for the 'primary' responses to these substances found in Chapter six.

The other important finding in Chapter six, was that plasma showing chain-reactive S.A. to exogenous ADP were stimulated to start this reaction by thrombin, adrenaline and collagen. Thus all sources of ADP (platelet release reaction and cell damage) are able to initiate an aggregation chain-reaction *in vitro*.

Chapter seven reports the many investigations carried out into the effect of laboratory manipulation and time of sampling on the S.A. process. It was found that for reproducible estimation of S.A. it was necessary to anticoagulate blood with citrate, to store the plasma at body temperature, and to test for S.A. at this temperature and within 75 mins of venepuncture (Experiment One, Two and Nine). Anticoagulation of whole blood with heparin and EDTA produced inhibition of the S.A. reaction (Experiment Three). Contamination of the plasma with red and white blood cells did not affect their S.A. activity (Experiment Four and Five).

The comparability of the plasma platelet population to that of whole blood, and the effect of changes in platelet count, were studied in Experiment Eight. It was found that the percentage extraction of platelets into the supernatant plasma varied widely. A graph of the changes in S.A. activity produced by lowering the platelet count of a plasma by dilution, showed very similar slopes for plasma derived from nine subjects. So accurate corrections can be made for differences in platelet count. Evidence was also found that the plasma platelet population may be a good mirror of the whole blood platelet population, for plasma of comparable density showed the same slope of decrease in S.A. activity, irrespective of whether the density was reduced by dilution, or by centrifuging down the more sedimentable (? more reactive) platelets. More work is needed on this point.

The S.A. activity of healthy volunteers (Experiment Nine) remained remarkably steady through a day and from day to day in any one individual, while showing considerable variation between individuals.   This suggested that single estimates derived from a healthy population of volunteers would give a satisfactory measure of the normal range of S.A. activity.

Chapter Eight reports the S.A. activity measurements made in seventy-four blood donors who were evenly distributed over both sexes and the age range 21 to 60 years.   Care was taken to exclude any donor who was on drugs (whether prescribed or from self-medication) and who had an intercurrent illness, no matter how minor.

The histogram of the results showed a mode value for the S.A. point of 3µM ADP and there was a preponderance of results at the active S.A. end.   The S.A. point in this normal population varied from 0.75µM ADP (one) to S.A. absent (three).   The S.A. was self-limiting or absent in 84% but in the remaining 16% was of the chain-reactive type.

The levels of S.A. point found in the donor survey are comparable to those found by Mills (115, 118) in thirty-three volunteers, and lower than those found by Hardisty et al (65) for the reasons noted earlier.

In both the survey made by Hardisty et al and the donor survey in Chapter Eight, S.A. activity was higher in young adult women.   Hardisty ascribed his results to the lower PCV values he found in women, but this explanation was only a small part of the reason for the difference in the donor survey.   No explanation was found for this increased S.A. activity.

Chapter nine describes a study of the changes which occur in S.A. activity in the 'thrombogenic' period which follows acute illness. During this thrombogenic period many tests of platelet function show changes (see Table 9.1), and a previous study of the _total_ aggregation response of plasma from eighteen patients undergoing an operation has shown

an increase in total aggregation at nine days post-operation over the
pre-operation levels (Emmons and Mitchell 42). These authors found
the total aggregation response varied widely from day to day.

The purpose of the present study was to look for changes in
S.A. activity from the steady day to day pattern found in normals, and
for the development of chain-reactive S.A. A pilot study of the day to
day changes in four patients with acute illness (Chapter nine) showed
little variation was occurring around the mean trend, and so a baseline
reading and three further readings over the next twelve days were
recorded.

In a control group of non-acutely ill patients kept in bed, and
of ambulant volunteers, there was little change in the S.A. activity over
the twelve day period. In forty acutely ill patients (20 'medical',
20 'surgical') a rise in S.A. activity occurred in thirty-seven. This
rise was detected at four days in most and by 7-8 days in all of the thirty-
seven.

The peak S.A. level was reached by 7-8 days by most 'medically'
ill patients and a little later by the 'surgical' patients (N.S.). At
this peak point chain-reactive S.A. was present in over 70% of the acutely
ill group (of only 18% in matched blood donors). Also, during acute illness
very high S.A. points of 0.25 to 0.75µM ADP were frequent (of only one donor
at 0.75µM ADP). The increase in chain-reactive S.A. was highly significant,
and the level of chain-reactive S.A. at the peak was significantly higher
than that of chain-reactive S.A. in the donor population. Thus not only had
the frequency of chain-reactive S.A. increased, but also the aggregation
chain-reaction potential had reached levels which were not seen in normal
people.

A closer examination of the results showed that 'major' illnesses
produced a higher peak S.A. activity than 'minor' illnesses (Emmons and

Mitchell failed to show this in their smaller group), and that the peak level was higher in patients aged over 60 years. An interesting finding was that a rise in S.A. of expected degree occurred in the two patients who were receiving effective oral anticoagulant treatment.

Analysis also showed that the increase in S.A. activity was only in very small degree due to the increase in the plasma platelet count. The mechanism of the S.A. activity increase was therefore not detected by the present study. The time relations of the rise in S.A. activity show that it would be possible for the proportion of new platelets in the plasma to have increased to high levels, and further studies on this possibility are desirable. The inability of plasma transfer to influence S.A. activity (found in Chapter five), suggests that the change will be found to reside in the platelets themselves rather than in the plasma.

As wide a spectrum of acute illness as was possible was studied and the results showed that rises in S.A. activity occurred in all these types of diseases. The two largest groups of disease were myocardial infarctions and appendicectomies, and in these groups the rises parallelled the severity of the illness. The results suggest that a rise in S.A. activity will occur in nearly all acute illnesses of more than very minor severity, and that chain-reactive S.A. will develop in most 'major' acute illnesses. Since platelet-fibrin thrombi are the initial event of most deep vein thrombosis formation (Sevitt 163), rises in S.A. could be an important factor in the initiation of these thrombi.

The final study (Chapter ten) was a survey of mamalian laboratory animals and of primates. Many of the animals (rabbit, rat, pig) which are used in thrombosis and thrombo-atherosclerosis research failed to show S.A. One sub-variety of baboon and some guinea-pigs showed a self-limiting S.A. response, during which there was little or no release of aggregating activity by the platelets.

Chain-reactive S.A. was found only in the cat.　　It could
be significant that the cat is the only animal which, in it's natural
environment, is known to develop a platelet-fibrin mass large enough
to occlude a major artery (aorta) (Freak 46, Mitchell 120a).

The animal study also showed that the P.A. response of
platelets from man was no greater than that of the animal platelets,
so it was only by virtue of an S.A. reaction with release of ADP
that platelet aggregation in man (and the cat) was greater than that
of other mammals.

General Discussion

The work in this thesis has shown that the S.A. process generates large platelet aggregates which show marked inter-platelet adhesion and marked persistence. These firm persistent aggregates are releasing a stimulus for further similar platelet aggregation (ADP) and promoting the formation of fibrin around themselves by releasing Platelet factor 3 (196). As was pointed out earlier in the discussion, this powerful self-propagating in vitro thrombogenic process would be most able to express it's potential in vivo at sites of reduced blood flow, where ADP and Platelet factor 3 will accumulate (e.g. sites of haemostasis), and in areas of turbulent blood flow, which enhance platelet to platelet contact, and again allow the thrombogenic substances released by the platelets to accumulate (e.g. diseased arteries and around venous valves).

The study of S.A. is still in it's infancy and so in vivo studies are few and are of associations. Hereditary absence of S.A. associated with a mild bleeding tendency has been detected by Weis et al (182) and this association has also been shown by Hardisty and Hutton (63). Aspirin and other anti-inflammatory agents inhibit S.A., and a mild bleeding tendency can occur in this instance (O'Brien 142, 143), but as aspirin also affects other parts of the haemostatic process the interpretation of this finding is difficult. S.A. may thus be contributing to haemostasis in man.

Associations between S.A. and thrombosis, are the marked increase in frequency and potency of chain-reactive S.A. in the 'thrombogenic' period which follows acute illness (Chapter nine), and the presence of chain-reactive S.A. in the two animals, cat and man, known to produce large platelet-fibrin masses in their natural environment (Chapter ten, Macmillan and Sim 110). The associational

nature of this evidence indicates the need for further _in vivo_ studies in animals, especially the cat.   It is encouraging to remember that _in vivo_ work has so far confirmed _in vitro_ observations (see review).

While suggestions as to the place S.A. has in disease in man are, for the present, speculative, some can be made.   The increasing evidence that thrombosis on plaques in arterial walls plays a major part in the pathogenesis of atherosclerosis, was described in the review (Chapter one).   While many animals have been induced to form small to moderate sized atherosclerotic plaques with hyperlipidaemic diets (Hartroft and Thomas 70, Constantinides 28), no animal model has been found which shows more than occasionally the _occlusive_ thrombo-atherosclerosis found in man.   This failure of experimental research has occurred inspite of the use of 'thrombogenic' diets, hypertension, and even Russells Viper Venom, in conjunction with diets of fat (70, 28). The discrepancy between the animal models and man has sometimes been ascribed to the relatively short duration of the animal experiments, but a further possibility is the absence in animals of a factor essential for atherogenesis.   The morphologic evidence (Lindsay and Chaikoff 101a, 70, 28) suggests that such a factor would be a thrombogenic one.   The marked thrombogenic potential of S.A. shows that it could easily be this 'missing link'.   Thus it may in time prove to be significant that S.A. is absent (rabbit, rat, pig), or minimal (baboon) in the animals used at the present time for studies of atherogenesis.

The hypothesis that an active S.A. process may play an essential part in the genesis of thrombo-atherosclerosis could be tested in an animal which both responded to lipid diets with early atherosclerosis and exhibited S.A. with ADP release, but it may be that only man shows _both_ these factors.   An alternative line of investigation is through

epidemiological studies.   Most sub-varieties of animals show variations
in the way their blood platelet aggregate (Chapter ten, Constantine 27,
Hawkey and Simons 71) even to the extent of presence and absence of S.A.
(baboon sub-varieties Chapter ten),   There is thus a very real
possibility that S.A. may be absent in some races of man and, when
present, it may vary in it's activity in different races.   The author
is assisting in the planning of such a comparative study.

Another type of study that is desirable is one to find out
if the S.A. activity of patients with atherosclerotic arterial disease
is higher than that of 'normal' people (i.e. with no detectable evidence
of the disease).   Even more important, is to include the measurement of
S.A. activity in the battery of tests used in the prospective studies
which are investigating the factors associated with the development of
ischaemic heart disease.   Such prospective studies are the best method
of determining whether a high level of S.A. activity increases a persons
risk of developing arterial disease, and also of determining the inter-
relationship between S.A. activity and hyperlipidaemia or hypertension.
This relationship could be additive or synergestic.   A simplified
method of measuring the S.A. point (ADP) of a plasma would be of
considerable help in prospective studies because of the large numbers
involved, and one is being developed by the author.

Since many occlusive thrombi have several layers of apparently
different age (Crawford 30), it may prove revealing to follow the S.A.
activity of patients with ischaemic heart disease at regular intervals.
In this way a period of high S.A. activity preceding a myocardial
infarction, which could have been responsible for the initial layer of
the thrombus, would be detected.

Friedman and his co-workers (52) have shown that the incidence of myocardial infarction is higher in people with a striving personality and prone to emotional stress. Von Euler et al (43) have demonstrated a correlation between emotional stress and the release of catechol amines, and catechol amines are able, by way of the S.A. reaction, to produce a marked aggregation response from blood platelets. It is thus possible that stress may, through catecholamine release, induce a 'hyperthrombotic' state. Catechol amines have a further important action on platelets. They produce a potentiation of ADP induced platelet aggregation which persists without diminishing for as long as the catechol amines persist (Mills et al 117, Macmillan 108). This persisting potentiation is to be contrasted with the short-lived ability of platelets to aggregate when they are stimulated by ADP, for in less than a minute they become refractory to the ADP, and their response to further additions of ADP is impaired (Macmillan 108). Harrison et al (67) failed to find an increase in the total aggregation response of plasma collected from students undergoing the stress of examinations. But the effects of emotional stress on the S.A. activity of an individual have yet to be investigated and this would seem to be desirable.

Finally, what is the place of anti-platelet drugs (including inhibitors of the S.A. reaction) in the therapy of thrombotic disease? The theoretical potential for anti-platelet drugs as effective anti-thrombotic agents, is higher than that for any other measure yet considered. Platelet adhesion and aggregation is the first event of the thrombotic process (see review) and platelets form nearly all the mass of a white thrombus. Thus prevention of this process will probably prevent an obstruction ever occurring. Conventional anticoagulants have a more difficult task, for they do not act until after the platelet aggregates have formed, and by this stage the 'coral-like' structure

of a platelet aggregate (Poole and French 151) contains stagnant columns of plasma, rich in the platelet factor 3 released by the platelets.

There are three spheres of the treatment of atherosclerosis where anti-platelet drugs could be useful; prophylaxis in healthy people with markers of an increased proneness to atherosclerosis, treatment of patients with established atherosclerotic disease, and as an adjunct to arterial surgery, where they may make thrombo-atherosclerotic endarterectomy a highly successful procedure, by deterring reblockage.

The problem will be to decide which type of anti-platelet aggregation drug to use. Total inhibition of either adhesion or of aggregation (i.e. of P.A.) is virtually certain to produce a severe haemostatic defect. P.A. inhibition would have an effect comparable to severe thrombasthenia and so a high incidence of side effects, especially bleeding complications, would probably occur. In contrast, absence of S.A. produces only a mild haemostatic defect (Weis et al 182).

Only inhibition of S.A. would be probably acceptable for prophylaxis against atherosclerosis, for the other types of inhibition would almost certainly have too high a complication rate. The degree of inhibition of platelet P.A., S.A. and adhesion necessary for effective therapy of established atherosclerotic disease will have to be established by clinical trial. A possible ideal may be the combination of S.A. inhibition with partial inhibition of platelet adhesion to collagen.

These thoughts on the therapeutic use of anti-platelet aggregating agents are no idle look into the future, for, in addition to the inhibitors of P.A. (114, 115) and S.A. (142, 143) already reported in the journals, many more are being studied by the thrombosis research groups of I.C.I. (Haslam), Glaxo (Ireland), Beechams (Sim), Pfizer (Constantine) and CIBA; and some of the more promising inhibitors

are already undergoing toxicology trials in animals.

The outlook for a therapeutic breakthrough in the prevention and treatment of atherosclerotic and other thrombotic disease in man is therefore promising.

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