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Continuous measurement of shear stress induced platelet activation

Giorgio, Todd Donald, Ph.D.
Rice University, 1987
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Continuous Measurement of Shear Stress
Induced Platelet Activation

by

Todd Donald Giorgio

A Thesis Submitted
in Partial Fulfillment of the
Requirements for the Degree

Doctor of Philosophy

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ABSTRACT

A rotational viscometer was modified to permit continuous measurement of platelet aggregation, dense granule release and intracellular calcium ion concentration during exposure to uniform shear stress in either the presence or absence of added chemical agonists.

The shear stress intensity applied to a platelet suspension influences both the concentration of single, unaggregated platelets and the population volume distribution of formed aggregates in samples with and without added ADP. Direct evidence supports the postulate that large platelet aggregates formed in response to chemical and/or mechanical stimulus can dissociate into smaller particles upon additional shear stress exposure.

The rate of platelet dense granule release is approximately a constant function of applied shear stress intensity in the absence of added chemical agonists. Addition of 0.2 or 2.0 μM ADP accelerates the rate of dense granule release in a concentration dependent way which is also potentiated by increased shear stress intensity. Application of shear stress alone results in bulk phase concentrations of ADP which are insufficient to induce aggregation in a stirred platelet suspension. Platelet ADP surface concentrations estimated from the continuously measured luminescence response indicate that application of
shear stress alone results in local ADP concentrations which
are sufficient to cause platelet aggregation which is
consistent in rate and extent with that observed in response
to ADP added to a stirred platelet sample.

Increased intracellular calcium ion concentration
([Ca$^{2+}$]$_i$) is correlated with the formation of irreversible
platelet aggregation. Platelet aggregation which occurs in
response to 0.2 μM added ADP and/or low intensity shear
stress is reversible and not accompanied by a rise in
[Ca$^{2+}$]$_i$.

Inhibitors of ADP induced platelet activation (creatine
phosphate/creatine phosphokinase, apyrase,
5'-p-fluorosulfonylbenezoyl adenosine) were effective in
reducing, but not abolishing, shear stress induced
aggregation.

A mechanism for shear stress induced aggregation is
postulated which implicates elevated platelet surface
concentration of ADP as the primary mediator. This
postulate is extended to indicate that the initiation step
in this response is a shear stress dependent local elevation
of [Ca$^{2+}$]$_i$. 
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CHAPTER I
INTRODUCTION

A. Platelet Function

Blood platelets are discoid cells with a diameter of 2 μm and a volume of $10 \times 10^{-15}$ liters (107). Normal humans circulate $180 - 350 \times 10^3$ platelets per μl of blood through the vasculature and retain an additional 20 percent of the total number in the spleen (53). Once formed from megakaryocyte stem cells, platelets remain in the blood for an average of 10 days (107).

Platelets actively participate in the normal hemostatic mechanism which prevents blood loss from intact vessels and arrests bleeding from severed vessels. They are normally nonadherent to each other, to other blood cells and to the endothelial cells which form a continuous monolayer inside the vessel wall. At the site of an injury, platelets can adhere to structures beneath the endothelium. This process initiates changes which render platelets attractive to other platelets. The latter process, known as platelet aggregation, occurs though the action of specific biochemical agents. Some of these activators are contained within the platelet in vesicles known as the dense and alpha granules which can be released to the extracellular fluid upon activation. Additional platelet activating agents can be enzymatically synthesized by the cell. These platelet
derived agonists serve to accelerate the reaction rate and stabilize the aggregate structures formed.

The specific biochemical mechanism through which platelet activation occurs is a function of the agent which induces the response. There is increasing evidence that, despite differences in membrane binding sites, many chemical agents activate the same initial sequence of platelet biochemical activity. Subsequent to chemical agent binding, the reaction sequence generally begins with activation of phospholipase C. This enzyme cleaves phosphatidylinositol 4,5-bisphosphate, producing inositol 1,4,5-triphosphate and 1,2-diacyl-sn-glycerol. The former is able to mobilize calcium ion from the platelet dense tubular system; the latter activates protein kinase C (21,92). The phosphorylation of myosin light chain which results is responsible for platelet shape change and organization of dense granules in preparation for secretion. The increased concentration of intracellular calcium ion activates phospholipase A$_2$, which acts to release arachidonic acid from the inner half of the external platelet membrane. Intracellular calcium ion acts as a universal "second messenger" in the stimulus-induced response of many cell types. Every naturally-occurring biochemical platelet agonist induces an increase in intracellular calcium ion concentration as a prerequisite to aggregation and granule release. Arachidonic acid is the substrate for platelet cyclo-oxygenase and lipoxygenase enzymes which result in the
production of prostaglandins G₂, H₂ and thromboxane A₂, all of which are powerful platelet aggregating agents (78). The relative extents of platelet shape change, dense and alpha granule release and arachidonic acid metabolite production are responsible for the different aggregation characteristics induced by various chemical agonists.

Two regimes of platelet activation can be defined. The events occurring on the enzymatic level which induce shape change, granule release and/or aggregation can be termed "microscopic" in contrast to the "macroscopic" results of platelet-platelet aggregation and the actual release of granule components. The entire biochemical activation scheme, depending on the chemical initiating agent, requires only a fraction of a second (35,36,37,113). The macroscopic events continue for many seconds to minutes depending on, among other parameters, the type and intensity of stimulus and the availability of additional platelets for reaction.

The same biological activity which make platelets useful also allows them to play significant roles in a variety of undesirable processes. The importance of platelets has been identified or implicated in the initiation and growth acceleration of atherosclerotic lesions (14,112), in cancer metastasis (58), and in thrombotic complications associated with blood contact in devices fabricated from non-biological materials (88).

Platelet function is dependent on many factors which include cell-surface interactions and the fluid dynamics
around the response site. The importance of the fluid dynamics is exhibited by the finding that some chemical agents will not cause platelet aggregation in the absence of flow induced platelet-platelet collisions. In contrast, platelets can be activated by application of fluid shear stress in the absence of chemical aggregating agents. Shear stress is the only known physical force which induces many of the same platelet responses as chemical agonist induced activation. This behavior is of obvious importance during the normal function of aggregation at the wall of an injured vessel. In addition, this effect may be the major component of platelet participation in atherosclerosis and in thrombotic complications associated with vascular prosthesis and artificial organs. Although much work has been accomplished in characterizing the response of platelets to different levels of shear stress and various exposure times, the essential knowledge of the biochemical reaction mechanism has only been poorly described.

B. History of Shear Stress Induced Platelet Activation

The relationship between blood flow rate and coagulation has been investigated since the 1940's when stasis was postulated to be a prerequisite for blood clotting.

The study of platelet response to shear stress was accidentally initiated in 1962 by G.V.R. Born. Using a turbidimetric technique with platelet rich plasma in a
cylindrical cuvette containing a magnetic stir bar, he obtained an increase in light transmitted through the sample (indicating the formation of platelet aggregates) without the addition of any platelet agonist (17).

"The tendency of platelets to aggregate seems to depend, amongst other things... on the rate of stirring." - G.V.R. Born

This effect can be primarily attributed to the shear stress imparted to the platelets by the rotating stir bar. The geometry of such a system produces complex shear stress distributions which can not easily be quantified. The need to study platelet behavior in a well defined shear field was thus realized.

Early investigation (1962-1973) utilized qualitative measurements of blood cell interactions in cone and plate viscometers. The motion of platelets in the shear field was described through the use of high speed cinematography (63-65,87,89,90). Important observations include the existence of a critical shear rate below which no platelets are seen to activate. At shear rates slightly above critical, experiments seemed to indicate that polymerization of fibrinogen to insoluble fibrin was followed by shear induced platelet activation whereas at even greater shear rates, shear induced platelet activation began at the start of shear application and fibrinogen polymerization followed
later. Shear induced platelet aggregation (SIPAG) was also measured as an increase in light transmitted through the sample and fibrin polymerization as an increase in apparent viscosity. The extent of SIPAG was observed to go through a maximum with respect shear stress, implying that platelets aggregate at low shear stress and that these aggregates could be broken apart by the increased hydrodynamic forces at higher shear stresses. The mechanism of shear induced platelet activation (SIPAC) had not been investigated, and results describing the relative timing of fibrinogen polymerization were later disputed, but the existence of SIPAC and its shear stress-exposure time behavior was documented (26,87).

The second period of SIPAC investigation ranges from 1973 to 1978 and is characterized by both hydrodynamic and biochemical advances. A second type of platelet suspension shearing apparatus, having a flow pattern which also produces a well-defined, quantifiable shear stress, was developed (19). This device consisted of a Couette flow region between two concentric cylinders with a cone and plate region at the bottom and cone and cone geometry at the top. It is important to note that this apparatus did not permit flow visualization as did all previous designs. Thus, different methods were utilized to determine the response of the platelets to shear stress. These new techniques were primarily biochemical and thereby allowed
quantitation of results and re-opened the field to biologically oriented investigators.

Measurement of platelet aggregation by cell counting (and a limited degree of particle sizing) was performed previous to and after application of shear stress. The qualitative results obtained previously were quantified and the ability of shear stress induced aggregates to separate minutes and hours after removal from the shear field indicated that SIPAG, to some degree, is reversible. To test the viability of platelets which have been exposed to shear stress, the response of those cells to chemical agents such as ADP and collagen was compared to the response of unsheared control platelets (3,5). Results indicated that platelet response to chemical agents decreased in a dose dependent fashion after exposure to shear stress and that those cells which had disaggregated after shear exposure also increased their reactivity to chemical agents.

Markers were employed to detect activation of the platelet release reaction solely in response to shear stress. Serotonin, at low concentrations, is actively uptaken by the platelet and stored in the dense granules. The level of radioactivity in the platelet supernatant is a direct indication of specific, active release or non-specific lysis of platelets preincubated with radiolabelled serotonin (3,5,57). Supernatant acid phosphatase was used as a marker for platelet lysozyme release (or lysis) (57). The lack of specificity of the
release assays initiated use of supernatant lactate dehydrogenase (LDH) as a specific marker for lysis (4,57). LDH is contained only in the platelet cytosol and not in any granules or lysozymes. Extracellular radioactivity derived from platelet pre-incubation with $^3$H-ATP (which exchanges only with the metabolic (cytosolic) ATP pool) was also used as an indicator of lysis (19).

A threshold shear stress, which is a function of the exposure time, was identified for incipient platelet dense granule release and lysis. The lysis threshold was found to be greater than that of the release reaction, indicating that platelet aggregation and release could occur as a result of shear stress in the absence of platelet damage (3,5,50,57,77,95).

The duration of shear stress exposure in rotational viscometers was generally limited to times of thirty seconds or greater due to inertia of the rotating element and the requirement of a well developed flow field. Some of the devices developed during this period specifically for application of short exposure time shear stress did not have a shear field which could be accurately estimated (12,60,109). The most successful device for short exposure time shearing was the capillary viscometer (22). Although the applied shear stress in this type of instrument is not uniform throughout the fluid sample, an average shear stress can be readily calculated. Measurement of platelet response after shear exposure times of physiological significance was
possible. The hydrodynamic conditions of the normal vasculature were found to be below the threshold required to induce incipient platelet granule release (3,85). Certain types of prosthetic heart valves, however, were found to be in the shear stress-exposure time regime where platelet activation occurs in the bulk flow (3,85). This result is in agreement with clinical observations of decreased platelet lifespan, increased platelet turnover, and thromboembolic complications in patients fitted with these devices.

The extent of surface activation as compared to bulk flow (shear stress) activation was also investigated. Some workers found that the degree of platelet aggregation, release and lysis was a strong function of the surface to volume ratio of the shearing device, thus indicating a significant role for surface induced effects (50). Others, using non-thrombogenic surfaces, were able to demonstrate that the extent of platelet activation in the shear field was independent of surface to volume ratio (at least under conditions of relatively high shear rate), implying that the effects measured were as a result of shearing stresses alone (4). The latter finding has been independently confirmed by other investigators (50). The study of platelet response at a thrombogenic surface requires a well-defined shear field and is a field separate, but related to, SIPAC.

The advances achieved through 1979 were limited to the determination of platelet response to shear stress in an
overall, or functional, manner. The more fundamental questions regarding the biochemical mechanism responsible for platelet activation in the shear field remained unanswered until suitable techniques became available in the late 1970's. Chemical agents were identified which alter platelet biochemistry in a predictable, specific way. The response of treated platelets can be compared to that of control, untreated platelets exposed to the same shearing conditions. The significance of the inhibited pathway in shear stress induced activation was reflected in alteration of the measured response of the treated platelets. Aspirin, a popular in vivo platelet inhibitor, irreversibly acetylates the active site of the enzyme cyclooxygenase, thereby preventing the conversion of arachidonic acid to prostaglandin \( G_2 \) (PGG\(_2\)), prostaglandin \( H_2 \) (PGH\(_2\)), and, subsequently, thromboxane \( A_2 \) (TXA\(_2\)). The use of aspirin-treated platelets has consistently shown that arachidonic acid metabolism and endoperoxide synthesis is not an important mechanism in shear stress induced platelet activation (1,44,94).

The successful use of aspirin spawned the use of more sophisticated inhibitors of platelet function in the determination of biochemical mechanisms. Prostaglandins \( E_1 \) and \( I_2 \) (PGE\(_1\) and PGI\(_2\) or prostacyclin) stimulate adenyl cyclase which increases production of the platelet function inhibitor cyclic adenosine monophosphate (cAMP). In addition, theophylline, an inhibitor of phosphodiesterase,
prevents degradation of cAMP to AMP. The effects of theophylline alone on SIPAC are slight (44). Incubation of platelets with either PGE\textsubscript{1} plus theophylline, or PGI\textsubscript{2} plus theophylline, before exposure to shear stress, however, reduces, but does not abolish, SIPAG at all stress-exposure levels. The shear stress threshold required to induce granule release and lysis are unaltered by this pre-treatment, but the levels of release and lysis are increased, indicating increased platelet fragility and/or enhanced membrane permeability (44-46,56,75). Modification of platelets by this method renders the platelets non-reactive to ADP or collagen, suggesting that SIPAC is not completely mediated by the same pathways. Similar results have been reported after treatment with dibutyryl cAMP (a lipophilic analog of cAMP) and theophylline (44-46).

Creatine phosphate/creatine phosphokinase (CP/CPK) is a substrate/enzyme system which scavenges ADP in the bulk phase through phosphorylation to ATP. Use of this system was intended to demonstrate the role of release or lysis generated ADP in SIPAC. Some investigators, using the chemical platelet agonist \( \gamma \)-thrombin, have concluded that the major effect of added CP/CPK is the reduction of background concentrations of ADP which can suppress the effect of subsequent chemical agents (54). Shear stress induced aggregation of platelets treated with CP/CPK was decreased, but not abolished (49,59,76). Preincubation with ATP, a competitive inhibitor of ADP binding, potentiated the
effect of CP/CPK on SIPAG, presumably by blocking ADP binding at the platelet membrane. Platelet aggregation in response to shear stress was almost completely blocked by the combination of CP/CPK and ATP, leading one set of workers to conclude that released ADP is the primary, if not the sole, mediator of SIPAC (76).

Further investigation of the biochemical mechanism of SIPAC involved hirudin, a strong inhibitor of thrombin induced aggregation, and colchicine, which prevents granule release by disrupting platelet microtubules (43,73,93,108). A certain hirudin concentration was demonstrated to completely block thrombin induced aggregation and dense granule release in the aggregometer and have no effect on ADP induced responses. Platelets pretreated with this hirudin concentration showed no deviation from the shear induced aggregation, release, and lysis of controls. Thrombin generation was concluded to be unimportant in SIPAC. Colchicine can be used to block the "second wave" of ADP induced aggregation and prevent dense granule release. At higher ADP concentrations, the colchicine treated platelets aggregated to a slightly lesser extent than control platelets and exhibited 33% less release, thus demonstrating that the concentration of colchicine used inhibited platelet release, but was not lethal. Colchicine treated platelets exposed to the shear field aggregated to a lesser extent than control platelets, but released and lysed
to the same degree. A hypothesis of ADP leakage as the primary initiator of SIPAC was presented (43).

Recent evidence indicates that the reaction time of the platelet biochemical triggering mechanism in response to physiological levels of shear stress may be a few seconds to less than one second (22,35-37,113). The results of this activation process, platelet aggregation and granule release, have been the traditional methods of quantifying shear stress induced platelet reaction and are usually measured after exposure times of many seconds to minutes.

Rotational viscometers of cone and plate or cup and bob geometry have the potential to apply a uniform, well-characterized shear field to a fluid sample, but they have the disadvantage of yielding only before-and-after measurements. As a result, shear stress exposure times in these devices are necessarily at least many seconds to allow for start-up and stopping of the moving component. Other devices such as capillary tubes, jet orifices, and oscillating wires have been used to obtain short exposure times (12,22,60,109). These methods produce non-uniform, but somewhat characterized shear fields. A flow-through viscometer has been described which eliminates inertial lag time and retains a relatively well-described shear stress distribution (51). Accurate measurements of shear stress induced platelet response at short exposure times, even with these devices, are cumbersome, if at all possible, due to artifacts introduced by the time between sampling and
measurement, or by the technique used for rapid preservation of the sheared sample characteristics (glutaraldehyde fixation, etc.).

It is now apparent that further investigation of the biochemical mechanism of shear stress induced platelet activation must focus on the intracellular events which occur prior to the traditionally measured macroscopic responses. The use of traditional rotational viscometers is, however, contradicted by the shear stress exposure times of interest. New methodologies are required to measure the response of platelets during, rather than after, exposure to shear stress in the rotational viscometer. Platelet reactions can then be studied as they occur on time scales of seconds or less.

C. Current Work

The goal of this work was the development of methods which can be used to describe platelet aggregation, dense granule release and intracellular calcium ion ([Ca$^{2+}$]$_i$) mobilization in a continuous fashion during application of controlled, well characterized and reproducible shear stress to a platelet suspension. Initial feasibility work which focused on the selection of a suitable indicator of intracellular free calcium ion concentration was expanded to include a study which determined that [Ca$^{2+}$]$_i$ mobilization is an important step in SIPAC (Chapter II). Additional preliminary work resulted in the design and construction of
a cone and plate viscometer modification which allowed continuous monitoring of platelet response in the shear field (Chapter V). This instrument was used to obtain information regarding the kinetics of platelet response to various levels of shear stress with and without the addition of extracellular ADP (Chapter VI). The scope of this effort also included determination of the relative importance of extracellular ADP in SIPAC through the use of the ADP analog 5'-p-fluorosulfonylbenzoyladenosine (FSBA) and two ADP scavenging enzyme systems, CP/CPK and potato apyrase (Chapter IV). FSBA is reported to bind irreversibly at the platelet membrane ADP recognition site without inducing platelet activation (11,23,31,74). CP/CPK, which has been used in sheared platelet suspensions (47,54,59,76), serves as a reference for the effects of apyrase on SIPAC. Interpretation of the continuously measured response of platelets during exposure to uniform shear stress, together with the endpoint measurements made in this work and those reported by others, results in a proposed mechanism of shear stress induced platelet activation (Chapter VII).
CHAPTER II
GENERAL EXPERIMENTAL METHODS

Experimental techniques which were used throughout this work are presented in this chapter. Methods which were specific to any single family of experiments are included in subsequent chapters.

A. Preparation of Platelet Suspensions

1. Blood collection

Blood donors were nonsmokers, ranged from 22 to 29 years in age, and claimed no ingestion of medication (including aspirin and aspirin substitutes) during the 14 day period prior to donation. Blood was obtained from these apparently healthy volunteers through antecubital venapuncture with a 19 or 21 gauge winged infusion set (Terumo Corporation, Toyko) connected to a disposable syringe (Becton Dickinson and Company) of appropriate volume. Anticoagulant, at 37°C, was contained in the collection syringe providing prompt inhibition of unwanted platelet aggregation. The filled syringe was gently inverted several times to insure thorough mixing of the blood with anticoagulant.

2. Preparation of platelet rich plasma

Blood anticoagulated with either 10 USP units/ml sodium heparin (porcine intestinal type; Elkins-Sinn, Inc.) or 0.38% (w/v) sodium citrate (Mallinckrodt, Inc.) was placed
into tightly capped polypropylene tubes. The anticoagulated blood was centrifuged for 15 minutes at 180 x g and 24°C. This treatment produced a layer of platelet rich plasma (PRP) which typically contained 280,000 to 500,000 platelets per µl as determined by electronic particle counting. The PRP was gently aspirated into a disposable polypropylene pipette and placed into a tightly capped polypropylene centrifuge tube. Contamination from other blood cell types was carefully avoided.

The unharvested volume remaining after centrifugation was gently inverted and centrifuged a second time for 20 minutes at 1200 x g and 24°C. The top layer produced by this treatment, platelet poor plasma (PPP), was used to dilute the PRP to a final concentration of 300,000 per µl. The final PRP was stored in tightly capped polypropylene tubes to prevent surface activation of the platelets and to minimize loss of CO₂ which can alter platelet reactivity though changes in suspension pH.

3. Platelet labeling in PRP

Labeling of the platelet dense granules was performed by incubating the PRP with 7.32 nM (200 nCi/ml) ³H-hydroxytryptamine creatinine sulfate (³H-serotonin; New England Nuclear). The exact amount of radiolabel used was determined from the volume of platelet suspension calculated on the basis of 300,000 platelets per µl. After 60 minutes at 24°C, platelets have been shown to uptake more than 80% of the radiolabeled serotonin (42).
For experiments involving the measurement of intracellular calcium ion mobilization, platelets were also loaded with either quin2/AM (Sigma Chemical Co., Inc.) or indo-1/AM (Molecular Probes, Inc.), both of which are fluorescent, tetracarboxylyate calcium ion chelators. A final concentration of 10 μM quin2/AM, based on 300,000 platelets per μl, was added to the PRP. Incubation for 60 minutes at 24°C was performed simultaneously with radiolabeling. Gentle mixing of the platelet suspension was provided during labeling by the orbital motion of an Adams Nutator (Clay-Adams). All sample tubes containing platelet suspensions treated with fluorescent photoprobes were wrapped in aluminum foil to protect the indicator dye from unnecessary photobleaching. Platelet loading with indo-1/AM was identical in procedure to that described with quin2/AM except the final concentration of indo-1/AM was typically 1 μM (based on 300,000 platelets per μl) and the incubation duration was 90 minutes.

4. Preparation of washed platelet suspensions

Whole blood, anticoagulated with 14.3% (v/v) acid-citrate-dextrose (ACD), was placed into polypropylene tubes and centrifuged for 15 minutes at 180 x g and 24°C. The top PRP layer was aspirated using a disposable polypropylene pipette. Contamination from other cell types was carefully avoided. The anticoagulated PRP was placed into a polypropylene tube of sufficient diameter to allow insertion of a pH electrode. The pH electrode/meter (Fisher
Scientific; model 620) was calibrated daily at pH = 4.00, pH = 7.00 and pH = 10.00 with a standard technique. The pH of the PRP was adjusted to 6.50 with the dropwise addition of ACD. The acidified platelet suspension was centrifuged for 15 minutes at 900 x g and 24°C. A platelet pellet was formed at the bottom of the centrifuge tube as a result of this treatment. The supernatant typically contained 2 - 5% of the original platelet concentration and was discarded. The platelet button was resuspended in HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer with the following composition: NaCl, 145 mM; KCl, 5 mM; HEPES (Research Organics, Inc.), 10 mM; Na₂HPO₄, 0.5 mM; MgCl₂, 1 mM; CaCl₂, 0.1 mM; glucose (Aldrich Chemical Co., Inc.), 5.5 mM; bovine albumin, fraction V (Sigma Chemical Co., Inc.), 3.5 mg/ml; potato apyrase, grade V (Sigma Chemical Co., Inc.), 2.5 ADPase units/ml; sodium heparin (Elkins-Sinn, Inc. or Organon, Inc.), 50 USP units/ml. Some preparations used 10 ATPase units/ml of potato apyrase, grade III (Sigma Chemical Co., Inc.) which is roughly equivalent to the concentration of potato apyrase, grade V used subsequently. The buffer pH was adjusted to 7.00 with 2 N NaOH in distilled water. This buffer was prepared with fresh glucose and albumin daily and filtered through a membrane of 0.2 μm diameter pore size before use.

Platelet labeling was typically performed in the first washed platelet suspension. Use of ³H-serotonin, quin2/AM
and indo-1/AM was identical to that described for platelets suspended in plasma.

After labeling, the platelets were centrifuged at 900 x g and 24°C for 10 minutes. A second platelet pellet was formed with 4 - 8% of the platelets discarded in the supernatant. This pellet was resuspended in the same HEPES buffer as used in the first wash, with the following exceptions: sodium heparin is omitted, potato apyrase is omitted, pH = 7.35. Where noted, the final resuspension buffer contained 0.025 ADPase units/ml potato apyrase, grade V.

After resuspension of the second button, human fibrinogen (Calbiochem) was added back to a final concentration of 1 mg/ml. Calcium concentration in the buffer was then returned to the physiological level of 1 mM through addition of CaCl₂. The cells were allowed to equilibrate in this final suspension for at least 30 minutes prior to experimentation. Except where noted, the final platelet concentration was 300,000 per µl.

B. Shear Stress Exposure Using The Unmodified Viscometer

All shear stress experiments utilized a Ferranti-Shirley cone and plate viscometer (Ferranti Electric; model 781). Some studies employed a modification of this instrument in which the standard, stainless steel lower platen was replaced by an specially constructed aluminum mounting base having an optically transparent platen surface. The design and use of the modified
viscometer, referred to as the Rice Optical Measurement Viscometer (ROMV), is described in Chapters V and VI.

1. Viscometer preparation

The stainless steel cone and plate surfaces of the viscometer were treated with a siliconizing agent (Prosil-28, PCR Research Laboratories) during the overnight period preceding a shearing experiment. The surfaces were rinsed with normal saline and dried before the first shear exposure. The plate height was adjusted to contact the cone apex. This action completes an electrical circuit integral to the apparatus in which a resistance heater then automatically controls the cone apex to plate distance through thermal expansion of the cone mounting column. The manufacturer claims gap height extremes of 0 to 10 μm during operation. A thermostatically controlled water bath (Lauda; model K-4/RD) was used to circulate a mixture of 50% water / 50% ethylene glycol through the hollow center of the lower viscometer platen. The temperature selected (either 24°C or 37°C) was regulated to ± 0.1°C.

2. Calculation of applied shear stress magnitude

For cone angles sufficiently small, such as the 0°20'11" cone angle used in this work, the applied shear rate was uniform throughout the sample volume defined between the cone and the flat lower platen (8). The shear rate in the sample can be calculated from the small cone angle geometry and the speed of the cone relative to that of the plate through
\[
\gamma = \frac{2 \cdot \pi \cdot \omega}{60 \cdot \Psi}
\]  

[II-1]

where \( \gamma \) is the shear rate in seconds\(^{-1} \), \( \omega \) is the cone rotational rate in revolution per minute (RPM) and \( \Psi \) is the cone angle in radians. The maximum theoretical shear rate which can be applied by this instrument fitted with a 0.333 degree (nominal) cone angle was 17,790 seconds\(^{-1} \). The maximum shear rate used was 12,500 seconds\(^{-1} \) due to practical restrictions and theoretical considerations of secondary flow (30). The shear stress experienced by the sample was then estimated, for Newtonian fluids such as PRP or WP suspensions, from Newton's Law of Viscosity

\[
\tau = \gamma \cdot \eta
\]  

[II-2]

where \( \tau \) represents shear stress and \( \eta \) is the viscosity of the fluid. The viscosity of PRP and washed platelet suspension was estimated to be 1.8 and 1.2 centipoise, respectively (7,29,80,81).

3. Shear stress application and recovery of sheared sample

The desired cone rotational rate was selected and 600 \( \mu l \) of platelet suspension was placed on the lower platen. The lower platen of viscometer was raised, trapping the sample beneath the cone. Cone rotation was initiated by simultaneously depressing the switches labeled "sweep" and "fast up" on the viscometer control panel. The desired cone speed was typically achieved in one second. Use of the "hold speed" function allowed the cone rotational rate to
remain constant until the appropriate shear stress exposure time was reached (10 to 300 seconds). The "hold speed" toggle was then released and the "fast down" button engaged, thus stopping cone rotation within one second. The platen was then lowered without delay and 3.33 µl of the sheared suspension was recovered and diluted into 10 ml of Isoton®II containing 0.5% (v/v) gluteraldehyde. Gluteraldehyde addition to the diluent prevented the particle size distribution of platelet aggregation from changing due to the break up of reversible platelet aggregates (8).

The sheared suspension remaining on the lower platen was recovered and placed in a polypropylene tube at 4°C containing 1 µl of 0.5 mM imipramine hydrochloride (Sigma Chemical Co.) dissolved in normal saline. Addition of imipramine and the reduced temperature were used to prevent platelet re-uptake of serotonin released in the shear field. The sample was then centrifuged for 7 minutes at 1450 x g and 4°C. The platelet-free supernatant was reserved for subsequent assay of $^3$H-serotonin radioactive decay and lactate dehydrogenase activity. Two unsheared samples were reserved from each experiment as controls and were centrifuged in the same manner as the sheared specimens: one aliquot was withdrawn directly from the stock platelet suspension, the other was placed between the stationary viscometer platens for the same exposure time as the sheared samples. An additional control sample was drawn directly from the platelet preparation but was not centrifuged. All
reserved samples were stored at 4°C for no longer than 72 hours before performing lactate dehydrogenase and $^3$H-serotonin assays.

C. Measurement Of Platelet Response To Applied Shear Stress

The techniques described in this section were applied to sheared platelet suspensions without regard to the viscometer configuration (unmodified or ROMV).

1. Particle size analysis

Particle size distributions of the sheared samples and corresponding controls were determined with a Coulter Counter (model ZBI) and Coulter Channelyzer with a Logarithmic Range Expander (all three instruments from Coulter Electronics). The platelet suspension, diluted 1:3000 with Isoton®II containing 0.5% gluteraldehyde is drawn through a 50 μm diameter aperture and past electrodes which count the number and measure the intensity of the impedance inhomogeneities. This information is converted into a population size distribution of 100 particle volume "bins" or "channels". The Channelyzer is connected to an Apple II plus microcomputer (Apple Computer, Inc.) via a Coulter Computer Interface (Coulter Electronics), thus allowing permanent storage of the population size distributions. Basic statistical operations such as averaging duplicate counts, removal of background noise, and conversion from a particle number versus particle volume histogram to a integrated volume versus particle volume
histogram can be performed (8). The operating parameters and calibration procedure used for particle size analysis are detailed in Appendix B. The measurement of large platelet aggregates was done with a 110 µm diameter aperture and an aspirated sample volume of 500 µl.

Each shear exposure generated a single sample of platelet suspension diluted for particle size analysis. Control, unsheared platelet suspension was sampled at the start and completion of each experiment to demonstrate the stability of the before-shear population size distribution. A blank sample of Isoton® plus gluteraldehyde containing no cells was counted and used as background. All samples were counted in duplicate and recorded on the microcomputer. Two sizes of calibration spheres, diluted in Isoton®II, were also counted each day for calibration purposes.

Duplicate counts were averaged and background subtraction was performed on each sample. Calibration of the channel volumes was performed as indicated in Appendix B. The mode channel of the unsheared control sample was taken to represent the average volume of a single, unaggregated platelet. The calculated volume distribution was then normalized with respect to the average single platelet volume. Thus, the mode channel volume, in dimensionless form, was defined to have the value of one.

2. Calculation of percent platelet aggregation

Measurement of platelet aggregation is normally described by the loss of single, unaggregated platelets to
the formation of platelet aggregates of two or greater. Resolution between a platelet singlet and a aggregate of two platelets is more difficult in an electronic particle counter than in a hemocytometer due to the heterogeneity of single platelet volumes. Thus, a reproducible method for identifying the singlet platelet volume fraction of the total platelet volume was required. The singlet platelet volume was defined as that volume between 0.5 and 1.5 average platelet volumes. This definition does not depend on the absolute particle volume which can vary significantly between individuals, but does require, for experiment to experiment consistency, that the platelet volumes of control samples are distributed around the mode volume in a reproducible manner. Percent aggregation of any sheared sampled was calculated as the loss of single platelets compared to the number of single platelets in the unsheared control sample as

\[
\text{Percent platelet aggregation} = \frac{x_i - x_f}{x_i} \cdot 100 \quad [II-3]
\]

where \( x_i \) and \( x_f \) are the number of particles between 0.5 and 1.5 average platelet volumes of the unsheared and the sheared samples, respectively.

Experiments in which entire population size distributions were determined using both 50 and 110 \( \mu \)m diameter apertures were represented by four subsets of the entire measured volume fraction. The volume fractions selected correspond to those used elsewhere (97): 2.55 to
8.97 μl³; 8.97 to 51.07 μl³; 51.07 to 490.3 μl³; 490.3 to 8,580 μl³. Each volume fraction was normalized through division by the entire integrated volume from 2.55 to 8,580 μl³. As a result, the four volume fraction percentages sum to 100 percent of the total measured volume.

3. Dense granule release measurement; \(^3\)H-serotonin method

   a. background

   Serotonin is a naturally occurring derivative of tryptophan which can be actively uptaken against a concentration gradient by platelets (42). The cells store serotonin in vesicles known as dense granules. Uptake and storage of serotonin is not altered by radiolabeling (either \(^3\)H or \(^14\)C) part of the molecule (42). Platelet dense granule release, which occurs in response to some stimuli, can be measured in a platelet population previously labeled with \(^3\)H-serotonin through determination of the supernatant radioactivity level. Serotonin can also be discharged to the supernatant as a result of platelet lysis. The level of serotonin in the supernatant reflects both the extent of active dense granule release and the degree of cellular lysis.

   b. experimental method

   The sheared sample supernatants and unsheared controls were warmed to 24°C. Triton® X-100 (J.T. Baker Chemical Co.) was added to the uncentrifuged control aliquot to a final concentration of 0.1% (v/v). The platelet buttons reserved after centrifugation of the other unsheared control
samples were resuspended in normal saline containing 0.1% (v/v) Triton®. All three aliquots were individually sonicated for 10 seconds at 60% full power using a sonic dismembrator (Artek Systems Corp.). This treatment forced all of the ³H-serotonin and cytocellular enzymes contained inside the platelets to be released to the continuous phase.

100 µl of each sample was diluted into 10 ml of complete liquid scintillation counting solution specifically prepared for aqueous samples (National Diagnostics) contained in a polypropylene vial (Sarstedt). All samples were prepared in duplicate. Each vial was counted for 20 minutes in a liquid scintillation counter (Packard Instrument Co., Inc.; model 4430). The decay energy counting range was limited between 2 keV and 19 keV. The number of counts recorded per minute (CPM) was converted to decays per minute (DPM) using a calibration for machine counting efficiency and correcting for quenching effects.

c. calculations

Total DPM of the sample \( \text{DPM}_{\text{tot}} \) was calculated by averaging the uncentrifuged sample DPM with the sum of the unsheared control supernatant DPM \( \text{DPM}_{\text{bkgd}} \) and the unsheared control button DPM. The percent release of serotonin of any single sample was calculated

\[
\% \text{ serotonin release} = \frac{\text{DPM}_s - \text{DPM}_{\text{bkgd}}}{\text{DPM}_{\text{tot}} - \text{DPM}_{\text{bkgd}}} \cdot 100 \quad [11-4]
\]

where \( \text{DPM}_s \) is the decays per minute in the supernatant of the sample.
4. Determination of platelet lysis; LDH assay
   a. background

   Lactate dehydrogenase (LDH) is an enzyme which catalyzes the conversion of pyruvate to lactate. This reduction is coupled with the oxidation of nicotinamide adenine dinucleotide, reduced form (NADH) plus a proton (H+) to nicotinamide adenine dinucleotide (NAD+).

   \[
   \text{pyruvate} + \text{NADH} + H^+ \rightarrow \text{lactate} + \text{NAD}^+ 
   \]

   NADH has a relatively high specific absorbance at 340 nm compared to the specific absorbance of NAD+ at the same wavelength. This property can be used to monitor the reaction rate which can then be used to determine the LDH activity in the sample. The method used to determine LDH activity has been described (110) and is available in commercial form (Sigma Chemical Co., Inc.; assay kit UV-340).

   LDH is contained in the platelet cytosol and is not actively released. The relative amount of LDH in the supernatant of a platelet suspension indicates the level of cellular lysis which has occurred independent of dense or alpha granule release.

   b. experimental method

   The sheared sample supernatants and unsheared controls were warmed to 24°C. \(\beta\)-NADH (Sigma Chemical Co.) was dissolved in either pH = 7.50 phosphate buffer (Sigma Chemical Co.) or pH = 7.50 normal saline buffered with 10 mM
HEPES. Stock β-NADH concentration was 71.4 μg/ml; 2.80 ml of this solution was placed into a disposable cuvette (Sarstedt) with 1 cm optical pathlength. Although disposable, these cuvettes transmit 88% of incident light at 340 nm. 100 μl of sample was added to the β-NADH in the cuvette and allowed to incubate for at least 30 minutes. 100 μl of a sodium pyruvate solution (20 mM in pH = 7.50 buffer) (Sigma Chemical Co.) was added to the cuvette to start the reaction. The absorbance of the mixture was measured and recorded with a Gilford 2600 spectrophotometer (Gilford Instrument Laboratories, Inc.). The instrument was allowed to warm up for at least 30 minutes before the first measurement; absorbance of a cuvette filled with distilled water was set to zero at a wavelength of 340 nm. Sample absorbance was automatically measured every 12 seconds for a total of 180 seconds. A microprocessor inside the spectrophotometer calculated the rate of absorbance decrease between every three consecutive absorbance measurements. The LDH activity of each reserved sample was measured in duplicate and averaged.

c. calculations

Sample LDH activity was calculated from the absorbance rate of change via

\[
\text{LDH units/ml} = \frac{\Delta A_{340 \text{min}} \cdot \text{TCF}}{0.001 \cdot L \cdot \text{vol}}
\]

[II-5]

where TCF is a factor which corrects for the temperature dependence of LDH activity, L is the optical path length of
the cuvette, vol is the volume of added supernatant sample and 0.001 represents the $\Delta A_{340}$ equivalent of one LDH unit of activity in a total volume of three ml with a one cm optical path length at 25°C. The percent supernatant LDH activity was calculated from

$$\% \text{ LDH activity} = \frac{\text{LDH}_s - \text{LDH}_{bkgd}}{\text{LDH}_{tot} - \text{LDH}_{bkgd}} \cdot 100 \quad \text{[II-6]}$$

where $\text{LDH}_s$ is the supernatant LDH activity of the sample, $\text{LDH}_{tot}$ is the LDH activity of the uncentrifuged, sonicated sample and $\text{LDH}_{bkgd}$ is the LDH activity of the unsheared, viscometer control supernatant.

5. Determination of intracellular quin2$^{4-}$ concentration

Three ml of platelet suspension were reserved for determination of intracellular quin2$^{4-}$ concentration. Triton®X-100 (0.5% (v/v)) was added to the sample before duplicate 10 second exposures to sonication at 60% full power in a sonic dismembrator. The calcium ion concentration of the sample was reduced to nearly zero through addition of EDTA to a final concentration of 6 mM. The sample was transferred to an acrylic cuvette which was placed into a spectrofluorometer (Perkin-Elmer; model MPF-2A). Excitation wavelength was 339 nm with a bandwidth of 6 μm. Fluorescence of the sample was measured at wavelengths scanned smoothly between 400 nm and 600 nm with a bandwidth of 6 μm. Free calcium ion concentration was then increased above the quin2$^{4-}$ saturation level of 100 μM $\text{Ca}^{2+}$ through addition of 30 μl of 1000 mM CaCl$_2$ stock
solution. Fluorescence intensity of the sample was again measured using the same spectrofluorometer settings. Fluorescence peak height at 492 nm was used to quantify the minimum (zero free Ca\(^{2+}\)) and the maximum (> 100 μM free Ca\(^{2+}\)) response of the quin2/AM trapped and enzymatically converted by the platelets into quin2\(^{4-}\) \(^{\infty}\). The fluorescence of a platelet sample treated in the same way but not exposed to quin2/AM was interpreted as cell autofluorescence and was subtracted from the results using quin2\(^{4-}\) \(^{\infty}\) loaded cells.

A series of six calibration solutions was prepared containing 0, 1.0, 2.0, 3.0, 4.0, 5.0 μM quin2\(^{4-}\) (Sigma Chemical Co.) in HEPES platelet washing buffer containing 2 mM CaCl\(_2\). The fluorescence of each of these solutions was measured and the fluorescence intensity at 492 nm was recorded. The unknown bulk quin2\(^{4-}\) concentration in the lysed platelet samples ([quin2\(^{4-}\)\(_{\text{bulk}}\)]\()\) was estimated by simple interpolation of the fluorescence intensity at 492 nm with that of the calibrations solutions having a known quin2\(^{4-}\) concentration. The total cellular volume of the platelet sample was determined from integration of the Coulter Counter generated particle volume distribution. The average intracellular quin2\(^{4-}\) concentration ([quin2\(^{4-}\)\(_{\text{i}}\)]\()\) was calculated from

\[
[\text{quin2}^{4-}]_{\text{i}} = [\text{quin2}^{4-}]_{\text{bulk}} \cdot \frac{1}{x_v}
\]

[II-7]

where \(x_v\) is the volume fraction of cells in the spectrofluorometer cuvette.
CHAPTER III
THE EFFECTS OF QUIN2/AM AND INDO-1/AM LOADING
ON THE RESPONSE OF PLATELETS TO CHEMICAL AND
SHEAR STRESS ACTIVATION

A. Introduction

Quin2/AM is an uncharged molecule which can equilibrate across plasma membranes with little difficulty. Once inside the cell, enzymes cleave the esters which mask four carboxylic acid functional groups. The resultant molecule (quin2$^{4-}$) shares the backbone structure and divalent cation chelation properties of ethylenediaminetetraacetic acid (EDTA). Quantitative estimates of the calcium ion concentration are obtained by measuring the fluorescence intensity of the quin2$^{4-}$-Ca$^{2+}$ complex (99,100).

Several workers have reported investigations on the use of quin2$^{4-}$ as a quantitative probe for platelet calcium ion transients in the activation response to added chemical agonists (6,20,28,41,48,61,62,82-84,86,103,115). The value of quin2/AM in the study of platelet activation induced by the controlled shear field of the rotational viscometer is, however, uncertain.

Quin2/AM was used to study the role of intracellular calcium ion concentration in mediating shear-induced platelet activation. Washed platelet suspensions were subjected to various levels of uniform, known shear stress in a cone and plate viscometer in the absence of added chemical agonists. Additional samples were aggregated in
response to chemical platelet agonists in a conventional aggregometer.

The results of this study prompted additional work to determine the effects of another fluorescent tetracarboxylate chelator (indo-1/AM) on platelet function. Indo-1$^{4-}$ offers the following advantages over quin2$^{4-}$: 30 times greater fluorescence intensity; major changes in the emission wavelength (not simply the intensity) upon Ca$^{2+}$ chelation; slightly lower affinity for Ca$^{2+}$; and improved selectivity for Ca$^{2+}$ over other divalent cations (40).

B. Experimental Methods

Washed platelet suspensions were exposed to various levels (0 - 150 dynes/cm$^2$) of well-defined shear stress at 24 °C in the traditional cone and plate viscometer. Particle counts and size distributions were performed as described in Chapter II, section C and Appendix B. Platelet dense granule release was measured from supernatant $^3$H-serotonin radioactivity (Chapter II, section C) and cellular lysis was determined from supernatant lactate dehydrogenase (LDH) activity (Chapter II, section C). Shear field exposure times were 30 and 300 seconds. Experimental work was performed with washed platelets treated with 5 µM quin2/AM dissolved in dimethylosulfoxide (DMSO). Control platelet suspensions were treated with the same concentration of DMSO only (0.1 % v/v). Subsequent work was performed with washed platelets treated with 1 µM indo-1/AM
dissolved in DMSO. Additional control platelets prepared with 0.1 % v/v DMSO were also tested.

Aliquots (0.25 ml) of the washed platelet preparations were assayed for response to adenosine diphosphate (ADP) (10 μM, Sigma Chemical), collagen (2 mg/liter, Hormon-Chemie) and human thrombin (0.25 U/ml, Calbiochem) at 37°C by a turbidimetric method in a commercial aggregometer (Whole Blood Aggregometer, ChronoLog). Three parameters were quantified from the optical signal vs. time recorder trace: lag time to shape change, maximum rate of reaction and total percent decrease in optical signal (79).

C. Results

1. response to chemical agonists

Figure III-1 displays the results of ADP, collagen and thrombin addition to washed platelet suspensions in the aggregometer. Platelets treated with quin2/AM exhibited significantly longer reaction lag times than did untreated controls (Student's paired t-test, p < 0.01) after exposure to any of the three agonists. With two of the agonists, a statistically significant difference between loaded and control cells was also observed in the maximum slope of the optical density vs. time recording. However, the quin2⁴⁻-containing cells produced the same ultimate decrease in optical density in response to all three aggregating agents. Final intracellular quin2⁴⁻ concentrations averaged 956 ± 167 μM.
Figure III-1  Response of quin-2<sup>4</sup>- loaded washed platelets (■■■■) and control washed platelets (□□□□) to added agonists in a turbidimetric aggregometer. Platelet aggregation was measured 300 seconds after introduction of the agonist. Data: mean ± standard deviation.

*, p < 0.01; **, p < 0.001 (Student's paired t-test)
Figure III-2 presents the response of indo-1/AM treated platelets to chemical agonists. The lag time to incipient reaction was the same for control and indo-1/AM treated platelets (data not shown). In addition, the optically measured percent aggregation and the reaction rate of platelets containing indo-1-4' in response to any of the chemical agents was not significantly different than the response of unlabeled control platelets. Final intracellular indo-1-4' concentration averaged 178 ± 37 μM.

2. response to shear stress

Platelet suspension response to shear stress is shown in Figure III-3 (30 second exposure time), and Figure III-4 (300 second exposure time) for control and quin2/AM treated samples. Platelet aggregation, as measured by the percentage reduction of platelet-sized particles, increased monotonically with shear stress except at the highest shear for 300 seconds exposure.

Viscometer controls exposed to no shear stress for 300 seconds exhibited a reduction in singlet count corresponding to 20 percent aggregation. This unexpected result is not found with anticoagulated PRP samples. A series of experiments were initiated where either sodium heparin (10 U/ml), sodium citrate (0.38%) or both were added to the washed platelet suspension and the results of a 300 second viscometer control run were compared to those obtained with the standard, un-anticoagulated washed platelets. No difference was found between the treated and untreated
Figure III-2. Response of indo-1^- loaded washed platelets (XXXX) and control washed platelets (■■■■) to added chemical agonists in a turbidimetric aggregometer. Platelet aggregation was measured 300 seconds after introduction of agonist. Data: mean ± standard deviation.

*, p < 0.01; **, p < 0.001 (Student's paired t-test)
Figure III-3. Response of quin2− loaded washed platelets (□) and control washed platelets (△) to shear stress in the absence of added chemical agonists in the rotational viscometer. The exposure time to the shear field was 30 seconds. Data: mean ± standard deviation.

*, p < 0.01; **, p < 0.001 (Student's paired t-test)
Figure III-4. Response of quin2^4- loaded washed platelets (□) and control washed platelets (△) to shear stress in the absence of added chemical agonists in the rotational viscometer. The exposure time to the shear field was 300 seconds. Data: mean ± standard deviation.

*, p < 0.01; **, p < 0.001 (Student's paired t-test)
platelets. This result suggests that the reduction in platelet number under these conditions may be the result of simple settling rather than platelet surface activation. The $^3$H-serotonin release results at 0 dynes/cm$^2$ (Figures III-3b, III-4b) are elevated slightly, perhaps due to a settling-related response.

Platelets treated with quin2/AM responded to shear stress in a fashion qualitatively similar, but quantitatively different than the corresponding controls. For all shear stress values which produced significant aggregation in the control sample, the quin2$^{4-}$-containing platelets responded with much less aggregation. This finding is consistent for both 30 second (Figure III-3a) and 300 second (Figure III-4a) times of exposure to the shear field. The viscometer control, however, produced the same percentage reduction in platelet singlets regardless of quin2$^{4-}$ loading.

After 30 seconds of shear exposure, the treated cells produced supernatant serotonin and LDH values which are indistinguishable from those generated by controls (Figures III-3b, III-3c). However, during 300 seconds of shear exposure time, quin2/AM-treated cells released a much greater percentage of their serotonin and LDH than did corresponding controls (Figures III-4b, III-4c).

Shear-induced platelet lysis and serotonin release values reported here are greater than those found with PRP under corresponding conditions (4,5,43-46,77). Washed
platelets prepared by this method appear to be more fragile than platelets suspended in native, anticoagulated plasma. Figures III-5 and III-6 display the shear stress induced responses of control and indo-1/AM treated platelet suspensions after 30 and 300 seconds of exposure, respectively. The endpoint measurements obtained from indo-1/AM treated platelets exposed to shear stress are not significantly different than those of control platelets. Platelet aggregation was greater after 300 seconds than after 30 seconds of exposure to 50 dynes/cm$^2$. The reverse is true at increased levels of applied shear stress. Percent platelet dense granule release was greater than percent cell lysis under all shear stress-exposure time conditions. In addition, platelet dense granule release and cellular lysis both occur to a greater extent with increasing shear stress exposure time.

Addition of 0.025 ADPase units/ml of grade V apyrase to the final platelet resuspension solution was experimentally determined to improve platelet suspension reactivity to added chemical agonists and to shear stress alone. Differences in the response of control washed platelet suspensions used in the quin2/AM and the indo-1/AM studies are directly attributable to this modification of the platelet washing technique performed on the latter.

D. Discussion and Conclusions
Figure III-5. Response of indo-1$^{4-}$ loaded washed platelets (□) and control washed platelets (△) to shear stress in the absence of added chemical agonists in the rotational viscometer. Exposure time to the shear field was 30 seconds. Data: mean ± standard deviation.

*, p < 0.01; **, p < 0.001 (Student's paired t-test)
Figure III-6. Response of indo-1²⁻ loaded washed platelets (□) and control washed platelets (△) to shear stress in the absence of added chemical agonists in the rotational viscometer. Exposure time to the shear field was 300 seconds. Data: mean ± standard deviation.
* p < 0.01; ** p < 0.001 (Student's paired t-test)
The results of Figure III-1 show that platelet aggregation is reduced in quin2\(^4^-\)-containing platelets thirty seconds after addition of an agonist. Three hundred seconds after addition of the agonist, the indicated aggregation of the quin2/AM-treated platelets is indistinguishable from that of controls. The ability of quin2/AM-treated platelets to eventually achieve the same extent of aggregation in response to chemical agonists as controls indicates that neither quin2/AM nor its reaction products have toxic effects on the platelets.

Chemical aggregating agents cause a rise in the concentration of intracellular free calcium ion which precedes shape change and aggregation (82,83). The magnitude of this increase is such that a millimolar loading of intracellular quin2\(^4^-\) is able to act as a finite calcium-ion sink. This effect delays the onset of activating levels of free calcium-ion and is responsible for the increased lag time seen in response to ADP, collagen, and thrombin. Quin2\(^4^-\) delays, but does not eliminate, intracellular calcium ion concentration transients. The calcium ion buffering effects of quin2\(^4^-\) are expected to become insignificant after times which are long compared to calcium ion mobilization time.

Figure III-2 indicates that indo-1/AM treatment does not measurably perturb platelet function in response to chemical agents. The primary difference between quin2/AM and indo-1/AM treatment is the intracellular concentration
of the fluorescent indicator. Apparently, the lower intracellular concentration of indo-1\(^{4-}\) as compared to quin2\(^{4-}\) is responsible for the reduced extent of platelet inhibition. This result is in agreement with the postulated quin2\(^{4-}\) inhibition mechanism. Because indo-1\(^{4-}\) is 30 times more fluorescent than quin2\(^{4-}\) and generates a shift in emission wavelength upon Ca\(^{2+}\) chelation, the reduced concentration of indo-1\(^{4-}\) actually represents an intracellular calcium ion measurement technique which is more sensitive than the greater concentration of quin2\(^{4-}\).

For the case of shear-induced activation, the picture is less clear. Shear field exposure induces much greater aggregation in the control platelet suspensions than in the platelets which were quin2/AM-treated. To determine whether this effect was solely as a result of the increased lag time to calcium mobilization, samples were also sheared for 300 seconds. Under these conditions, the quin2\(^{4-}\)-containing platelets again appeared to aggregate much less than did control cells. The result at 300 seconds is clouded by additional information which reveals that quin2/AM-treated platelets had significantly greater values of lysis and release than did controls. Evidence from prior work (9,10) suggests that the apparent reduction in platelet aggregation is due, at least in part, to an increase in the number of platelet fragments as a result of increased lysis.

These results indicate that quin2\(^{4-}\) has effects distinct from alteration of intracellular calcium ion
concentration transients. EDTA, a chemical relative of quin2\(^{4-}\), has been shown to irreversibly alter components of the cellular membrane (91,116,117). It is possible that quin2\(^{4-}\) has similar effects on the plasma membrane resulting in increased cellular fragility as measured by elevated platelet lysis and release. It seems clear that quin2\(^{4-}\) is a suitable intracellular calcium ion probe for platelets only under relatively moderate shear stress and exposure time conditions.

Indo-1/AM treatment does not result in any detectable changes in platelet aggregation, dense granule release or lysis in response to either 30 or 300 seconds of shear stress application. The relatively low concentration of this tetracarboxylate chelator required to obtain a strong fluorescent signal in response to changing platelet intracellular calcium ion concentration results in a greatly reduced degree of platelet response modification. Indo-1/AM appears to be an appropriate choice for measurement of platelet intracellular calcium ion concentration changes in response to chemical or physical stimulus.
CHAPTER IV

INFLUENCE OF EXTRACELLULAR ADP ON SHEAR STRESS INDUCED PLATELET ACTIVATION

A. Introduction

Consistent experimental results have directly and indirectly indicated a significant role for ADP participation in shear stress mediated platelet aggregation (42,76). Isolation of ADP induced platelet response in the shear field is complicated by at least four factors. 1) The time history and level of background ADP concentration in the platelet suspending fluid is important. Background ADP can serve as an accelerator of platelet aggregation induced by other means (54). Slightly altered conditions can cause the platelet suspension to become refractory to ADP and lose reactivity with time. 2) ADP is actively released along with the other components contained in platelet dense granules. 3) Metabolic ADP can be discharged to the platelet suspension as a result of cellular damage and lysis. 4) Platelet response is strongly dependent on the local, rather than the bulk, concentration of chemical agonist. Low bulk phase ADP concentrations may be the result of ADP diffusion from a high local concentration near the individual cells (after dense granule release or lysis) to the platelet free volume which constitutes 99.7% of the suspending medium.
Creatine phosphate/creatine phosphokinase is a substrate/enzyme system which phosphorylates ADP to ATP. Previous studies have used this reaction to rapidly consume release or lysis generated ADP during platelet exposure to shear stress (49,76). This technique has the secondary effect of producing ATP which is a competitive inhibitor of ADP induced platelet aggregation.

Apyrase enzymatically dephosphorylates ATP to ADP and ADP to AMP. Use of apyrase in removing extracellular ADP has been limited to platelet washing applications where resuspension of a platelet button formed after centrifugation can induce a low level of dense granule release and cellular lysis.

Use of neither CP/CPK nor apyrase, both bulk phase scavengers, address the relative importance of local ADP concentrations in shear induced platelet activation (SIPAC). A chemical analog of ADP, 5'-p-fluorosulfonylbenzoyladenosine (FSBA), has been reported to covalently modify a single protein in intact platelets (11,31). Platelets treated with FSBA, as compared to identical but non-treated cells, demonstrate inhibited shape change, aggregation and fibrinogen binding in response to added ADP (31). Platelet response to other chemical activators such as calcium ionophore A23187 and human thrombin was shown to be unaffected by FSBA pre-treatment. The FSBA technique was modified to include adenosine deaminase (AD) during incubation (74) to convert any
adenosine, a platelet function inhibitor produced as a spontaneous decomposition product of FSBA, into inosine.

B. Purpose

A more exhaustive investigation of the importance of released ADP on shear induced platelet response was indicated on the basis of published findings (43,76). Both CP/CPK and apyrase were used as bulk phase inhibitors of ADP-mediated platelet activation. The effect of local ADP concentration was studied by blocking the ADP binding sites at the platelet membrane with FSBA. The effects of inhibitor treatment were also monitored in response to added ADP with a commercial platelet aggregometer to determine extent of inhibition. Collagen and thrombin were also employed as chemical agonists to estimate the significance of released ADP on other biochemical mechanisms of platelet aggregation. All experiments performed at 24°C were duplicated at 37°C. Comparison of control sample results provided direct evidence for temperature effects on SIPAC. The effectiveness of inhibition as a function of temperature was also observed.

C. Experimental Methods

Washed platelets were prepared and radiolabeled with \(^3\)H-serotonin as described in Chapter II. The final platelet suspension, which contained 0.025 ADPase units/ml of grade V apyrase, was divided into four aliquots. Aliquot one was incubated for five minutes before testing with 2.5 ADPase
units/ml of grade V apyrase (Sigma Chemical Co.) dissolved in normal saline. Number two was treated with CP (5 mM) and CPK (10 units/ml; both from Sigma Chemical Co.) dissolved in HEPES platelet washing buffer (pH = 7.35). Incubation time before testing was five minutes. Aliquot three was incubated for 60 minutes prior to testing with FSBA (45 μM) dissolved in dry DMSO and AD (2 units/ml; both from Sigma Chemical Co.) dissolved in HEPES platelet washing buffer (pH = 7.35). The fourth sample was an untreated control to which HEPES platelet washing buffer (pH = 7.35) was added. Each platelet suspension was sheared in the unmodified cone and plate viscometer at 0, 50, 100 or 150 dynes/cm² for 60 seconds. A subset of the shear exposures were 300 seconds to allow more direct comparison with other studies. Platelet suspensions were sheared and stored at either 24°C or 37°C; both temperatures were employed for each experiment. The sheared sample was reserved for population size measurement and particle counting, assay of dense granule release from supernatant ³H-serotonin scintillation counting and platelet lysis determined from supernatant LDH activity. All assays are detailed in Chapter II.

Platelet response to added chemical agonists was performed as described in Chapter III, section B with the following alterations. The reaction lag time was not determined and four different samples were assayed (control and three treated aliquots). Two concentrations of added ADP were used (2 μM and 10 μM) and all experiments performed
at 37°C were duplicated at 24°C. One 10 μl sample of the platelet suspension was removed and placed into 10 ml of Isoton containing 0.5% gluteraldehyde. A duplicate sample was reserved 30 seconds after addition of 2 μM ADP. The population size distributions of these preparations were determined as described in Chapter II, section C.

D. Results

1. Platelet response to added chemical agonists; 37°C

Figure IV-1 displays the ultimate percent increase in transmitted light intensity after addition of chemical aggregating agents to each of the four platelet suspension aliquots. Figure IV-2 shows the maximum rate of reaction recorded during these experiments.

Addition of ADP to the control platelet suspension resulted in a dose-dependent extent of aggregation and rate of reaction. Platelets treated with either of the ADP scavenging enzyme systems or with FSBA/AD did not respond measurably to the same concentrations of added ADP. Addition of apyrase or CP/CPK resulted in inhibition of collagen induced reaction rate and extent of reaction as compared to untreated controls. FSBA/AD treatment abolished platelet response to added collagen. Apyrase, CP/CPK and FSBA/AD inhibited the rate of reaction to added thrombin by 6.4, 20.2 and 49.0 percent, respectively, compared to the control washed platelet suspension. Within five minutes of thrombin addition, however, the ultimate reaction extent of
Figure IV-1. (top) Percent transmitted light intensity recorded by a turbidimetric aggregometer after addition of chemical agonists at 37°C. Samples treated as described in text. Data: mean ± standard deviation.
* p<0.05, ** p<0.01, *** p<0.001 (Student's paired t-test versus control)

Figure IV-2. (bottom) Maximum slope of the percent transmitted light intensity versus time curve recorded by a turbidimetric aggregometer at 37°C. Samples as described in text. Notation as given in Figure IV-1.
the treated platelets could not be distinguished from that of control platelets.

2. Platelet response to shear stress; 37°C

Figures IV-3, IV-4 and IV-5 exhibit, respectively, the percent reduction in platelet singlet concentration, the percent dense granule release and the percent platelet lysis after 60 second exposure to the indicated levels of shear stress for the control sample and the three treated platelet suspensions.

Percent aggregation of the control washed platelet suspension increased monotonically with applied shear stress intensity. The levels of shear induced platelet dense granule release and cellular lysis in the control sample also rose smoothly with increasing shear stress.

Platelets treated with CP/CPK exhibited no measurable aggregation in response to applied shear stress. The same aliquots demonstrated increased ability to release dense granule components at 100 and 150 dynes/cm² (Student's paired t-test; \( p < 0.05 \)) as compared to control samples. Shear stress application induced slightly more cellular damage (\( p < 0.05 \)) in the CP/CPK treated sample than in the control suspension at 50 dynes/cm², but not at higher shear stress levels.

Addition of apyrase to the platelet suspension reduced aggregation in a statistically significant way only at 150 dynes/cm² (\( p < 0.05 \)). The dense granule release of the platelet suspensions with added apyrase was greater than the
Figure IV-3. Percent aggregation of platelet suspensions exposed to 60 seconds of shear stress at 37°C. Samples treated as described in text. Notation as shown in Figure IV-1.
Figure IV-4. (top) Percent dense granule release of platelet suspensions exposed to 60 seconds of shear stress at 37°C. Samples treated as described in text. Notation as shown in Figure IV-1.

Figure IV-5. (bottom) Percent lysis of platelet suspensions exposed to 60 seconds of shear stress at 37°C. Samples treated as described in text. Notation as shown in Figure IV-1.
corresponding response of untreated control platelets at all non-zero levels of applied shear stress. Percent dense granule release in the apyrase containing sample also increased smoothly with increasing shear stress level. Cellular lysis in the apyrase treated suspension differed from the control value only at 50 dynes/cm² and never exceeded 5%.

Incubation of the platelet sample with FSBA/AD resulted in aggregation response to applied shear stress which was independent of the shear field intensity. Aggregation was statistically suppressed at both 100 and 150 dynes/cm² but not at 50 dynes/cm². FSBA/AD treatment, as compared to untreated controls, resulted in increased extent of platelet dense granule release at all non-zero shear stress levels. The percent release measured was also a monotonic function of shear stress. Platelet lysis was unaffected by FSBA/AD incubation at all shear stresses studied.

In every non-zero shear stress/sample treatment case, the extent of dense granule release exceeded the percent platelet lysis. The viscometer controls of each aliquot, having higher supernatant LDH activity, were the exception. The increased level of platelet dense granule release as a result of inhibitor addition is interesting to note in relation to the reduced or unaltered degree of platelet aggregation in the treated samples.

3. Platelet response to added chemical agonists; 24°C
Results of chemical agonist addition to control and treated samples are contained in Figure IV-6 (final percent aggregation) and Figure IV-7 (maximum reaction rate). Comparing the response of untreated platelets to addition of ADP at 24°C (Figures IV-6 and IV-7) and at 37°C (Figures IV-1 and IV-2) reveals a statistically significantly higher percent aggregation (p < 0.001; 2 μM and 10 μM), but not rate of reaction, at the lower temperature.

Percent aggregation and reaction rate in response to 10 μM ADP were measurable, but significantly less than control values, for all three treated aliquots. The 2 μM dose of ADP stimulated non-zero response from all but the CP/CPK treated aliquot. In all cases, both measures of platelet response proceeded to a greater extent with the addition of 10 μM ADP as compared to 2 μM ADP.

The extent of response of control platelets to the addition of collagen or thrombin was relatively unaltered at 37°C as compared to 24°C. Control platelet suspension reaction rate to collagen and thrombin was significantly greater at 37°C than at 24°C. The response of control platelets to added ADP exhibited the reverse temperature dependency; the reaction rate was relatively temperature independent as compared to the increase in reaction extent measured at 37°C as compared to 24°C. The two bulk phase ADP scavengers induced small, but statistically significant (p < 0.01), reductions in both the reaction extent and rate in response to the addition of collagen. FSBA/AD treatment
Figure IV-6. (top) Percent transmitted light intensity recorded by a turbidimetric aggregometer after addition of chemical agonists at 24°C. Samples treated as described in text. Notation as given in Figure IV-1.

Figure IV-7. (bottom) Maximum slope of the percent transmitted light intensity versus time curve recorded by a turbidimetric aggregometer at 24°C. Samples treated as described in text. Notation as given in Figure IV-1.
completely abolished collagen induced response. All three treated aliquots were inhibited in response to thrombin with respect to both percent aggregation and reaction rate except the reaction extent of the CP/CPK sample.

4. Platelet response to shear stress; 24°C

Figures IV-8, IV-9 and IV-10 display the percent loss of single platelets, the percent dense granule release and the percent cellular lysis, respectively, of the four aliquots after 60 second exposure to the indicated level of shear stress. Aggregation of the control sample exceeded 90% when the applied shear stress was equal to or greater than 50 dynes/cm². All three treated aliquots exhibited reduced aggregation in comparison with the control platelet suspension (p < 0.001) at all three shear stresses studied. The aggregation response of these treated samples did, however, increase with increasing shear stress level. The aliquot treated with CP/CPK aggregated to a lesser extent than any of the other samples.

Lysis of the control sample as a result of shear field exposure was greater than that experienced by any of the treated samples. The extent of cellular lysis in the treated samples never exceeded 2% and was a monotonic function of applied shear stress magnitude except in the case of apyrase treatment where the lysis value was not a function of shear stress.

An elevated level of cellular damage was also indicated in the generally higher values of dense granule release from
Figure IV-8. Percent aggregation of platelet suspensions exposed to 60 seconds of shear stress at 24°C. Samples treated as described in text. Notation as shown in Figure IV-1.
Figure IV-9. (top) Percent dense granule release of platelet suspensions exposed to 60 seconds of shear stress at 24°C. Samples treated as described in text. Notation as given in Figure IV-1.

Figure IV-10. (bottom) Percent lysis of platelet suspensions exposed to 60 seconds of shear stress at 24°C. Samples treated as described in text. Notation as given in Figure IV-1.
the control aliquot. Differences in the dense granule release between untreated and treated platelet suspensions were significant at 50 dynes/cm$^2$ and, for the apyrase treated aliquot, also at 150 dynes/cm$^2$. Dense granule release from each of the samples treated with an inhibitor was a smooth function of applied shear stress intensity.

5. Platelet response to shear stress; 300 second exposure

Figure IV-11 contains the aggregation, dense granule release and lysis response of control and CP/CPK treated platelets exposed to 300 seconds of shear at 37°C. The degree of control platelet aggregation increased with increasing shear stress in contrast to the CP/CPK treated platelets which exhibited no measurable aggregation at any shear stress studied. The levels of dense granule release and platelet lysis were monotonic functions of applied shear stress intensity for both the treated and untreated samples. Cellular lysis and, correspondingly, apparent dense granule release were greatly elevated after 300 seconds of shear stress exposure as compared to 30 seconds (Figures IV-4 and IV-5). Except for the CP/CPK treated aliquot sheared at 100 dynes/cm$^2$, the percent dense granule release and extent of lysis response of the two samples were identical. Note that CP/CPK addition completely abolished shear induced platelet aggregation under these conditions without measurably altering dense granule release or cellular lysis.

6. Volume distribution of ADP induced aggregates
Figure IV-11. Percent aggregation (a), percent dense granule release (b) and percent lysis (c) of platelet suspensions exposed to 300 seconds of shear stress at 37°C. Control (□), CP/CPK treated (△). Samples treated as described in text. Notation as given in Figure IV-1.
Figure IV-12 shows the percentage of total particle volume represented by four volume subsets of the treated and control platelet samples before addition of 2 μM ADP. Figure IV-13 is the same measurement made 30 seconds after addition of the agonist.

All samples were composed of particles having the same volume distribution before ADP addition. Approximately 45% of the total volume was represented by individual particles with volumes between 2.55 μm³ and 8.97 μm³. An additional 45% of the total volume was contributed by particles of 8.97 μm³ to 51.1 μm³. The remaining 10% was represented by particles greater than 51.1 μm³.

In the untreated sample, the fraction of particles larger than 51.1 μm³ increased from 9% to 75% of the total volume after addition of ADP. The largest volume fraction measured represented 4% of the volume before ADP treatment and 62% after. Some aggregates in this sample exceeded the maximum measurable volume of 8580 μm³ (as determined from the total particle volume from 2.55 μm³ to 8580 μm³).

Thirty seconds after ADP addition to the apyrase treated aliquot, 86% of the total volume was represented by particle volumes from 51.1 μm³ to 8580 μm³. Before agonist treatment, 94% of the particle volumes were between 2.55 μm³ and 51.1 μm³. The largest particles measured comprised 46% of the total volume after ADP addition.

CP/CPK treatment abolished the formation of aggregates and the concomitant reduction of small volume particles in
Figure IV-12. (top) Particle distribution by volume of platelet samples before addition of 2 μM ADP at 37°C. Samples treated as described in text. Notation as given in Figure IV-1.

Figure IV-13. (bottom) Particle distribution by volume of platelet samples 30 seconds after addition of 2 μM ADP at 37°C. Samples treated as described in text. Notation as given in Figure IV-1.
response to added ADP. Particles smaller than 51.1 μ³ represented 89% of the total volume before agonist addition as compared to 87% after. The largest measured volume fraction changed from 7% to 4% of the entire platelet volume after ADP addition.

The population volume distribution of FSBA/AD treated platelet suspensions exhibited a shift of particle volumes from the two fractions less than 51.1 μ³ (87% before, 70% after) to particles 51.1 μ³ through 490 μ³ (4% before, 25% after). The increase in volume fraction of aggregates 490 μ³ to 8580 μ³ was 2% of the total volume (from 4% before to 6% after).

The percent increase in transmitted light intensity (percent aggregation) of the platelet suspensions at the time of sampling was 29% for the control platelets and 5%, -5% and -3% for the platelet suspensions treated with apyrase, CP/CPK and FSBA/AD, respectively.

E. Discussion and Conclusions

Finding that platelets are more responsive to added ADP at 24°C than at 37°C only reconfirms previous knowledge. Due to this temperature induced change in responsiveness, treatments which were shown to abolish platelet aggregation in response to the addition of 2 μM or 10 μM ADP at 37°C provide a reduced, but significant, level of inhibition under the same conditions at 24°C.
At 24°C the number of single platelets incorporated into aggregates as a result of shear stress application is higher than at 37°C. The temperature effects on platelet aggregation induced by added ADP are qualitatively the same as those measured after shear stress induced aggregation. This observation may be no coincidence and, therefore, may represent further indirect evidence supporting a positive role for platelet derived supernatant ADP in SIPAC. This temperature sensitivity behavior should be useful in the design of future biochemical mechanism studies of shear stress induced platelet response.

Mechanical damage of the large aggregates formed during shear stress exposure at 24°C was presumably responsible for the elevated values of cellular lysis and dense granule release exhibited by the control platelet suspension. This physical disruption of platelets can result from enhanced surface contact forces which occur when the mean aggregate size approaches the cone-to-plate distance. Exceptionally large platelet aggregates can get "pinched" or "squeezed" between the rotating cone and platen. Aliquots treated with any of the three inhibitors produced aggregates of smaller mean volume than the controls in response to applied shear stress. These treated samples did not exhibit elevated platelet lysis values.

Platelet response to addition of collagen was moderately impaired by the two bulk phase inhibitors indicating that a portion of the collagen induced response
depends on ADP released from dense granules. The finding of complete inhibition by FSBA of collagen induced response at both temperatures has recently been confirmed by others (32). Platelet synthesis of thromboxane A$_2$ in response to collagen is reported to be unaffected by FSBA/AD modification (32), leading to the postulate that released ADP was required for platelet aggregation to collagen. This theory is unlikely in light of the collagen induced platelet reaction measured in the presence of either bulk ADP scavenging enzyme system. Production of TXA$_2$ indicates that FSBA/AD treatment does not abolish recognition and binding of collagen at the platelet surface receptor. The well-documented ability of FSBA to block platelet fibrinogen binding, however, is likely responsible for the observed inhibition. Without surface-bound fibrinogen, platelet aggregation, regardless of activation extent, is weak and reversible.

FSBA may, through inhibition of fibrinogen binding, prevent the formation of large platelet aggregates but be ineffective at preventing the creation of small aggregates. In this situation, the degree of platelet aggregation reported by an optical aggregometer would be nearly zero because the transmitted light intensity measurement technique is insensitive to the formation of small aggregates (97). Experimental evidence using a Coulter Counter to determine the particle size distribution in response to added agonist indicates that small aggregates do
form in FSBA/AD treated samples. The information presented in Figures IV-12 and IV-13 emphasizes the limitation of the optical aggregometer to discriminate only between the formation of large aggregates and small aggregates; no information can be extracted about the incorporation of single platelets into small aggregates.

The effects of bulk phase ADP consumption on thrombin induced response indicate that released ADP participates by increasing the reaction rate with little impact on the ultimate extent of aggregation. In contrast to earlier findings (31,74), FSBA/AD treatment reduces platelet reaction rate to added thrombin. This result supports the postulated mechanism of FSBA/AD inhibition resulting from direct modification of platelet fibrinogen binding. The unaltered percent aggregation of the FSBA/AD treated platelets to added thrombin is due to platelet adhesion to the bulk phase clot formation which results from the thrombin induced polymerization of fibrinogen to fibrin.

Each of the three inhibitor systems effectively prevented platelet aggregation (measured in the aggregometer) to added ADP at 37°C, but only CP/CPK treatment abolished shear stress induced platelet aggregation. The difference in inhibition effectiveness was not due to reduction of either shear induced dense granule release or cellular lysis which were both greater in all treated samples than in the control preparation. Under the experimental conditions used, the rate of CP/CPK reaction
was able to prevent formation of a membrane ADP concentration sufficient to promote platelet activation.

At 24°C, CP/CPK was unable to completely abolish aggregation induced by shear stresses greater than 50 dynes/cm². This reduction in inhibition is not the result of temperature induced enhancement of dense granule release but due to a lower platelet threshold for aggregation at 24°C than at 37°C. Thus, the same low concentration of released ADP which remains at the platelet surface despite CP/CPK addition is able to induce shear stress mediated response at 24°C but not at 37°C. ADP diffusion from the platelet surface after release occurs more quickly at 37°C than at 24°C and may play a role in the observed temperature effects.

Apyrase, the other bulk phase ADP scavenger, was not as effective as CP/CPK at preventing platelet aggregation to either addition of ADP or to shear stress exposure. The concentration of apyrase may have been insufficient to remove released ADP before significant platelet-ADP binding could occur. The apyrase concentration was restricted by the observation that high doses of apyrase alone could support platelet aggregation. This effect was noted in five different commercial preparations of potato apyrase and has been observed by other workers (16). The specific apyrase preparation used and the concentrations employed were selected to be below the threshold of detectable platelet aggregation. Another consideration is the relative kinetics
of ADP degradation and ATP degradation by apyrase. Platelet dense granules contain two parts ATP per three parts ADP. The selectivity of the enzyme for ADP over ATP and the relative rates of reaction may play additional roles in limiting the effectiveness of apyrase in this system. Platelet inhibition due to ATP production from the reaction between CP/CPK and ADP could serve to increase the inhibition ability of the CP/CPK system relative to that of apyrase.

Treatment with FSBA/AD provides inhibition of platelet reaction in response to local (rather than bulk) ADP concentrations. This inhibition is independent of enzymatic reaction rates since FSBA/AD acts by preventing ADP from binding to the active site on the platelet membrane. The importance of released ADP in SIPAC does not appear to be supported by the response of FSBA/AD treated platelets. FSBA/AD strongly inhibited platelet response to added ADP. However, FSBA/AD was much less effective in inhibition of shear induced aggregation. Platelet binding of FSBA is slow, requiring a 60 minute incubation for maximal effect. Shape change is known to expose platelet membrane binding sites which are unavailable before activation. Shear stress application is also believed to induce exposure of previously unavailable binding sites which, therefore, would not be modified by previous FSBA/AD incubation. The time scale of inhibition through FSBA binding is much longer than the time scale of platelet membrane binding of released ADP.
In addition, aggregation results obtained from the light transmission technique are relatively insensitive, and do not reflect the formation of small aggregates which occurs after ADP addition to FSBA/AD treated samples. Thus, ADP may play a role in the observed shear induced aggregation in FSBA/AD treated platelet suspensions.

The inhibited response of platelet samples treated with CP/CPK was measured after 60 seconds of shear stress exposure. CP/CPK treatment was also an effective inhibitor of shear induced platelet response after 300 seconds of exposure. Shear stress exposure time of 300 seconds corresponds to conditions reported in previously published work (76). CP/CPK treatment abolished shear stress induced platelet aggregation despite identical dense granule release and lysis results as compared to the control platelet suspension. The concentration of single platelets in the control suspension present after 300 seconds of shear exposure was greater than that after 30 seconds due, in part, to disaggregation in the shear field. In addition, cellular lysis fragments, which were more numerous after the longer shear stress exposure, can be misinterpreted as single platelets in the electronic particle counter. This artifact increased the number of platelet sized particles counted and artificially depressed the calculated percent aggregation. The inhibitory effect of CP/CPK on shear stress induced platelet activation in this study is stronger
than, but in qualitative agreement with, the results of other workers (76).

Clearly, the response of CP/CPK treated platelet suspensions is further evidence supporting a significant role for ADP in shear stress induced platelet activation. The specific role of ADP in the shear field remains unknown. Specifically, 1) can the low bulk concentration of ADP measured represent local concentrations high enough to induce platelet reaction alone or 2) does extracellular ADP act to potentiate other, as yet unidentified, biochemical reactions in platelet response to applied shear stress? Information regarding the source of supernatant ADP (dense granules or cell lysis), the kinetics of release and the mass transfer characteristics of the system are required before an estimate of the local ADP concentration in the shear field can be obtained.
CHAPTER V

DESIGN OF THE RICE OPTICAL MEASUREMENT VISCOMETER

A. Purpose of modification

The study of platelet response to applied shear stress has been carried out in the past by measurement of some index of platelet function or state before-and-after subjecting a specimen to shear stress. Platelet response to shear stress is a dynamic event which is not adequately represented by discrete measurements, especially during the first few seconds of shear stress exposure when the activation mechanism is thought to initiate. These first few seconds are the least well studied for shear stresses of physiologic significance.

A cone and plate device which was able to measure changes in platelet suspension apparent optical density as an indication of platelet aggregation during shear stress exposure was developed (63,64,87). A large cone angle (3 degrees) was employed to satisfy the optical geometry of this instrument. As a result, published studies include results of platelet behavior only at shear rates below 115 seconds$^{-1}$. Although shear stress induced platelet activation has not been detected under these conditions subsequently, a decrease in the apparent optical density, indicating platelet aggregation, was recorded by this instrument. The measured alteration in platelet suspension optical characteristics was likely to have resulted from
alignment of the asymmetric platelets in the uniform shear field.

Thus, the primary goal of this research project was development of methodology for continuous measurement of platelet aggregation, dense granule release and intracellular calcium ion mobilization in controlled shear fields of at least 4167 seconds$^{-1}$.

B. General Description of Viscometer

An existing cone and plate viscometer was chosen for modification (Ferranti Electric; model 781) (Figure V-1). This instrument provides selection of controlled cone rotational rates (0 - 1000 revolutions per minute), easily interchangeable cone diameters and angles, accurate control of cone to platen gap height, and rapid step changes in cone speed without overshoot or instability. The design goal was instrument modification to allow application of continuous measurement techniques without compromising the flexibility or accuracy of shear field application. In addition, the modifications were to be easily reversible for continuation of studies using the viscometer in the traditional configuration.

C. Continuous Measurement Techniques

Platelet aggregation can be monitored using a turbidometric technique as employed in most commercial "platelet aggregometers". This optical method has been adapted for use in viscometers to measure microrheology
Figure V-1. Ferranti-Shirley rotational viscometer before modification. Measurement unit, far right; motor and speed control electronics, center; temperature control bath, left.
parameters of platelet suspensions (33,39). A collimated beam of light, often monochromatic, is focused on a platelet suspension. A suitable photosensitive device measures the intensity of incident light which passes through the platelet sample. Initially, the suspension is composed of relatively many small particles which scatter a substantial fraction of the incoming light away from the detector. Platelet aggregation induced by chemical or mechanical stimulus results in the loss of single platelets and the formation of relatively few aggregates. The particle sizes formed during chemically induced platelet aggregation scatter less light, resulting in an increase in the transmitted light intensity recorded by the photodetector. This technique was applied as a measure of platelet aggregation in the viscometer during shear stress application.

Platelet dense granules contain serotonin, ATP, ADP, calcium ion and pyrophosphate. An extremely sensitive luminescence assay for ATP has been described which uses the firefly luciferase enzyme (105,106). Activation of d-luciferin with the magnesium salt of ATP (MgATP) results in the formation of luciferase bound d-luciferyl adenylate and free pyrophosphate. The d-luciferyl adenylate is then oxidatively decarboxylated to AMP, CO₂ and electronically excited oxyluciferin. When the electronically excited oxyluciferin returns to the ground state, a photon is emitted with an overall quantum yield of 0.88 (71).
Luciferase can catalyze additional reaction cycles after the release of AMP and oxyluciferin from the active enzymatic site. The bound oxyluciferin persists on the enzyme and is considered a noncompetitive inhibitor of the luminescent reaction. The luminescent intensity is proportional to reaction velocity which is a function of the oxyluciferin concentration. The time course of ATP release can be calculated from a continuous measurement of luciferin-luciferase luminescence using the Michaelis-Menten rate equation with correction for presence of the noncompetitive inhibitor (Chapter VI, section B).

A technique for measurement of intracellular calcium ion concentration in small, intact cells has been described using a family of tetracarboxylate calcium ion chelators which change their fluorescent properties upon calcium ion binding (40,100). Quin2/AM and indo-1/AM are two members of this family which have been esterified at each of their four carboxylic acid sites. This treatment renders the molecules membrane permeant. Once inside the cell, naturally occurring esterases cleave the acetoxyethyl esters, producing the cell impermeant, four anionic (quin2$^{4-}$, indo-1$^{4-}$) calcium ion chelators. Quin2$^{4-}$ shares the same backbone structure and divalent cation chelation properties of ethylenediaminetetraacetic acid (EDTA) (100). Quin2$^{4-}$ shows a five-fold increase in fluorescent intensity upon calcium ion chelation and has an apparent dissociation constant in platelets of 115 nM (101). Indo-1$^{4-}$ is a second generation
member of this family; it offers numerous performance improvements over quin2⁺₄⁻ including greater fluorescent intensity, a shift of emission wavelengths rather than a simple change in fluorescent intensity, better selectivity for Ca²⁺ over other divalent cations and a higher apparent dissociation constant (250 nM) (40). Platelets have been successfully loaded with these indicators and resting as well as transient intracellular calcium ion concentrations have been determined (8,20,28,41,48,61,62,82-84,86,103,115).

D. Overview Of Viscometer Modifications

The optical nature of the three measurement techniques and the cone and plate viscometer geometry dictated replacement of the existing stainless steel lower platen. An aluminum platen base was designed and constructed to mate with the existing micrometer-driven flange available after removal of the standard lower platen. The base was provided with precision machined surfaces for both the micrometer flange interface and the platen floor, thus insuring minimum horizontal alignment error (Figure V-2). The platen may be constructed of any optically transparent material of uniform thickness and four inch diameter. The material selected for this work was 0.125 inch thick Vycor 7913 (ESCO Products, Inc.). This platen was modified to include a centered, smooth through bore of 0.0635 inch diameter (Optical Instruments, Inc.). The end of a 0.75 inch long carbon steel drill bit shank (0.0625 inch diameter) was lapped flat
and square (Optical Instruments, Inc.). The drill bit was fixed into the platen bore with the precision lapped surface flush with the Vycor platen surface (Figure V-3). The metallic contact area of the drill bit was used as the cone-to-platen contact sensor. Contact of the rotating cone with the drill bit sensor completed an electrical circuit which included a resistance heater mounted on the cone support pillar. Thermal expansion of this pillar caused the cone to platen distance to increase, thereby breaking the circuit and allowing the resistance heater to cool. This self-correcting technique cycles the gap height between contact and 0.001 inch.

Light enters and leaves the platen base parallel to the Vycor platen surface. Each light path is collimated, rotated 90 degrees and passed through the Vycor platen into the fluid sample by three identical optical assemblies inside the platen base (Oriel Corp.) (Figure V-2). A mounting plate fixes the position of the 90 degree collimating beam probes. Ports in the wall of the platen base both support the probes in the vertical direction and allow connection of suitable optical cables to each probe (Figures V-2, V-3). The optical cables terminate at either a light source or a photosensitive device for light intensity measurement. The electrical signal produced from the photodetectors is amplified and sent to a computer for analog to digital conversion and storage. The modified viscometer is shown in Figure V-4.
Figure V-2. Bottom view of optical viscometer platen. Precision tolerance mounting surface, center; beamsplitter with fitting to accept liquid light guide and collimating beam probe for fluorescence excitation input, right; fiber optic cable with rectangular optical terminus, top; electrical connection for cone-to-plate distance regulation, upper left; bifurcated fiber optic cable for light transmission measurement, lower left.
Figure V-3. Top view of optical viscometer platen. Transparent Vycor platen mounted in aluminum base, center; cone contact sensor for cone-to-plate distance regulation, center of platen; 90° collimating beam probes (3), one visible beneath upper center of Vycor platen.
Figure V-4. Ferranti-Shirley rotational viscometer after optical modification. Filtered mercury vapor arc lamp in housing (a); quartz-halogen source in housing (b); photodiode detector in housing with filter and offset electronics (c); transimpedance amplifiers (d); photomultiplier tubes for fluorescence detection (e); optical platen mounted on rotational viscometer (f); photomultiplier tube for luminescence detection (g).
E. Specifics Of Modification

1. transmitted light intensity measurement

Incident light was collected from a 100 watt quartz-halogen lamp (Osram) powered from a precision-regulated DC supply (Hewlett Packard; model HP 6264B) (Figure V-5). The light was filtered (Schott Glass Technologies, Inc.; model RG 695) and focused onto the polished surface of a bifurcated glass fiber optic cable. The illumination continued into the common leg of the optical cable which was fitted into a 90 degree collimating beam probe mounted in the platen base. The incident beam passed through the Vycor platen and platelet suspension. The polished cone reflected this light directly back through the sample a second time and into the collimating beam probe. The twice transmitted exit beam was focused onto the common leg and transmitted down the second branch of the bifurcated optical cable. The exit beam was focused through a second, identical red glass filter onto the active surface of a large area photodiode (United Detector Technology; model PIN 10DPI/SB) contained in a light-tight cylinder. The photodiode was operated in the photovoltaic mode; neither offset nor reverse bias current was supplied. Photodiode output current was connected to a transimpedance signal amplifier (United Detector Technology; model UDT 101C) operating at a gain of $10^5$. The analog voltage output of this device was digitized and stored on an IBM CS/9000 computer. The amplified photodiode response was
also occasionally processed with a 2 or 0.2 Hz 6th order Butterworth low pass active filter with adjustable output offset.

2. luminescence intensity measurement

Light from the luminescent reaction of ATP with firefly luciferase/ luciferin in the platelet suspension was collected and focused onto a specially constructed glass fiber optic cable (Cuda Products Corp.) by a second 90 degree collimating beam probe mounted beneath the modified viscometer platen surface (Figure V-6). Fibers of the custom optical cable were arranged in a circular pattern at the viscometer end for insertion into the unmodified beam probe, but were arranged into a rectangle at the other end. This geometry provided efficient optical coupling with the rectangular photocathode of a photomultiplier tube (PMT) (Hamamatsu Corp.; model 1P21) contained in an light-tight housing at ambient temperature (Pacific Instruments Inc.; model 3150). Illumination from the optical cable was filtered through a three cavity optical bandpass filter centered at 570.0 nm (ESCO Products, Inc.) before arriving at the photodetector. Operating voltage (Harrison Laboratories; model 6522A) was supplied to the photomultiplier tube through a voltage divider circuit contained inside the PMT housing. Photomultiplier tube output current was connected to a transimpedence signal amplifier (United Detector Technology; model UDT 101B) operating at a gain of $10^{+9}$. The analog voltage output of
Figure V-5. Schematic of light transmission measurement system of the Rice Optical Measurement Viscometer.
this device was digitized and recorded on an IBM CS/9000 computer.

3. fluorescent intensity measurement

Excitation illumination for fluorescence measurements was provided by a 100 watt mercury vapor arc lamp (Oriel Corp.) (Figure V-7). The lamp was mounted in a stable housing provided with vertical and horizontal lamp position adjustments (Oriel Corp.; model 66170). A regulated, adjustable constant current power supply was used to operate the lamp (Oriel Corp.; model 68740). An ignitor, built into the power supply, was used to establish the arc and start the lamp. A fused silica condensing lens collected the lamp output and produced a beam focused through a three cavity optical bandpass filter centered at 340.0 nm (ESCO Products, Inc.) onto the end of a liquid filled light guide (Oriel Corp.). The opposite end of the liquid light guide was fitted with a collimating beam probe (Oriel Corp.) which was mounted into a custom designed beamsplitter. Fifty percent of the collimated 340 nm beam intensity was reflected 90 degrees from incident with a partially silvered glass beamsplitter (Oriel Corp.). This excitation beam passed into the viscometer platen base and through the 90 degree collimating beam probe mounted there. This light finally passed through the Vycor platen and into the platelet suspension which had been labeled with a fluorescent intracellular calcium ion chelator. A fraction of the excitation light is absorbed by the chelator and is
Figure V-6. Schematic of luminescence measurement system of the Rice Optical Measurement Viscometer.
returned as fluorescence at one of two longer wavelengths. The fluorescent intensity is collected by the 90 degree collimating beam probe beneath the platen and directed toward the beamsplitter. Fifty percent of the fluorescent intensity is transmitted through the beamsplitter without modification. The fluorescent signal passes through a colored glass filter (Schott Glass Technologies, Inc.; model GG 395) to remove stray excitation intensity and enters the common leg of a custom fabricated bifurcated fiber optic cable (Cuda Products Corp.). Each branch of the optic cable terminates at a photomultiplier tube housing (Pacific Instruments, Inc.; model 3150). The illumination from one leg passes through a broad band interference filter centered at 400 nm (Oriel Corp.) before reaching the photodetector. Fluorescence from the second leg passes through both a colored glass filter (Schott Glass Technologies; model GG 455) and a short wave pass filter (Oriel Corp.) with a cutoff wavelength of 500 nm before reaching the photodetector. Each photomultiplier tube (Hamamatsu; model 1P21) was powered by an independent, adjustable supply of regulated high voltage (Pacific Instruments, Inc.; model 229). Photomultiplier tube outputs were connected to independent transimpedence amplifiers operating at a gain of $10^7$ each. The analog voltage output was digitized and stored on an IBM CS/9000 computer.
Figure V-7. Schematic of fluorescence measurement system of the Rice Optical Measurement Viscometer.
CHAPTER VI
CONTINUOUS MEASUREMENT OF PLATELET RESPONSE

A. Introduction

The apparatus modifications described in Chapter V were used to obtain continuous measurement of platelet aggregation, dense granule release and intracellular calcium ion ([Ca$^{2+}$]$_i$) mobilization in a well defined shear field. Response was measured in washed platelet suspensions at 24°C stimulated through application of various levels of shear stress with or without the addition of 0.2 µM ADP or 2.0 µM ADP. The ADP studies were performed at different shear rates to determine the influence of controlled hydrodynamics on ADP induced platelet response.

B. Experimental Methods

1. shear stress application using the Rice Optical Measurement Viscometer
   a. viscometer preparation

   Before each experiment, the standard viscometer platen was removed and the optical lower platen was installed. The cone to platen gap height was adjusted and both the Vycor platen and the stainless steel cone were treated with 2% aqueous solution of Prosil®-28 and dried overnight at room temperature. Optical connections were made between the light sources, the photodetectors and the modified viscometer platen. Electrical connections were established
between the photodetectors, power supplies, transimpedance amplifiers and the analog to digital (A/D) conversion board of the computer. Lamps were ignited at least 30 minutes before the start of shearing and the photomultiplier tubes were powered up at the same time.

b. calculation of applied shear stress magnitude

The 3.5 cm diameter cone made a one degree angle with the lower platen. The shear rate in the sample was calculated from \([\text{II-1}}\) and the shear stress was estimated from Newton's Law of Viscosity (equation \([\text{II-2}}\)) using the values of 1.8 and 1.2 centipoise for the viscosity of PRP and washed platelet suspension, respectively \((7,80,81)\). The maximum theoretical shear rate which can be applied with this instrument fitted with a one degree cone angle is 6,000 seconds\(^{-1}\). The maximum shear rate used was 4,164 seconds\(^{-1}\) due to practical restrictions and theoretical considerations of secondary flow \((30)\).

c. shear stress application and recording of data

The cone rotational rate corresponding to the desired shear rate was selected and the data acquisition program was accessed from the computer keyboard. Run parameters such as duration of shear exposure, number of optical detectors to be employed and the analog to digital conversion rate were entered. The platelet sample was placed on the platen and the viscometer cone and plate brought into contact. The room was darkened and photomultiplier tube housing shutters were opened. Data acquisition was started simultaneously with
cone rotation. Addition of any reagent during shear was accomplished by placing the tip of a precision microliter syringe (Hamilton Co.) in the shearing fluid and infusing the syringe contents. The cone was stopped after the appropriate shear exposure time, all shutters were closed, room lights were turned on and platelet suspension samples were recovered for determination of particle size distribution and supernatant LDH activity. The digitized data was stored with a preselected filename on a 20 Mb hard disk for subsequent recall and analysis. The viscometer surfaces were cleaned with normal saline followed by a rinse with 0.4% (w/v) poly(ethylene oxide) (Aldrich Chemical Co., Inc.; average molecular weight = 100,000) dissolved in normal saline. This treatment was used to prevent the possibility of glass-induced surface contact platelet modification (38). The cone was polished after each experiment with a slurry consisting of equal parts Alconox (American Scientific Products) and polishing cleanser (American Household Products Corp.) dissolved in distilled water.

2. continuous measurement of SIPAC

Particle size analysis, scintillation counting of released $^3$H-serotonin and assay of supernatant LDH activity was performed as described in Chapter II, section C.

a. transmitted light intensity

The intensity of light transmitted through a sample of unaggregated platelet suspension sheared at 8.25 dynes/cm$^2$
was interpreted as 0% light transmission. Platelet free suspending medium, exposed to the same conditions, was interpreted to represent 100% light transmission (Figure VI-1). The intensity of light transmitted through the sample platelet suspension during shear application was converted to percent aggregation through linear interpolation between the 0% and 100% control values.

b. luminescent light intensity

ATP released from platelet dense granules is consumed by the luciferase reaction and the oxyluciferin product is a powerful non-competitive inhibitor of the enzyme reaction (Figure VI-2) (52). The following relationships are valid for this luminescent reaction:

\[
[\text{ATP}]_{\text{tot}} = [\text{ATP}]_t + [I]_t \quad \text{(VI-1)}
\]

\[
V_t = \frac{V_{\text{max}} \cdot [\text{ATP}]_t}{(K_m + [\text{ATP}]_t) \cdot (1 + \frac{[I]_t}{K_i})} \quad \text{(VI-2)}
\]

where \([\text{ATP}]_{\text{tot}}\) is the total concentration of released ATP at time \(t\), \(V_t\) is the instantaneous velocity of the luciferase reaction, and \([\text{ATP}]_t\) and \([I]_t\) are the ATP and oxyluciferin concentrations at time \(t\), respectively (52). The velocity of the reaction \((V_t)\) is measured directly by the intensity of luminescent emission and \([I]_t\) is proportional to the area under the \(V_t\) versus time curve integrated from time zero to time \(t\). The Michaelis constant \((K_m)\), the maximum reaction velocity \((V_{\text{max}})\) and the inhibition constant \((K_i)\) are all
Figure VI-1 Light intensity transmitted through platelet-free HEPES buffer (◊) and through an unactivated washed platelet suspension (□) during exposure to 8.3 dynes/cm² of uniform shear stress in the ROMV. The light intensity transmitted through the platelet-free sample was interpreted to represent 100% aggregation and the corresponding result with washed platelet suspension to represent 0% aggregation.
Figure VI-2 Schematic of firefly luciferase-luciferin luminescent reaction cycle. (1) Initial activation of d-luciferin (L<sub>red</sub>) with MgATP (ATP) results in the formation of enzyme (E) bound d-luciferyl adenlyate (L<sub>red</sub>-AMP) and free pyrophosphate (PP<sub>i</sub>). (2) Oxidative decarboxylation of d-luciferyl adenlyate to AMP, CO<sub>2</sub> and electronically excited oxyluciferin (L<sup>*</sup>). (3) Decay of excited oxyluciferin to ground state oxyluciferin (L<sub>ox</sub>) with the emission of a photon with an overall quantum yield of 0.88. (4) Release of AMP and oxyluciferin from the active site of the enzyme. Diagram from Lundin (71).
kinetic constants which must be determined. If the concentration of released ATP is small compared with $K_m$ ($K_m = 150 \mu M$ (52)), then

$$v_t = \frac{V_{max} \cdot [ATP]_t}{K_m \cdot (1 + \frac{[I]_t}{K_i})} \quad \text{[IV-3]}$$

The final relationship can be derived as

$$[ATP]_{tot} = \frac{v_t}{V_{max} \cdot \frac{1}{K_m} \cdot (1 + \frac{[I]_t}{K_i}) + [I]_t} \quad \text{[IV-4]}$$

Substitution of the directly measured values $v_t$ and $[I]_t$ resulted in quantitative determination of the total concentration of dense granule released ATP after any shear exposure time.

Kinetics of the luminescent reaction were determined using a standard sample of platelet suspension containing 10 $\mu l$ of the firefly luciferase-luciferin preparation described in Appendix A. The sample was sheared at 8.25 dynes/cm$^2$ to provide mixing without inducing platelet dense granule release. After 10 seconds of shear exposure in the darkened room, a known final concentration of ATP was added to the sample and the luminescence was recorded for a total of 300 seconds (Figure VI-3). The initial reaction rate represented by the height of the rapid luminescent output peak ($v_o$) can be directly related to $V_{max}/K_m$ through

$$v_o = \frac{V_{max}}{K_m} \cdot [ATP]_o \quad \text{[IV-5]}$$

where $[ATP]_o$ is the initial ATP concentration after
Figure VI-3 Luminescent reaction intensity of 200 nM ATP added to a washed platelet suspension containing 10 µl of firefly 2 luciferase-luciferin during exposure to 8.3 dynes/cm² of uniform shear stress in the ROMV. ATP injection occurred at exposure time = 10 seconds.
injection into the calibration sample. From equations [VI-3] and [VI-5]

\[
\frac{v_0}{v_t} = \frac{[\text{ATP}]_0}{[\text{ATP}]_t} \cdot \left(1 + \frac{[I]_t}{K_i}\right) \quad [\text{IV-6}]
\]

Reaction conditions were such that \([\text{ATP}]_0 = [\text{ATP}]_t\) for times equivalent to the longest shear exposure time used. Physically, this statement means that the consumption of ATP could be disregarded compared to the initial ATP concentration during the calibration procedure. Thus, a plot of \(v_0/v_t\) versus \([I]_t\) yields a straight line with slope \(1/K_i\). The value of \([I]_t\) is proportional to the definite integral of the reaction velocity versus time curve. Exact calibration of \([I]_t\) was done by allowing the luminescent reaction to go to completion; the total area under the reaction velocity versus time curve represents \([I]_t\) for the known concentration of ATP injected.

Ten \(\mu l\) of luciferase/luciferin reagent (Appendix A) were added to each platelet suspension just before shear application. When the appropriate shear exposure time was reached, a pulse of known ATP concentration was added to the sample. Shearing and data collection continued for an additional 10 seconds. The luminescent reaction velocity measured as a result of the ATP injection was used to compensate the dense granule release results for differences in luciferase/luciferin concentrations via

\[
\epsilon = \frac{v_{0,c}}{v_{0,s}} \cdot \frac{[\text{ATP}]_0,s}{[\text{ATP}]_0,c} \quad [\text{IV-7}]
\]
where $v_{o,c}$ is the initial luminescent reaction velocity resulting from addition of a known concentration of ATP ($[\text{ATP}]_{o,c}$) in the calibration-only experiment and $v_{o,s}$ and $[\text{ATP}]_{o,s}$ are the corresponding values obtained in any particular platelet suspension shearing experiment. The released ATP concentration was calculated from equation [VI-4] multiplied by the correction factor $\epsilon$.

The concentration of ADP as a result of dense granule release was estimated as $3/2$ the released ATP concentration given that, in the platelet dense granules, the ratio of ADP to ATP is 3 to 2 (107). Estimations of percent dense granule release were made by dividing the measured ATP concentration with the bulk ATP concentration which would result if all platelets had released 100% of their dense granule components (67). Experimental determinations of the ATP concentration resulting from 100% dense granule release are often performed with platelet suspensions pre-labeled with $^3$H-serotonin and exposed to high doses of strong inducers of the platelet release reaction (67). Residual radioactivity in the platelet mass after this treatment indicates the extent of dense granule release.

c. fluorescent light intensity

An aliquot of platelet suspension which had not been fluorescently labeled was sheared at 8.25 dynes/cm$^2$ to obtain the background illumination level and cell autofluorescence intensity. These values were subtracted individually from each of the two detectors of fluorescent
light intensity. A second calibration run was performed with a sample of indo-1/AM labeled platelets to which a final concentration of 6 mM EDTA had been added. This suspension was lysed through duplicate 10 second exposures to sonication at 60% full power. The sample was placed in the optical viscometer and sheared at 16.5 dynes/cm$^2$ to provide mixing. The fluorescence intensities measured, after background and cell autofluorescence subtraction, were interpreted as corresponding to a free calcium ion concentration of essentially zero (Figure VI-4).

Without stopping the cone rotation, a final concentration of 10 mM CaCl$_2$ was added to saturate the available dye with free Ca$^{2+}$. The fluorescent intensities measured under these conditions were interpreted to represent the maximum measurable free calcium ion concentration.

The two fluorescent wavelengths and two dye species employed by this technique require four proportionality constants: $S_{f1}$ for the calcium ion free dye at wavelength $\lambda_1$, $S_{f2}$ for the free dye at $\lambda_2$, $S_{b1}$ for the dye chelated with a single calcium ion at $\lambda_1$ and $S_{b2}$ for the bound dye at $\lambda_2$ (40). Each optically filtered photomultiplier tube measured the fluorescent intensity at either $\lambda_1$ or $\lambda_2$. Thus, the EDTA treated sample yielded $S_{f1}$ and $S_{f2}$; after CaCl$_2$ addition values for $S_{b1}$ and $S_{b2}$ were measured. For a mixture of free and Ca$^{2+}$ bound dye at respective concentrations $c_f$ and $c_b$, the total fluorescence intensities at $\lambda_1$ ($F_1$) and $\lambda_2$ ($F_2$) are described by
Figure VI-4  Fluorescence of a platelet suspension loaded with 1 μM indo-1/AM, washed, and lysed as measured during exposure to 16.6 dynes/cm² of uniform shear stress in the ROMV. During the first 60 seconds of exposure, the sample contained 6 mM EDTA to provide a suspension essentially free of calcium ion. At 60 seconds, a final concentration of 10 mM CaCl₂ was added to the suspension providing a free calcium ion concentration sufficient to saturate the indo-1 indicator.
\[ F_1 = S_{f1} \cdot c_f + S_{b1} \cdot c_b \quad [IV-8] \]

\[ F_2 = S_{f2} \cdot c_f + S_{b2} \cdot c_b \quad [IV-9] \]

Indo-1\(^{4-}\) chelates \(Ca^{2+}\) in a 1:1 ratio, therefore

\[ c_b = \frac{c_f \cdot [Ca^{2+}]}{K_d} \quad [IV-10] \]

where \(K_d\) is the effective dissociation constant and \([Ca^{2+}]\) is the free calcium ion concentration. The fluorescence ratio \((F_1/F_2 = R)\) can be calculated from

\[ R = \frac{S_{f1} + S_{b1} \cdot [Ca^{2+}]}{K_d} \quad \frac{S_{f2} + S_{b2} \cdot [Ca^{2+}]}{K_d} \quad [IV-11] \]

Solving for the calcium ion concentration yields the following calibration equation

\[ [Ca^{2+}] = K_d \cdot \left| \frac{S_{f1}}{S_{f2}} - \frac{S_{b1}}{S_{b2}} \right| \quad [IV-12] \]

\(S_{f1}/S_{f2}\) is the limiting value of \(R\) when the calcium ion concentration is zero \((R_{\text{min}})\) and \(S_{b1}/S_{b2}\) is the limit at calcium ion saturation of the dye \((R_{\text{max}})\).

Calibration experiments and calculation of the proportionality constants were performed for each indo-1/AM labeled platelet suspension. Platelet intracellular calcium ion concentration was calculated using equation \([VI-12]\) and was plotted as a function of shear field exposure time.
C. Results

1. shear stress stimulation only; no added ADP

Figures VI-5 through VI-8 display typical platelet response to shear stress application of 50 dynes/cm$^2$ in terms of transmitted light intensity (Figures VI-5 and VI-6), dense granule release (Figure VI-7) and intracellular calcium ion concentration (Figure VI-8). Corresponding results at 33 dynes/cm$^2$ are contained in Figures VI-9 through VI-12 and at 17 dynes/cm$^2$ in Figures VI-13 through VI-16. Each figure was selected as representative of 2-6 independent trials. Selected discrete parameters characteristic of the continuous response are presented in Table VI-1.

The points which comprise Figures VI-5 through VI-16 represent actual, unsmoothed results recorded directly from the photodetector. Individual data points have been averaged over one second and every other point is plotted for clarity. The left ordinate displays the absolute value of the measured output for the transmitted light intensity and luminescent light intensity measurements. The left ordinate of the intracellular calcium ion determination is presented as the ratio of the fluorescent light intensity measured at 410 nm to the fluorescent light intensity measurement at 485 nm. The right ordinate has been scaled to display the output in terms of a calibrated parameter as described in section B of this chapter.
Figure VI-5 (top) Continuous measurement of light intensity transmitted through a washed platelet preparation during exposure to 50 dynes/cm² of shear stress. Platelet concentration = 300,000 per μl

Figure VI-6 (bottom) Continuous measurement of light intensity transmitted through a washed platelet preparation during exposure to 50 dynes/cm² of shear stress. Platelet concentration = 400,000 per μl
Figure VI-7 (top) Continuous measurement of firefly luciferase-luciferin-ATP luminescence intensity in a washed platelet preparation during exposure to 50 dynes/cm of shear stress.

Figure VI-8 (bottom) Continuous measurement of the fluorescence intensity ratio (410 nm / 485 nm) of an indo-1/AM labeled washed platelet preparation during exposure to 50 dynes/cm of shear stress.
Figure VI-9 (top) Continuous measurement of light intensity transmitted through a washed platelet preparation during exposure to 33 dynes/cm² of shear stress. Platelet concentration = 300,000 per μl.

Figure VI-10 (bottom) Continuous measurement of light intensity transmitted through a washed platelet preparation during exposure to 33 dynes/cm² of shear stress. Platelet concentration = 400,000 per μl.
Figure VI-11 (top) Continuous measurement of firefly luciferase-luciferin-ATP luminescence intensity in a washed platelet preparation during exposure to 33 dynes/cm² of shear stress.

Figure VI-12 (bottom) Continuous measurement of the fluorescence intensity ratio (410 nm / 485 nm) of an indo-1/AM labeled washed platelet preparation during exposure to 33 dynes/cm² of shear stress.
Figure VI-13 (top) Continuous measurement of light intensity transmitted through a washed platelet preparation during exposure to 17 dyne/cm$^2$ of shear stress. Platelet concentration = 300,000 per μl

Figure VI-14 (bottom) Continuous measurement of light intensity transmitted through a washed platelet preparation during exposure to 17 dyne/cm$^2$ of shear stress. Platelet concentration = 400,000 per μl
Figure VI-15 (top) Continuous measurement of firefly luciferase-luciferin-ATP luminescence intensity in a washed platelet preparation during exposure to 17 dynes/cm of shear stress.

Figure VI-16 (bottom) Continuous measurement of fluorescence intensity ratio (410 nm / 485 nm) of an indo-1/AM labeled washed platelet suspension during exposure to 17 dynes/cm of shear stress.
# SHEAR STRESS STIMULATION

Shear Stress, dynes/cm²

<table>
<thead>
<tr>
<th>Platelet Aggregation, percent</th>
<th>50</th>
<th>33</th>
<th>17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>-10 ± 5</td>
<td>-11 ± 4</td>
<td>-11 ± 3</td>
</tr>
<tr>
<td>Maximum</td>
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</tr>
<tr>
<td>Final</td>
<td>-10 ± 5</td>
<td>-11 ± 4</td>
<td>-11 ± 3</td>
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Supernatant ADP concentration, nM

<table>
<thead>
<tr>
<th></th>
<th>60 seconds</th>
<th>180 seconds</th>
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</thead>
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<tr>
<td>Concentration</td>
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<td></td>
</tr>
<tr>
<td>60 seconds</td>
<td>464 ± 45</td>
<td>219 ± 46</td>
</tr>
<tr>
<td>180 seconds</td>
<td>1409 ± 103</td>
<td>557 ± 112</td>
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</table>

Intracellular Ca²⁺ concentration, nM

<table>
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<tr>
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<th>Baseline</th>
<th>Peak</th>
<th>Time to Peak, sec</th>
<th>Final</th>
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<tr>
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<td>Baseline</td>
<td>195 ± 84</td>
<td>195 ± 84</td>
<td>195 ± 84</td>
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<td>Peak</td>
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<td>Time to peak, sec</td>
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<tr>
<td>Final</td>
<td>956 ± 417</td>
<td>250 ± 101</td>
<td>191 ± 111</td>
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Platelet aggregation, percent (400,000 per μl)

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<tr>
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<th>Minimum</th>
<th>Maximum</th>
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</thead>
<tbody>
<tr>
<td>Concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
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<td>-10 ± 11</td>
<td>-13 ± 2</td>
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<tr>
<td>Maximum</td>
<td>19 ± 10</td>
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<td>0</td>
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<tr>
<td>Final</td>
<td>3 ± 11</td>
<td>-7 ± 15</td>
<td>-13 ± 2</td>
</tr>
</tbody>
</table>

Table VI-1: Discrete results obtained from the continuous response measured in the ROMV. Time to [Ca²⁺]ₙ peak in seconds after shear stress addition.
Data = average ± standard deviation, n = 4-8.
Two transmitted light intensity curves are presented for each shear stress due to the qualitative differences exhibited for platelet concentrations of 300,000 per μl (n = 6-8) as compared to 400,000 per μl (n = 3). Suspensions containing 300,000 platelets per μl did not exhibit an increase in transmitted light intensity upon shear stress application. Response of this system was a monotonic decrease in transmitted light intensity which reached a constant minimum value representing approximately 10% apparent platelet aggregation after 60-100 seconds of shear stress exposure. The extent of this response was not affected by the magnitude of applied shear stress; if a threshold for this response exists, it is below 17 dynes/cm².

The response of washed platelet suspensions treated with colchicine or cytochalasin E₁ is shown in Table VI-2. Platelet aliquots treated with either reagent exhibited a dose dependent decrease in extent of shape change induced by addition of 2 μM ADP. Platelets treated with the same level of colchicine or cytochalasin E₁ also demonstrated statistically significant reduction in the decrease in transmitted light intensity measured after shear stress application.

Samples prepared with 400,000 platelets per μl yielded an increase in transmitted light intensity during application of 50 or 33 dynes/cm², but not at 17 dynes/cm². The measured increase occurs with a lag time of 20-30
Platelet Shape Change

<table>
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<tr>
<th>treatment</th>
<th>addition of 2 μM ADP</th>
<th>application of 33 dynes/cm²</th>
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</thead>
<tbody>
<tr>
<td>control</td>
<td>10</td>
<td>100%</td>
</tr>
<tr>
<td>colchicine, 2 mM</td>
<td>4</td>
<td>74% ± 23%</td>
</tr>
<tr>
<td>colchicine, 4 mM</td>
<td>2</td>
<td>46% ± 13% *</td>
</tr>
<tr>
<td>cytochalasin E₁, 10 μM</td>
<td>1</td>
<td>39% ± 1% **</td>
</tr>
<tr>
<td>cytochalasin E₁, 50 μM</td>
<td>0</td>
<td>25% ± 7% ***</td>
</tr>
</tbody>
</table>

Table VI-2. (center column) Extent of platelet shape change measured in response to added ADP using a platelet aggregometer (relative optical units). (right column) Reduction of the decrease in transmitted light intensity measured in response to shear stress using an optically modified cone and plate viscometer (percent decrease in response compared to untreated platelet suspension). All samples were treated with 4 mM EDTA to prevent aggregation. Incubation time was 60 minutes for each treatment. Data: average ± standard deviation. Student’s t-test: * p < 0.05, ** p < 0.01, *** p < 0.001 (compared to untreated control)

seconds after initial shear stress exposure. Application of 50 dynes/cm² consistently resulted in a reversal of the transmitted light intensity increase after 120 seconds of shear stress application. This reversal was never observed during exposure to 33 or 17 dynes/cm².

The concentration of supernatant ATP increased continuously with application of any shear stress intensity. Differences between the ATP supernatant concentration after 180 seconds and 60 seconds of exposure at any level of shear stress were significant (p < 0.001). The change in ATP concentration between 0 and 60 seconds of shear stress application was also significant for all tested shear stresses (p < 0.001). Application of 50 dynes/cm² induced
a statistically significant increase in the level of supernatant ATP after both 60 and 180 seconds of exposure compared to that measured at 33 dynes/cm²; the results at 17 dynes/cm² were significantly lower than at either of the two other shear stress levels, but were greater than zero. Response of the luminescent reaction was detectable after one second of shear stress exposure (one second is the shortest practical time scale for reproducible measurements with this apparatus).

Baseline $[\text{Ca}^{2+}]_i$ was 195 nM with significant scatter between different washed platelet preparations. Intracellular calcium ion concentration increased to 1371 nM (significantly greater than baseline, $p < 0.001$) with a lag time of approximately 10 seconds after shear stress application of 50 dynes/cm². The fluorescent signal was observed to decline in some samples after approximately 120 seconds of shear stress exposure. The increase in $[\text{Ca}^{2+}]_i$ during application of 33 dynes/cm² was not statistically significant. Shear stress exposure of 17 dynes/cm² resulted in no measurable alteration of the platelet intracellular calcium ion concentration. The initial platelet count for all samples was 300,000 per µl unless otherwise indicated to be 400,000 per µl.

2. shear stress stimulation + 0.2 µM added ADP

Figures VI-17 through VI-19 are representative, respectively, of the transmitted light intensity, luciferase-luciferin-ATP luminescence intensity and the
indo-1\textsuperscript{4-} fluorescence intensity results obtained after addition of 0.2 \textmu M ADP to washed platelet suspensions exposed to shear stress of 50 dynes/cm\textsuperscript{2}. Figures VI-20 to VI-22 display the same measurements made during exposure to 33 dynes/cm\textsuperscript{2} and Figures VI-23 to VI-25 correspond to application of 17 dynes/cm\textsuperscript{2}. Table VI-3 reports discrete parameters representative of the continuous response.

Addition of 0.2 \textmu M ADP to a washed platelet suspension sheared at 50 dynes/cm\textsuperscript{2} resulted in a decrease in transmitted light intensity which occasionally reversed after 140-160 seconds of shear stress exposure but never returned to the original baseline. Platelet suspensions exposed to 33 dynes/cm\textsuperscript{2} exhibited an increase in transmitted light intensity after addition of 0.2 \textmu M ADP. This response was often accompanied by a decrease in transmitted light intensity after 120-140 seconds of shear stress exposure. Platelet response to the same treatment at 17 dynes/cm\textsuperscript{2} was characterized by a longer lag time and a reduced extent of transmitted light intensity increase as compared to 33 dynes/cm\textsuperscript{2}. Partial reversal of this response after 120-140 seconds of shear stress exposure was observed in 50% of the washed platelet samples.

Velocity of the luminescent reaction increased non-linearly with increasing shear stress exposure time. The concentrations of supernatant ADP were significantly different between each of the three shear stresses after 60 seconds. At 180 seconds of shear stress exposure, response
Figure VI-17 (top) Continuous measurement of light intensity transmitted through a washed platelet preparation during exposure to 50 dynes/cm of shear stress. Addition of 0.2 μM ADP occurred after 10 seconds of shear stress exposure.

Figure VI-18 (bottom) Continuous measurement of firefly luciferase-luciferin-ATP luminescence intensity in a washed platelet preparation during exposure to 50 dynes/cm of shear stress. Addition of 0.2 μM ADP occurred after 10 seconds of shear stress exposure.
Figure VI-19 Continuous measurement of the fluorescence intensity ratio (410 nm / 485 nm) of an indo-1/AM labeled washed platelet preparation during exposure to 50 dynes/cm of shear stress. Addition of 0.2 μM ADP occurred after 10 seconds of shear stress exposure.
Figure VI-20 (top) Continuous measurement of light intensity transmitted through a washed platelet preparation during exposure to 33 dynes/cm² of shear stress. Addition of 0.2 μM ADP occurred after 10 seconds of shear stress exposure.

Figure VI-21 (bottom) Continuous measurement of firefly luciferase-luciferin-ATP luminescence intensity in a washed platelet preparation during exposure to 33 dynes/cm² of shear stress. Addition of 0.2 μM ADP occurred after 10 seconds of shear stress exposure.
Figure VI-22 Continuous measurement of the fluorescence intensity ratio (410 nm / 485 nm) of an indo-1/AM labeled washed platelet preparation exposed to 33 dynes/cm² of shear stress. Addition of 0.2 μM ADP occurred after 10 seconds of shear stress exposure.
Figure VI-23 (top) Continuous measurement of light intensity transmitted through a washed platelet preparation during exposure to 17 dynes/cm². Addition of 0.2 μM ADP occurred after 10 seconds of shear stress exposure.

Figure VI-24 (bottom) Continuous measurement of firefly luciferase-luciferin-ATP luminescence intensity in a washed platelet preparation during exposure to 17 dynes/cm² of shear stress. Addition of 0.2 μM ADP occurred after 10 seconds of shear stress exposure.
Figure VI-25 Continuous measurement of the fluorescence intensity ratio (410 nm / 485 nm) of an indo-1/AM labeled washed platelet preparation during exposure to 17 dyne/cm² of shear stress. Addition of 0.2 μM ADP occurred after 10 seconds of shear stress exposure.
<table>
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<tr>
<th>Shear Stress, dynes/cm²</th>
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<th>17</th>
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</thead>
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<td>platelet aggregation,</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>percent</td>
<td></td>
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<tr>
<td>minimum</td>
<td>-9 ± 14</td>
<td>-10 ± 4</td>
<td>-7 ± 3</td>
</tr>
<tr>
<td>maximum</td>
<td>0</td>
<td>11 ± 9</td>
<td>8 ± 5</td>
</tr>
<tr>
<td>final</td>
<td>-3 ± 6</td>
<td>2 ± 6</td>
<td>6 ± 4</td>
</tr>
<tr>
<td>supernatant ADP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>concentration, nM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 seconds</td>
<td>477 ± 15</td>
<td>332 ± 24</td>
<td>195 ± 37</td>
</tr>
<tr>
<td>180 seconds</td>
<td>1454 ± 317</td>
<td>929 ± 300</td>
<td>481 ± 81</td>
</tr>
<tr>
<td>intracellular Ca²⁺</td>
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<td></td>
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</tr>
<tr>
<td>concentration, nM</td>
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<td>baseline</td>
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<td>126 ± 61</td>
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<tr>
<td>final</td>
<td>1807 ± 512</td>
<td>176 ± 87</td>
<td>115 ± 2</td>
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Table VI-3 Discrete results obtained from the continuous response measured in the ROMV. Supernatant ADP concentration corrected for agonist addition. Time to [Ca²⁺]ᵢ peak in seconds after ADP addition. Data = average ± standard deviation. n = 4-8.
was significantly different between 17 and 50 dynes/cm\(^2\), but not for any other shear stress combination.

Addition of 0.2 µM ADP induced a shear stress dependent increase in platelet intracellular calcium ion concentration which was significantly different between all shear stress pairs except 33 and 17 dynes/cm\(^2\). In 50% of the samples exposed to 33 dynes/cm\(^2\) and all samples at 17 dynes/cm\(^2\), the final [Ca\(^{2+}\)]\(_i\) measured after 180 seconds of shear stress exposure was significantly less than the peak [Ca\(^{2+}\)]\(_i\). Peak [Ca\(^{2+}\)]\(_i\) was statistically greater than baseline [Ca\(^{2+}\)]\(_i\) for all shear stresses studied. The lag time of Ca\(^{2+}\) mobilization was < 2 seconds after ADP addition and was independent of shear stress.

3. shear stress stimulation + 2.0 µM added ADP

Figures VI-26 through VI-28 represent the three continuous optical measurements of washed platelet suspension response to application of 50 dynes/cm\(^2\) with the addition of 2.0 µM ADP. The same measurements are shown in Figures VI-29 through VI-31 during application of 33 dynes/cm\(^2\). Figures VI-32 through VI-34 contain the corresponding response at 17 dynes/cm\(^2\). Discrete values obtained from the continuous response are displayed in Table VI-4.

Addition of 2 µM ADP during application of shear stress at any level resulted in an increase in the intensity of light transmitted through the washed platelet suspension sample. The final extent of of light intensity change after
Figure VI-26 (top) Continuous measurement of light intensity transmitted through a washed platelet preparation during exposure to 50 dynes/cm² of shear stress. Addition of 2 µM ADP occurred after 10 seconds of shear stress exposure.

Figure VI-27 (bottom) Continuous measurement of firefly luciferase-luciferin-ATP luminescence intensity in a washed platelet preparation during exposure to 50 dynes/cm² of shear stress. Addition of 2 µM ADP occurred after 10 seconds of shear stress exposure.
Figure VI-28 Continuous measurement of the fluorescence ratio (410 nm / 485 nm) of an indo-1/AM labeled washed platelet preparation during exposure to 50 dynes/cm² of shear stress. Addition of 2 μM ADP occurred after 10 seconds of shear stress exposure.
Figure VI-29 (top) Continuous measurement of light intensity transmitted through a washed platelet preparation during exposure to 33 dynes/cm² of shear stress. Addition of 2 μM ADP occurred after 10 seconds of shear stress exposure.

Figure VI-30 (bottom) Continuous measurement of firefly luciferase-luciferin-ATP luminescence intensity in a washed platelet preparation during exposure to 33 dynes/cm² of shear stress. Addition of 2 μM ADP occurred after 10 seconds of shear stress exposure.
Figure VI-31 Continuous measurement of the fluorescence intensity ratio (410 nm / 485 nm) of an indo-1/AM labeled washed platelet preparation during exposure to 33 dynes/cm² of shear stress. Addition of 2 μM ADP occurred after 10 seconds of shear stress exposure.
Figure VI-32 (top) Continuous measurement of light intensity transmitted through a washed platelet preparation during exposure to 17 dynes/cm² of shear stress. Addition of 2 µM ADP occurred after 10 seconds of shear stress exposure.

Figure VI-33 (bottom) Continuous measurement of firefly luciferase-luciferin-ATP luminescence intensity in a washed platelet preparation during exposure to 17 dynes/cm² of shear stress. Addition of 2 µM ADP occurred after 10 seconds of shear stress exposure.
Figure VI-34  Continuous measurement of the fluorescence intensity ratio (410 nm / 485 nm) of an indo-1/AM labeled washed platelet preparation during exposure to 17 dynes/cm² of shear stress. Addition of 2 μM ADP occurred after 10 seconds of shear stress exposure.
### Shear Stress Stimulation + 2.0 μM ADP

**Shear Stress, dynes/cm²**

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<tbody>
<tr>
<td></td>
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<tr>
<td>platelet aggregation, percent</td>
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<tr>
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<td>-13 ± 9</td>
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<tr>
<td>maximum</td>
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<td>24 ± 7</td>
</tr>
<tr>
<td>final</td>
<td>27 ± 9</td>
<td>38 ± 8</td>
<td>24 ± 7</td>
</tr>
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</table>

**supernatant ADP concentration, nM**

<p>| | | | |</p>
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<tbody>
<tr>
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</tr>
<tr>
<td>60 seconds</td>
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<tr>
<td>180 seconds</td>
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**intracellular Ca²⁺ concentration, nM**

<p>| | | | |</p>
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<tr>
<th></th>
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<tr>
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<td>time to peak, sec</td>
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<tr>
<td>final</td>
<td>1532 ± 437</td>
<td>450 ± 231</td>
<td>135 ± 40</td>
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</table>

Table VI-4 Discrete results obtained from the continuous response measured in the ROMV. Supernatant ADP concentration corrected for agonist addition. Time to [Ca²⁺]ᵢ peak in seconds after ADP addition. Data = average ± standard deviation. n = 4-8.
180 seconds of exposure was greatest for treatment at 33 dynes/cm² but was statistically greater than baseline at both 50 and 17 dynes/cm². Reversal of this increase was not noted in any sample. Lag time to incipient transmitted light intensity increase was greatest and the rate of the optically measured reaction smallest when the sample was sheared at 17 dynes/cm². Shear stress of 33 dynes/cm² yielded the shortest lag time and the greatest reaction rate.

The concentration of supernatant ATP increased in a non-linear fashion with exposure time to shear stress. Shear stress applied for 180 seconds resulted in a statistically significant difference in supernatant ATP concentration between 17 and either 33 or 50 dynes/cm². Luminescent response was not a function of applied shear stress intensity after 60 seconds of exposure.

Intracellular calcium ion concentration of the washed platelet suspension increased with less than one second delay upon addition of 2 μM ADP during exposure to shear stress. The peak [Ca²⁺]ᵢ was significantly greater than baseline at all shear stress levels studied. 50% and 25% of the samples sheared at 17 and 33 dynes/cm², respectively, demonstrated a decrease in [Ca²⁺]ᵢ after achieving the peak value.

4. Endpoint determinations of platelet activation

Figure VI-35 presents platelet aggregation (electronic particle counting), dense granule release (³H-serotonin
supernatant radioactivity) and platelet lysis (supernatant lactate dehydrogenase activity) results after 180 seconds of exposure to various levels of shear stress. Table VI-5 indicates the statistical significance of the data presented in Figure VI-35(a).

Platelet aggregation without addition of ADP increased with increasing shear stress intensity. Addition of either concentration of ADP significantly increased platelet aggregation at any shear stress. Use of 2 μM ADP induced greater platelet aggregation than did addition of 0.2 μM ADP. The extent of aggregation measured by electronic particle counting after addition of ADP was not a function of applied shear stress.

The concentration of $^3$H-serotonin measured in the sheared supernatant increased with increasing shear stress for all platelet treatments. At any shear stress, samples treated with either concentration of ADP released dense granule components to the same extent, but exhibited more supernatant radioactivity than did the samples without added ADP. Without the addition of ADP, $^3$H-serotonin was not detected in the supernatant of samples exposed to 17 dynes/cm$^2$; platelet aggregation was statistically greater than zero at these conditions.

The activity of LDH in the platelet suspension supernatant after 180 seconds of exposure increased with increasing shear stress intensity. For any specific sample/treatment pair, the calculated percent lysis was less
Figure VI-35 Endpoint measurement of platelet aggregation determined by electronic particle counting (a), dense granule release determined by radioactive serotonin release (b), and platelet lysis determined by supernatant lactate dehydrogenase (LDH) activity (c). Shear stress exposure time = 180 seconds. Washed platelet suspensions exposed to shear stress without addition of chemical agonists (□), washed platelet suspensions with addition of 0.2 µM ADP after 10 seconds of shear exposure (△), washed platelet suspensions with addition of 2.0 µM ADP after 10 seconds of shear exposure (◇). Data = average ± standard deviation.
### Platelet Aggregation

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<td>X</td>
<td>***</td>
<td>***</td>
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<td>***</td>
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Table VI-5 Statistical significance of endpoint platelet aggregation data measured by electronic particle counting (Figure VI-35(a)).

* p < 0.05; ** p < 0.01; *** p < 0.001; -- p > 0.05

Analysis performed using Student's paired t-test. The significance level between the results of any two experimental conditions is given by the symbol located at the intersection of the appropriate row and column.
than the calculated dense granule release. ADP addition resulted in an increase in lysis as compared to application of shear stress alone. Supernatant LDH activity in samples with added ADP was not a function of ADP concentration.

Particle volume distributions of unsheared viscometer control washed platelet suspensions as well as aliquots which were exposed to 180 seconds of shear stress at 17 dynes/cm$^2$ and contained 0, 0.2 or 2.0 μM added ADP are displayed in Figure VI-36. Figures VI-37 and VI-38 present the same information after exposure to 33 and 50 dynes/cm$^2$, respectively. Statistical information regarding each of the four volume size fractions is presented in Tables VI-6 through VI-9.

The integrated total volume of all particles from 2.55 μ$^3$ to 8580 μ$^3$ was relatively unaltered after 180 second application of shear stress either alone (final total particle volume was 105% to 124% of unsheared control volume) or with addition of 0.2 μM ADP (final total particle volume was 89% to 113% of unsheared control volume). Total particle volume of samples treated with 2.0 μM ADP was significantly less than that of unsheared control platelet suspensions or platelet suspensions exposed to shear with or without addition of 0.2 μM ADP (final total volume of samples treated with 2 μM ADP = 54%, 38% and 36% of unsheared control volume after 50, 33 and 17 dynes/cm$^2$, respectively). As a result, the percent volume fractions reported for platelet suspensions sheared with 2 μM added
Figure VI-36 Endpoint measurement of percent total particle volume in four ranges of particle volume for samples treated as indicated. Suspensions were exposed to 180 seconds of controlled shear stress at 16.7 dynes/cm. Data: bars represent average values and are topped by lines representing one standard deviation.
Figure VI-37 Endpoint measurement of percent total particle volume in four ranges of particle volume for samples treated as indicated. Suspensions were exposed to 180 seconds of controlled shear stress at 33 dynes/cm². Data: bars represent average values and are topped by lines representing one standard deviation.
Figure VI-38 Endpoint measurement of percent total particle volume in four ranges of particle volume for samples treated as indicated. Suspensions were exposed to 180 seconds of controlled shear stress at 50 dynes/cm². Data: bars represent average values and are topped by lines representing one standard deviation.
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Table VI-6 Statistical significance of endpoint measurement of percent total particle volume represented by particles having volume between 2.55 and 8.97 µm (Figures VI-36, VI-37, VI-38).

* p < 0.05; ** p < 0.01; *** p < 0.001; -- p > 0.05
Analysis performed using Student's paired t-test.
Significance determined as described in Table VI-5.
### Table VI-7: Statistical significance of endpoint measurement of percent total particle volume represented by particles having volume between 8.97 and 51.1 \( \mu \text{m}^3 \)

(Figures VI-36, VI-37, VI-38).

* \( p < 0.05 \); ** \( p < 0.01 \); *** \( p < 0.001 \); -- \( p > 0.05 \)

Analysis performed using Student's paired \( t \)-test.

Significance determined as described in Table VI-5.
<table>
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PERCENT TOTAL VOLUME, 51.1 - 490 μ³

Table VI-8 Statistical significance of endpoint measurement of percent total particle volume represented by particles having volume between 51.1 and 490 μ (Figures VI-36, VI-37, VI-38).

* p < 0.05; ** p < 0.01; *** p < 0.001; -- p > 0.05

Analysis performed using Student's paired t-test. Significance determined as described in Table VI-5.
<table>
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<tr>
<th>SHEAR STRESS, DYN(^{-2}) CM(^{2})</th>
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Table VI-9: Statistical significance of endpoint measurement of percent total particle volume represented by particles having volume between 490 and 8580 \(\mu\) (Figures VI-36, VI-37, VI-38).

* \(p < 0.05\); ** \(p < 0.01\); *** \(p < 0.001\); -- \(p > 0.05\)

Analysis performed using Student's paired t-test. Significance determined as described in Table VI-5.
ADP must be interpreted with some care. The platelet volume fraction which appears to be lost is presumed to represent platelet aggregates larger than 8580 μ³ which are not counted.

Platelet aliquots exhibited a progressive decrease in the volume fraction of the smallest measured particles and a simultaneous gain in the volume fraction of larger particles with exposure to increasing shear stress intensity. This effect was significantly potentiated by the addition of either 0.2 or 2 μM ADP. Platelet suspensions exposed to any level of shear stress, regardless of added ADP concentration, contained a significantly reduced fraction of 2.55 to 8.97 μ³ particles and a significantly increased fraction of 490 to 8580 μ³ particles as compared to unsheared controls. Addition of either concentration of ADP resulted in a significant increase in the volume fraction of the largest measured particles as compared to platelet samples exposed to shear stress only.

D. Discussion

1. aggregation results

Platelets at a concentration of 300,000 per μl form aggregates (Table VI-1, Figure VI-35), in response to shear stress alone, which are of insufficient size (Figures VI-36 to VI-38) to cause a decrease in the apparent suspension optical density (Figures VI-5, VI-9, VI-13). Addition of 2 μM ADP in the shear field induces the formation of
aggregates larger than those produced in response to shear stress alone (Figures VI-36 to VI-38). The population size distribution which results is represented by an increase in light intensity transmitted through the platelet sample (Figures VI-8, VI-12, VI-16). Introduction of 0.2 μM ADP yields an intermediate aggregate size distribution (Figures VI-36 to VI-38) which causes the apparent optical density of these samples to decrease slightly with occasional reversal (Figures VI-7, VI-11, VI-15). Clearly, in the optically modified viscometer as in commercial platelet aggregometers, changes in transmitted light intensity are directly related to the formation of platelet aggregates at least as large as 8580 μ³. This finding is consistent with reports which conclude that the disappearance of single platelets and the formation of aggregates having volumes less than 490 μ³ makes no significant contribution to the increase in light intensity transmitted through a stirred platelet suspension treated with collagen, thrombin, ADP or epinephrine (34,68,97). At least one report indicates that, under similar conditions, the formation of aggregates larger than 8580 μ³ contributes significantly to the initial phase of light transmittance increase and is closely correlated with the overall change in transmitted light intensity (97).

The shear stress induced decreases in apparent optical density in suspensions containing 400,000 platelet per μl (Figures VI-6, VI-10, VI-14). This result indicates formation of platelet aggregates which are larger than those
formed at a concentration of 300,000 per µl. This difference is presumably explained by the increased concentration of platelet release and synthesis products expected to be present at the higher platelet concentration, in addition to enhanced platelet-platelet collision frequency. At a platelet concentration of 400,000 per µl, the increase in transmitted light intensity is greatest at 50 dynes/cm², non-existent at 17 dynes/cm² and incipient at 33 dynes/cm² (Table VI-1), thus establishing a direct relationship between shear field intensity and the extent and size distribution of shear stress induced platelet aggregates. In addition, reversal of the transmitted light intensity increase is observed, especially at 50 dynes/cm², indicating breakup of previously formed large aggregates. Continuous measurement of platelet suspension light transmission indicates the importance of both initial platelet concentration and the time dependence of aggregate size distribution in the study of shear stress induced platelet aggregation. Presentation of aggregation as percentage decrease in single platelet concentration at discrete shear stress exposure times may not adequately represent the actual extent of platelet response.

The insensitivity of the light transmission technique must be considered whenever platelet aggregation results are reported from optical aggregometers. Comparison of the aggregation measurements made by electronic particle counting (Figure VI-35) to the optically determined maximum
percent aggregation (Tables VI-2, VI-3 and VI-4) reveals that a 40% reduction in single platelet concentration may yield only incipient increases in light intensity transmitted through the platelet suspension. Furthermore, a shear stress independent decrease in single platelet concentration of approximately 90% can result in an optically measured percent aggregation ranging from 24% to 38% depending on the shear stress influenced particle size distribution. Despite the limitations of this method, the optically modified viscometer yields accurate, reproducible apparent platelet aggregation as a function of time during application of well characterized, uniform shear flow.

An interesting finding of the aggregation studies was that, in the absence of added agonists, application of the shear field caused a decrease in the light intensity transmitted through platelet suspensions having an initial platelet concentration of 300,000 per μl. This change in apparent optical density is in the direction opposite of that expected during platelet aggregation. This negative apparent aggregation observed upon application of shear stress is not due to platelet adhesion to either the Vycor platen (54 platelets/mm², 0.03% surface coverage) or to the rotating steel cone (14 platelets/mm², <0.01% surface coverage). The extent of light scattering and absorption provided by these adherent platelets is not expected to exceed 0.16% of the incident light intensity as estimated from Mie theory (20a). Thus, platelet surface adhesion does
not appear to offer a complete explanation of the approximately 10% change in transmitted light intensity measured under these conditions.

Randomly oriented oblate spheroids in suspension can be aligned in uniform shearing flow (39). The steady state distribution of particle orientation, however, results in an increase in light intensity transmitted through the sample for the optical geometry of the modified viscometer. Thus, particle orientation would result in a change in transmitted light intensity in the direction opposite to that observed.

Platelet transition from smooth disks to spiny spheres (shape change) causes a decrease in the intensity of light transmitted through a platelet suspension. The negative apparent aggregation would be consistent with a shear stress induced shape change analogous to the observation in conventional aggregometry in response to low levels of added chemical agonist. A series of experiments were carried out to examine this possibility. The first experiments involved shear stress exposure of gluteraldehyde fixed platelets. Washed platelets fixed by gluteraldehyde treatment can not undergo shape change. Suspensions of these fixed platelets were found not to exhibit a decrease in light transmission upon addition of chemical agonists or shear stress exposure.

A second series of studies was performed with platelets incubated with chemical cytoskeletal modifiers. The measured decrease in light intensity transmitted through a platelet suspension upon shear application is the result of
active platelet response which can be altered by known cytoskeletal modifiers. As shown in Table VI-2, the results of this work are consistent with those obtained with gluteraldehyde fixed platelets in support of the shear induced shape change hypothesis.

Optically determined platelet aggregation in response to 2 μM ADP is a maximum at the intermediate shear stress of 33 dynes/cm² (Table VI-4). Presumably, enhancement of the aggregation process through additional collisions caused by increasing the shear stress to 50 dynes/cm² is more than offset by the greater hydrodynamic forces acting to break particles apart. At 17 dynes/cm², the frequency of particle interactions is reduced sufficiently to cause a decrease in the rate of aggregate formation as compared to conditions at 33 dynes/cm². Platelet response to 0.2 μM ADP is weak and reversible. At this low dosage of added ADP, exposure to shear stress greater than 17 dynes/cm² greatly enhances the disaggregation process (Table VI-3).

2. Dense granule release results

ATP concentrations of less than 20 nM can be accurately measured with the optically modified viscometer under experimental conditions which do not affect platelet function in any detectable way (Appendix A). The luminescent response is linear to ATP concentrations of at least 5 μM. Thus, the useful dynamic range of this instrument is greater than 250:1 (48 dB). If the source of ATP measured is from dense granule release, the range of
this instrument allows detection of equivalent ADP concentrations over a range from below that of demonstratable significance to platelet response to above that which induces 100% dense granule release.

Calculation of percent release from the ATP concentration obtained with luciferase-luciferin luminescence requires the use of the bulk ATP concentration present after 100% dense granule release. An literature value of 6.9 μM bulk ATP after 100% release was experimentally verified (67). Using this result, dense granule release results obtained by ³H-serotonin release agree with those generated by the luciferase technique.

Dense granule release proceeds measurably and without lag upon platelet suspension exposure to any shear stress intensity tested (Figures VI-7, VI-11, VI-15). This type of continuous measurement has not been previously possible by the radiolabelled serotonin endpoint assay. The rate of ATP appearance in the platelet suspension supernatant is a constant with respect to shear stress exposure time and a monotonic function of shear stress intensity. Addition of ADP to the sample during shear exposure results in a non-uniform, accelerated rate of ATP discharge superimposed on the constant rate observed during shear stress application only, indicating that platelet response under these conditions is self-promoting through agonist induced dense granule release (Figures VI-18, VI-21, VI-24, VI-27, VI-30, VI-33).
The ATP:ADP ratio in the platelet metabolic pool is five times greater than in the dense granules (107). This source of adenine nucleotides could reach the supernatant fluid through platelet lysis. The rate of ADP concentration increase in the sheared samples is, however, too large to be accounted for by lysis (Figure VI-35c). The ADP concentrations reported in this thesis are calculated from the measured ATP concentration using the 3:2 ratio of ADP:ATP reported to be present in platelet dense granules (107).

At short shear stress exposure times, the bulk ADP concentration is below the threshold normally required for platelet aggregation (107). Yet, after 30 seconds of shear stress application, significant aggregation has been reported at 50 dynes/cm$^2$ (9,10). Three different explanations can be proposed: 1) the local concentration of ADP near the platelet surface may be significantly greater than the bulk concentration measured; 2) shear stress may reduce the ADP concentration threshold required for platelet aggregation by directly exposing additional platelet surface ADP binding sites which may also have a greater affinity for, or enhanced reactivity to, released ADP; 3) other biochemical mechanisms, independent of components released by dense granules, may be responsible for at least a portion of the initiation of shear stress induced platelet aggregation.
The validity of the first explanation can be investigated through estimation of the platelet surface ADP concentration induced by shear stress application in the absence of added chemical agonist. The limiting case Nusselt number for diffusion from a sphere during low Reynolds number flow is

\[ \text{Nu} = \frac{k_{\text{xm}} \cdot D}{c_f \cdot D_{\text{ABf}}} = 2 \]  \[ \text{[VI-13]} \]

where \( \text{Nu} \) is the Nusselt number, \( k_{\text{xm}} \) is the mass transfer coefficient for ADP, \( D \) is the platelet diameter, \( c_f \) is the total ADP concentration and \( D_{\text{ABf}} \) is the diffusion coefficient for ADP in the platelet suspension (13). Assuming spherical platelets with an average volume of 8 fl (27) and a \( D_{\text{ABf}} \) of 237 \( \mu^2 \)/second (55), estimation of the ADP surface concentration through \[ \text{[VI-13]} \] depends only on \( k_{\text{xm}} \). This mass transfer coefficient can be determined from the continuous measurement of bulk ADP concentration during platelet shear stress exposure as summarized in Table VI-1.

Platelet surface concentrations of ADP estimated by this method are 7.3 \( \mu \text{M} \), 2.9 \( \mu \text{M} \) and 0.8 \( \mu \text{M} \) during exposure to shear stress levels of 50, 33 and 16.6 dynes/cm\(^2\), respectively, in the absence of added chemical agents. These platelet surface concentrations of ADP are in the range known to induce platelet responses in an aggregometer which are similar to those measured in response to shear stress alone. These calculations indicate that the flux of ADP (and, therefore, the ADP concentration at the platelet
surface) is an important parameter in determining the extent of platelet response to applied shear stress.

Platelet dense granule release is potentiated through a dose-dependent relationship by ADP addition in the shear field (Tables VI-1, VI-3, VI-4). In addition, the extent of release after 180 seconds of exposure, at any level of added ADP concentration, is elevated by increasing shear stress intensity. Thus, the study of platelet response, even to chemical stimulus, should be performed in shear fields which are determinate.

Addition of 2 μM ADP largely determines the extent of platelet dense granule release response after 60 seconds of shear stress exposure. Longer exposure times increase the contribution of shear stress relative to that of the single 2 μM ADP addition in terms of platelet dense granule release. Influence of low dose ADP on dense granule release is greatest when shear stress effects alone are small (17 and 33 dynes/cm²). At greater shear stress intensity, the relative importance of 0.2 μM added ADP is reduced.

3. intracellular calcium ion concentration results

Platelets containing up to 200 μM indo-1⁴⁻ do not exhibit the altered responses to shear stress and chemical agents demonstrated by platelets loaded with 956 μM quin2⁴⁻ (Chapter III). Even for these low indo-1⁴⁻ intracellular concentrations, the optically modified viscometer is capable of continuous determination of intracellular calcium ion
concentrations from 0 to 3000 nM in platelet suspensions exposed to controlled, uniform shear stress.

Resting intracellular calcium ion concentration measured in the optically modified viscometer (168 nM) is slightly greater than values previously reported for platelets in anticoagulated native plasma (100 nM) \((18,82-84)\). The increased baseline \([\text{Ca}^{2+}]_i\) is presumably due to cellular modification introduced during the platelet washing procedure.

The rate of calcium ion concentration increase, which occurs after 9 seconds of shear stress exposure at 50 dynes/cm\(^2\), suggests that the platelet activation process, in response to shear stress alone, is initiated uniformly throughout the bulk phase. Evidently, the activation process does not begin as localized platelet activation which propagates via chemical agents through the sample to activate additional platelets. The bulk ADP concentration after 9 seconds of shear stress exposure at 50 dynes/cm\(^2\) is less than 40 nM, a level unlikely to induce an increase in \([\text{Ca}^{2+}]_i\) in a stirred platelet suspension. Shear stress application of sufficient intensity must modify platelet membrane permeability to calcium ion either directly, through passive concentration-driven calcium ion flux across the cell membrane, or as a result of enhanced active transport triggered by increased platelet reactivity to bulk phase chemical agents.
The shear stress dependent increase in platelet $[\text{Ca}^{2+}]_i$ declines when optically measured platelet disaggregation occurs. Thus, platelet disaggregation which occurs in the shear field may not be completely due to hydrodynamic effects, but may also involve reversal of biochemical platelet activation. The degree of $[\text{Ca}^{2+}]_i$ increase observed after addition of ADP is a function of the applied shear stress intensity, emphasizing the important role of hydrodynamics as a variable even in platelet response induced by strong chemical agonists.
CHAPTER VII

INSIGHT INTO THE BIOCHEMICAL MECHANISM OF SHEAR STRESS INDUCED PLATELET ACTIVATION

Shear stress induced platelet aggregation is the final product of many biochemical events which are linked and regulated by a complex network of messengers, inhibitors and enzymes. The response of platelets to applied shear stress is, therefore, a dynamic event which can not be accurately represented by before-and-after measurements.

During exposure to low intensity shear stress, platelets do not experience an increased intracellular concentration of free calcium ion and the extent of dense granule release is small. Reduction in single platelet concentration under these conditions is largely due to passive, transient aggregation caused by flow induced cell-cell collisions and mediated by a sticky protein or proteins such as fibrinogen. Greater levels of shear stress induce large changes in intracellular free calcium ion concentration and rapid elevation of supernatant ADP concentration. Exposed to these conditions, platelet aggregation is less reversible, the mode aggregate size represents incorporation of hundreds of single platelets, fibrinogen polymerizes to insoluble fibrin and von Willebrand factor-platelet binding is presumed to occur.

Supernatant ADP from dense granule release or lysis has been thought to be responsible for shear stress induced
platelet activation. Addition of 0.2 μM ADP to a platelet suspension exposed to shear stress of 17 dynes/cm² results in extensive response, including mild $[\text{Ca}^{2+}]_i$ increase, formation of large aggregates and enhanced dense granule release after only a few seconds. The same platelet suspension treated to shear stress alone achieves a supernatant ADP concentration of 0.2 μM after 180 seconds of exposure through dense granule release, but exhibits only passive aggregation without $[\text{Ca}^{2+}]_i$ mobilization. Clearly, the instantaneous concentration of ADP is not well correlated with these responses. What, then, determines the extent of reaction, the reversibility vs. irreversibility and the aggregate size distribution of shear stress induced platelet aggregation?

Platelets exposed to ADP concentrations which are below the threshold for initiation of aggregation demonstrate reduced response to subsequent ADP challenges; the cells are said to become refractory to ADP. At low shear stresses, the ADP concentration present near the beginning of shear stress exposure serves to inhibit platelet reaction at longer exposure times when the ADP concentration has risen above the level required for platelet activation if introduced as a single dose. The rate of ADP release during the initial stages of platelet exposure to high levels of shear stress is sufficiently great to increase the bulk ADP concentration quickly enough to raise $[\text{Ca}^{2+}]_i$ and induce aggregation through much the same biochemical mechanisms as
does ADP added in a stirred cuvette. Simply stated, the degree of shear stress induced platelet aggregation is not a function solely of the ADP concentration at any discrete point during the exposure time, but depends on the rate of ADP discharge to the supernatant and, therefore, the rate of platelet-ADP binding. Low rates of release favor inhibition by increasing platelet insensitivity to ADP whereas high rates of release are analogous to pulse ADP addition as performed in an aggregometer. Such high rates of release favor platelet aggregation. A hypothesis on the sequence of events is presented schematically in Figure VII-1.

Shear stress application (Figure VII-1a) induces the exposure of receptor sites on the platelet membrane (b) and ADP discharge to the supernatant (c). The rate of ADP binding to receptors is a function of both the rate of site exposure and the rate of ADP release; these rates are both related to the shear stress intensity (d). Receptor exposure, ADP discharge and ADP binding continue to occur (e,f) with increasing time of shear field exposure. This stage of the response is characterized by weak, reversible aggregation, much of which is due to passive, temporary adhesion of individual platelets mediated by flow induced collisions. If the rate of ADP binding exceeds a critical threshold (g), which is a function of the platelet exposure history to ADP, intracellular calcium ion concentration rises and other biochemical events occur (Chapter II) which lead to irreversible platelet aggregation (h).
Figure VII-1 Proposed mechanism of shear stress induced platelet response. (a) shear stress exposure. (b) additional membrane receptor site exposure. (c) ADP discharge. (d) ADP and fibrinogen binding. (e,f) receptor exposure, ADP discharge and binding occur continuously at a rate proportional to the applied shear stress. (g) ADP binding rate exceeds a critical threshold. (h) irreversible platelet activation.
The answer to the perplexing question regarding the initial step of platelet shear stress response is not explicitly revealed through this model. Shear stress may act on the platelet to directly cause exposure of additional membrane binding sites. The discharge of ATP is too great to be the result of lysis, and it may occur too early to be true dense granule release mediated by intracellular biochemical events.

Shear field exposure causes particles to rotate as a direct result of the unequal velocity vectors which act on the particle. Rotating platelets are expected to experience tensile and compressive forces which alternate with a period proportional to the applied shear rate. Through this action, shear stress may decrease platelet membrane stability and allow calcium ion, driven by the large concentration gradient which exists between the resting platelet (100 nM) and the suspending fluid (1000 μM), to enter the cytosol (Figures VII-2a and VII-2b). Initially, natural mechanisms responsible for intracellular calcium regulation are sufficient to actively remove the excess calcium ion against the concentration gradient. Ultimately, as shear stress continues to increase the rate of calcium ion influx, and through the contribution of other activating factors such as membrane receptor binding, the membrane calcium ion pump is overcome (c) and platelet activation rapidly follows the ensuing rise of \([Ca^{2+}]_i\) (d). This postulate is in agreement with the experimental results on
\([\text{Ca}^{2+}]_i\) kinetics of washed platelet suspensions exposed to 50 dynes/cm\(^2\) as shown in Figure VI-8. This type of activation, depicted in Figure VII-2, might be a contribution to shear stress induced platelet activation concurrent with events (b) and (c) of Figure VII-1. Alternatively, the initial steps of this mechanism, before a measurable rise in \([\text{Ca}^{2+}]_i\), might precede steps (b) and (c) of Figure VII-1 and, therefore, represent the initiation mechanism of shear stress induced platelet activation.

Increased calcium ion flux across the membrane may also induce locally high intracellular calcium ion concentrations despite the action of platelet homeostasis in removing or sequestering the additional \([\text{Ca}^{2+}]_i\). Locally elevated intracellular calcium ion concentration would presumably trigger a spatially limited extent of the normal platelet response to high \([\text{Ca}^{2+}]_i\) such as membrane receptor exposure, activation of phospholipases A\(_2\) and C as well as protein kinase C, and arachidonic acid metabolism. In addition, tensile and compressive membrane perturbations caused by shear stress application can enhance diffusion of metabolic ADP through the cell wall into the supernatant and may also directly cause exposure of platelet membrane binding sites.

The increased calcium ion transfer across cell membranes exposed to shear stress has been proposed for other cell types (32a). In addition, results comparing the response of quin2\(^{4-}\) (which employs the same mechanism as indo-1\(^{4-}\)) to the photoprotein aequorin indicate that the
Figure VII-2 Proposed mechanism of initiation of shear stress induced platelet activation. (a) shear stress exposure. (b) increased calcium ion flux down concentration gradient countered by increased rate of membrane calcium ion pumping and sequestering. (c) increased calcium ion flux down concentration gradient exceeds platelet ability to maintain homeostasis; intracellular calcium ion concentration increases. (d) platelet activation with additional membrane receptor exposure and ADP discharge.
fluorescent tetracarboxylate chelators may not respond to local calcium ion levels, but rather yield an average over the cell cytosol (86). Thus, locally increased regions of \([\text{Ca}^{2+}]_i\) may not be well detected in the present work.

The importance of ADP binding rate has been described in association with the activation of protein kinase C (2). There is evidence that the extent of some responses induced by extracellular ADP decay with time after initial ADP-cell binding. In addition, bound ADP appears to either remain at the membrane receptor or otherwise render the binding site permanently inactive. Irreversible inactivation of the finite number of platelet-ADP binding sites represents a logical mechanism for ADP insensitivity of platelets previously exposed to conditions during which the rate of ADP-receptor binding (not simply the ADP concentration) was too low to support platelet activation.

The hypothesis on the mechanism of shear stress induced platelet activation presented here accounts for the differences in the response of platelets exposed to a single low dose of ADP from the response of platelets exposed to the same ultimate ADP concentration during 180 seconds of shear stress. The hypothesis also seems to offer an explanation of the degree of reversibility or irreversibility of shear stress induced platelet response. Most importantly, a potential explanation of the first step of shear stress induced platelet activation has been proposed through the use of a measurement system capable of
monitoring platelet response in the shear field continuously.
CHAPTER VIII
FUTURE STUDIES

The Rice Optical Measurement Viscometer (ROMV) allows independent measurement of fluid sample luminescence, fluorescence and light transmittance during exposure to controlled, uniform shearing flow. The optical measurement characteristics of the ROMV are easily altered to accommodate new or alternate photoprobes through the exchange of colored and/or interference bandpass optical filters. In addition, the lower platen material is easily exchanged which allows use of the proper substrate for anchorage-dependent cells, non-thrombogenic surfaces for cells in suspension, and for most intermediate applications.

Modification of the ROMV is indicated, despite the designed-in flexibility of this instrument. The ROMV presently operates only at room temperature; addition of an air curtain incubator and suitable thermal isolation to permit operation at 37°C is essential. Application of fluid shear stress greater than 50 dynes/cm² would allow continuous measurement of platelet response under conditions which have been well-documented by endpoint techniques. Increased shear stress intensity can be achieved by decreasing the viscometer cone angle and/or increasing the viscosity of the platelet suspension. Better rejection of stray room light and more convienent operating conditions
are essential for continued practical use of the ROMV. These goals can be met by providing a laboratory location for the ROMV selected with consideration of the special constraints of this instrument.

Verification of the postulated platelet biochemical response presented in Chapter VII should be attempted. Platelet trans-membrane Ca\(^{2+}\) flux facilitation due to shear field exposure should be experimentally estimated. The platelet ATP-driven Ca\(^{2+}\) pump must be inactivated during this determination. Furthermore, excess intracellular Ca\(^{2+}\) can be removed from solution by Ca\(^{2+}\)-sequestering membranes. The Ca\(^{2+}\) probe chlortetracycline (CTC), which changes fluorescent emission characteristics upon binding to a Ca\(^{2+}\)-membrane complex, may be useful in estimating the contribution of Ca\(^{2+}\) chelation relative to that of Ca\(^{2+}\) pumping during intracellular Ca\(^{2+}\) hemostasis challenged by shear field exposure. Application of agents, known as calcium channel blockers, which greatly diminish the active Ca\(^{2+}\) hemostasis properties of both cell boundary and intracellular membranes, may be useful in a study of this type.

Another possible technique is the use of liposomes as artificial platelets. Although the physical properties of the liposomes may be different from those of platelets, these artificial membranes do not possess Ca\(^{2+}\) pumps or chelation sites which complicate the Ca\(^{2+}\) flux analysis in platelet suspensions. This work should be extended to
include continuous measurement of ADP or ATP transfer across liposome membranes during shear stress exposure using the luminescent techniques outlined in Chapters V and VI.

The intracellular calcium ion mobilization results reported in this study should be compared to those which can be obtained with the Ca$^{2+}$-sensitive photoprotein aequorin which is reported to be more sensitive to locally elevated Ca$^{2+}$ than indo-1$^{4-}$ (86).

More rigorous calculation of the ADP concentration at the platelet surface should be attempted and compared to the results obtained from the mass transfer correlation as computed in Chapter VI. Simple kinetic models of shear stress induced platelet activation, formulated from data contained in this work, can be used to predict platelet response in shear fields other than those used here. Modification of the shear stress induced platelet surface ADP concentration resulting from bulk phase ADP removal by apyrase or creatine phosphate/creatine phosphokinase could then be estimated.

Platelet pre-treatment with cytoskeletal modifiers such as colchicine or cytochalasin E appears to greatly reduce the extent of shear stress induced dense granule release. These agents may offer unique inhibition of intracellular platelet responses and may be of possible use in biochemical mechanism studies. Results obtained with platelets treated with colchicine or cytochalasin must be interpreted with
some care because the effects of these agents are somewhat non-specific.
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APPENDIX A

USE OF FIREFLY LUCIFERASE IN THE SHEAR FIELD

A. Introduction

Firefly luciferase catalyzes a reaction which produces light in the presence of adenosine triphosphate. A comprehensive review of the luminescent reaction mechanism has been published (69,72). Commercial devices are available which use this technique in the quantitative determination of ATP levels in a variety of systems. At least one such device, the ChronoLog Lumi-Aggregometer, measures the light intensity produced through this reaction as an indication of blood platelet dense granule release. Assay reagents are commercially available for use in the Lumi-Aggregometer. These preparations contain not only the luciferase enzyme, but also include the substrate, luciferin, as well as buffer salts and stabilizers.

Preliminary results, as will be shown, indicated that shear stress induced platelet activation was inhibited by addition of the recommended concentration of commercially obtained luciferase reagent. This inhibition was not as apparent during chemical agonist induced platelet response, but has been reported (96). A systematic study was initiated to determine the specific component of the luciferase reagent responsible for the inhibition.
The composition of Sigma Chemical Co. product number L-0633 is presented in Table A-1. At least two components of this reagent have documented platelet inhibition ability. EDTA is known to inhibit platelet function by chelating extracellular calcium ion (116). This inhibition is reported to be irreversible; if the calcium ion concentration is restored to normal after EDTA incubation, platelet activity remain perturbed (117). High concentrations of magnesium ion inhibit platelet reactivity, presumably by competing for calcium ion binding sites (91). Physiological magnesium ion concentration in plasma is 1 mM.

<table>
<thead>
<tr>
<th>component</th>
<th>mg/100mg bottle</th>
<th>final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>albumin, human serum</td>
<td>22.0</td>
<td>0.880 mg/ml</td>
</tr>
<tr>
<td>EDTA</td>
<td>3.4</td>
<td>0.405 mM</td>
</tr>
<tr>
<td>glycine buffer</td>
<td>73.2</td>
<td>39.0 mM</td>
</tr>
<tr>
<td>luciferase, firefly</td>
<td>0.200</td>
<td>0.008 mg/ml</td>
</tr>
<tr>
<td>luciferin</td>
<td>0.365</td>
<td>0.015 mg/ml</td>
</tr>
<tr>
<td>magnesium sulfate</td>
<td>12.0</td>
<td>4.0 mM</td>
</tr>
</tbody>
</table>

Table A-1. Composition of Sigma Chemical Co. firefly luciferase luciferin reagent (L-0633) for luminescent determination of ATP concentration. The final concentration presented represents the level present in the platelet suspension calculated after reagent dilution as recommended by Sigma Chemical Co.

B. Methods

Human blood was drawn into 10 units/ml heparin and centrifuged for PRP as in preparation for other shear stress induced platelet activation experiments (Chapter II). Aliquots of the PRP was treated with either EDTA or
magnesium sulfate at a final concentration identical to that obtained with luciferase reagent. Additional samples were treated with the luciferase reagent. Normal saline was added to the control samples. The PRP was sheared in the rotational viscometer at 25, 50, or 100 dynes per centimeter$^2$ for one minute at 24°C (Chapter II). The number of single platelets was determined by electronic particle counting (Chapter II, Appendix B).

C. Results

The results in Figure A-1 indicate significant inhibition of shear stress induced platelet aggregation in samples treated with the luciferase reagent. Addition of magnesium ion or EDTA to control platelet aliquots produces a level of inhibition which is not statistically different than that measured in the samples containing luciferase reagent. The only exception is the relative lack of inhibition provided by EDTA treatment during shear stress exposure of 50 dynes/cm$^2$.

D. Discussion

The indication that magnesium ion and EDTA are the primary causes of luciferase reagent induced platelet inhibition has been independently confirmed (96). The extent of this inhibition was considered to be too extensive to permit use of the commercial luciferase reagents during measurement of shear stress induced platelet activation.
Figure A-1. Shear stress induced platelet aggregation of samples treated with Sigma Chemical Co. firefly luciferase luciferin reagent (LUMI REAGENT), 4.0 mM MgCl₂ (Mg²⁺), 0.405 mM EDTA, or saline (CONTROL). Bar height represents average value; extension above bar represents one standard deviation. *p < 0.01; **p < 0.001 Student's paired t-test.
An alternative luciferin-luciferase preparation which did not contain platelet function inhibitors was required. Crude firefly lantern extracts and less purified luciferase produce more light per gram-mol of added ATP, but do so through the activity of contaminating adenylate kinases. Thus, some fractional component of the luminescence is due to ADP and AMP. These preparations are unsuitable for use during platelet activation due to the ADP scavenging effect (25,104). Concentration reduction of platelet inhibitors contained in the crude luciferase preparations via increased dilution is not effective (24). Commercial preparations lacking high concentrations of EDTA or magnesium ion are not available.

E. Preparation of Inhibition-free Luminescent Reagent

Two purified luciferase preparations from Sigma Chemical (L-5256, L-1759) were tested as was a synthetically prepared sodium salt d-luciferin (L-6882), also from Sigma. The chromatographically prepared luciferase (L-5256) proved to be unsuitable due to a lack of solubility in aqueous medium. The other luciferase was found to be highly soluble as was the luciferin.

Frozen solutions of luciferin or luciferase are relatively stable if certain conditions are met. EDTA has been found to prevent enzyme inactivation during storage (24). Luciferase is also stabilized by addition of protein in the diluent; bovine serum albumin is a common addition
to commercial preparations and, at low concentrations, has no effect on platelet function. Degradation of luciferin is primarily due to oxidation (24). Luciferin solution stored frozen and under nitrogen is stable for at least 24 weeks (24).

Separate stock solutions were prepared for the enzyme and the substrate. Stock reagent compositions are given in Table A-2. All aliquots were buffered at pH = 7.70 and were kept frozen at -70°C. The luciferin stock was prepared in 1.5 ml vials sealed with a screw cap fitted with a teflon and rubber septum. Nitrogen was purged through the septum into the luciferin filled vial for five minutes prior to storage. Aliquot volume was 80 µl for the luciferase and 20 µl for the luciferin. The final luminescence reagent is prepared by mixing one luciferase and one luciferin aliquot together. Nominal dilution of the mixed reagent is 1:120 with sample for determination of platelet ATP release.

**LUCIFERASE STOCK**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>bovine albumin</td>
<td>5 mg/ml</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.25 mM</td>
</tr>
<tr>
<td>HEPES buffer</td>
<td>10.0 mM</td>
</tr>
<tr>
<td>luciferase</td>
<td>2.5 mg/ml</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.9% (w/v)</td>
</tr>
</tbody>
</table>

**LUCIFERIN STOCK**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES buffer</td>
<td>10.0 mM</td>
</tr>
<tr>
<td>luciferin</td>
<td>50 mg/ml</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.9% (w/v)</td>
</tr>
</tbody>
</table>

Table A-2. Composition of firefly luciferase and luciferin in stock preparations.
F. Luminescent Reagent Testing

1. luminescence

The luminescent activity of the new reagent was compared to that of the commercial Sigma Chemical Co. preparation under identical conditions. Each luminescent reagent was diluted to the appropriate concentration in HEPES buffered saline (pH = 7.4) containing 1 mM MgCl₂. Aliquots (400 µl) of each solution were analyzed for luminescent activity in response to 0.01, 0.1, 1.0 and 10.0 µM ATP in the Lumi-Aggregometer. Table A-3 presents the maximum luminescent intensity (measured in mV) of the two reagents after addition of various ATP concentrations.

<table>
<thead>
<tr>
<th>added ATP µM</th>
<th>Sigma L-0633 luminescence</th>
<th>Inhibitor-free luminescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>$12.0\cdot10^0$</td>
<td>$34.9\cdot10^1$</td>
</tr>
<tr>
<td>0.10</td>
<td>$12.6\cdot10^1$</td>
<td>$37.8\cdot10^2$</td>
</tr>
<tr>
<td>1.0</td>
<td>$12.9\cdot10^2$</td>
<td>$37.2\cdot10^3$</td>
</tr>
<tr>
<td>10.0</td>
<td>$11.0\cdot10^3$</td>
<td>$38.1\cdot10^4$</td>
</tr>
</tbody>
</table>

Table A-3. Maximum luminescent reaction velocity of Sigma Chemical Co. L-0633 firefly luciferase-luciferin reagent and inhibitor-free prepared firefly luciferase-luciferin reagent in response to added ATP. Maximum velocity interpreted as luminescent peak height after ATP injection recorded in mV from a ChronoLog Lumi-Aggregometer.

2. effect on shear stress induced platelet response

Washed platelet suspensions prepared as described in Chapter II were exposed to 0, 16.7, 33 and 50 dynes/cm² of controlled shear stress in a rotational viscometer with and
without addition of 0.83% (v/v) inhibitor-free luminescence reagent. Platelet aggregation, dense granule release and cellular lysis were measured after 300 seconds of shear stress exposure. The results are presented in Figure A-2.

Platelet aggregation was not altered as a result of luminescence reagent treatment. Addition of the inhibitor-free luciferase-luciferin preparation did not cause statistically significant changes in the extent of platelet dense granule release or cellular lysis in response to shear stress stimulus.

G. Discussion and Conclusions Regarding Inhibitor-free Luminescence Reagent

$\text{Mg}^{2+}$ and EDTA are both known platelet function inhibitors. Direct evidence supports the postulate that $\text{Mg}^{2+}$ and EDTA participate in the inhibition of shear stress induced platelet response caused by commercial luminescence reagents. An inhibitor-free luminescence reagent was prepared without $\text{Mg}^{2+}$ and with greatly reduced EDTA concentration. Addition of this reagent (0.83% v/v) to platelet suspensions resulted in luminescent response 300-fold greater than that measured with commercial luminescence reagents. In addition, platelet suspensions containing the inhibitor-free preparation do not exhibit significant platelet function alteration in response to applied shear stress. It seems clear that use of commercial luminescence reagents which contain high concentrations of
Figure A-2. Response of control washed platelets (□) and washed platelets treated with 0.83% (v/v) inhibitor-free luciferase-luciferin (△) to shear stress. Exposure time is 300 seconds.
Data: average ± standard deviation
$\text{Mg}^{2+}$ and/or EDTA should be avoided in platelet function studies.
APPENDIX B

PLATELET COUNTING AND AGGREGATE SIZE DISTRIBUTION

A. Introduction

Determination of platelet concentration is most often performed with either a phase contrast microscope and a hemocytometer counting chamber or an electronic particle counter. Platelet counting with a hemocytometer is slow, subject to operator interpretation and error, and will yield a rough particle size distribution only after much effort. A Coulter electronic particle counter together with a Coulter Channelyzer can make rapid measurements of both particle concentration and particle volume distribution. Interpretation of Channelyzer results is complicated by the heterogeneity of single platelet volume when the sample has formed few and/or small (less than 10 platelet volume) aggregates.

The ability of the Coulter Counter to discriminate single platelets from small aggregates is greatly enhanced through the use of a Coulter Channelyzer and logarithmic scale expander. This technique allows the particle volume range of interest to be isolated and expanded into a 100 channel histogram representation of the population size distribution. A study was initiated to determine the optimum technique for the electronic measurement of small
changes in the concentration of single platelets induced after shear stress exposure.

B. Theory

The Coulter Channelyzer can resolve the number and intensity of impedance inhomogeneties measured by the Coulter Counter into a particle size distribution. The base channel threshold control and the window width control determine, respectively, the smallest and largest particle volumes which are included in the distribution. Judicious selection of the Channelyzer operating conditions allows selection of any particle volume range to be displayed as a 100 channel particle volume histogram. Precise calibration techniques are required for accurate interpretation of the particle histogram when the range of volumes is relatively narrow.

Polymer spheres of known, monodisperse, modal volume can be used to determine the particle volume which corresponds to a given channel in the Coulter Channelyzer. The volume in any of the other 99 channels can then be calculated:

\[
\log_{10}(V_C) = \log_{10}(V_{ref1}) + \frac{(N_C - N_{ref1}) \cdot (WW_{ref}/100)}{25} \quad [B-1]
\]

where \(V_{ref1}\) and \(N_{ref1}\) are the calibration sphere volume and the mode channel number of the calibration spheres as measured in the Channelyzer, respectively. \(V_C\) and \(N_C\) are the corresponding values for any other channel and \(WW_{ref}\) is
the window width parameter set on the Channelyzer. The use of two independent sets of calibration spheres, each having a different but accurately known modal volume, allows calibration of the channel volume size distribution without assuming knowledge of the window width. The window width is normally determined from the mechanical position of a dial and is responsible, in large part, for any error in the channel volume distribution calculation. Direct substitution into [B-1] gives:

\[
\log_{10}\left(\frac{V_{\text{ref2}}}{V_{\text{ref1}}}\right) = \frac{(N_{\text{ref2}} - N_{\text{ref1}}) \cdot (WW_{\text{ref}}/100)}{25} \tag{B-2}
\]

where \(V_{\text{ref2}}\) and \(N_{\text{ref2}}\) are the sphere volume and mode channel number corresponding to the second set of calibration spheres. Rearrangement yields:

\[
WW_{\text{ref}} = \frac{2500 \cdot \log_{10}\left(\frac{V_{\text{ref2}}}{V_{\text{ref1}}}\right)}{(N_{\text{ref1}} - N_{\text{ref2}})} \tag{B-3}
\]

The calculated window width can then be used in [B-1] with either set of calibration data to determine the Channelyzer volume distribution.

C. Experimental Methods

Calibration spheres (2.02\(\mu\) diameter, polyvinyl toluene; 4.92\(\mu\) diameter, polystyrene (Curtin Matheson Scientific)) were diluted 3000:1 in Isotón II. A 50\(\mu\) diameter aperture was used to aspirate 100 \(\mu\)l of diluted calibration spheres through the counting chamber. The Coulter settings used to
obtain these results were: lower threshold = 10, upper threshold = 100, aperture current = 2, amplification = 2, base channel threshold = 10, window width = 70. The channel number corresponding to the mode of the particle volume distribution was recorded for ten different counts performed with each set of spheres.

The second part of the experiment used this information, gathered at a variety of indicated window widths, to calculate the particle volumes represented by each of the 100 Channelyzer bins through application of equation [B-1]. The Channelyzer volume distribution was calibrated using the window width parameter calculated from [B-3] for 2.02 and 4.92 μ diameter spheres. Channel volumes were dedimensionalized through division by the average volume of a single platelet (taken to be 8 μ³ (27)).

D. Results

Figure B-1 displays three curves which represent the channel volume distributions calculated from equation [B-1] on the basis of 2.02μ diameter spheres and the mechanically indicated window width of 70, 4.92μ diameter spheres and the same window width, and the two sets of calibration spheres with a window width determined by equation [B-3]. The window width calculated from the latter is 80.5, which represents an error of 10.5 units or 15 percent. Table B-1 presents the difference between the mechanically indicated
window width and the same parameter calculated from calibration spheres.

Table B-2 displays the effect of window width on the particle volume range which is reported by the Channelizer. The smallest measurable particle volume is essentially unaffected by changing window width. The volume represented by channel 99 grows logarithmically with increasing window width. The number of channels corresponding to dimensionless particle volumes ranging from 0.5 to 1.5 decreases logarithmically with increasing window width. This range of particle volumes represents >67% of the entire volume of an anticoagulated platelet suspension prepared by a standard method (Chapter II).

E. Discussion and Conclusions

Increasing discrimination of single platelets from small aggregates through adjustment of Channelizer window width is accompanied by a reduction in the volume of the largest particle which can be simultaneously measured. Clearly, the selection of Coulter operating conditions represents a compromise which must be evaluated with consideration of the type of measurement desired. For most experiments in this thesis, accurate enumeration of single, unaggregated platelets before and after exposure to stimuli was a more important consideration than the population size distribution of the formed aggregates. This type of measurement is expected to be the most sensitive indicator
Figure B-1  Particle volume distribution represented by the Coulter Channelyzer, calculated from 2.02μm diameter calibration spheres (□) and window width = 70 (a); calculated from 4.92μm diameter calibration spheres (△) and window width = 70 (b); calculated from both sets of calibration spheres and the calculated window width of 80.5 (c).
<table>
<thead>
<tr>
<th>indicated window width</th>
<th>calculated window width</th>
<th>percent error</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>66.3</td>
<td>10.5</td>
</tr>
<tr>
<td>70</td>
<td>80.5</td>
<td>15.0</td>
</tr>
<tr>
<td>80</td>
<td>89.7</td>
<td>12.1</td>
</tr>
<tr>
<td>90</td>
<td>100.6</td>
<td>11.8</td>
</tr>
<tr>
<td>95</td>
<td>107.3</td>
<td>12.9</td>
</tr>
</tbody>
</table>

Table B-1 Comparison of the window width parameter indicated on the Coulter Channelyzer with that calculated from two sets of calibration spheres having uniform, well characterized diameters.

<table>
<thead>
<tr>
<th>indicated window width</th>
<th>dimensionless volume range</th>
<th>number of channels representing 0.5-1.5 platelet volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.28-1.71</td>
<td>60</td>
</tr>
<tr>
<td>30</td>
<td>0.25-3.87</td>
<td>40</td>
</tr>
<tr>
<td>40</td>
<td>0.26-9.82</td>
<td>30</td>
</tr>
<tr>
<td>50</td>
<td>0.26-25.1</td>
<td>24</td>
</tr>
<tr>
<td>60</td>
<td>0.25-59.7</td>
<td>19</td>
</tr>
<tr>
<td>70</td>
<td>0.24-143.</td>
<td>17</td>
</tr>
<tr>
<td>80</td>
<td>0.24-354.</td>
<td>15</td>
</tr>
<tr>
<td>90</td>
<td>0.25-865.</td>
<td>13</td>
</tr>
<tr>
<td>100</td>
<td>0.24-2115</td>
<td>11</td>
</tr>
</tbody>
</table>

Table B-2 Optimization of the mechanically indicated Channelyzer window width for single platelet counting. The dimensionless volumes are calculated as the channel volume divided by the average volume of a platelet singlet (8 μ). The number of channels which represent dimensionless particle volumes from 0.5 to 1.5 are presented as an indication of the relative discrimination between single platelets and small aggregates for each window width setting.

of the subtle single platelet concentration differences which occur after platelet suspension exposure to low shear stress intensity and/or for short shear exposure times. Maximizing sensitivity in this way presumably yields results most similar to those made by microscopic visualization and counting of single platelets in a hemocytometer such as has been reported by other workers (76,77). The mechanically
indicated window width used during these experiments was 35-40.

Additional experiments were designed to investigate the population size distribution of platelet aggregates formed in response to chemical agents (Chapter IV) or shear stress alone (Chapter VI). These experiments required the measurement of particles as large as 8580 μ³. The Channelyzer window width required to measure particles of this size resulted in reduced accuracy in the determination of single platelet concentration. Additionally, analysis of the population size distribution of these samples could not be effectively carried out with the 50 μ aperture tube even with a window width of 100 due to statistical variation resulting from the small number of large particles counted.

The measurement of both single platelets and large aggregates can be performed without compromise if the sample is independently analyzed with two aperture tubes of different size. Thus, the 50 μ aperture can be used to measure the concentration of particles from 2.55 to 490 μ³ without sacrificing much sensitivity to changes in single platelet number. Use of a 110 μ aperture tube allows aspiration of a larger sample volume (500 μl as compared to 100μl aspirated by the 50 μ orifice) which results in a five-fold increase in the number of large particles counted. The relatively poor small particle sensitivity of this large aperture tube is unimportant because information regarding particles less than 490 μ³ is obtained by the 50 μ aperture
tube. Thus, the larger orifice is used to determine the population size distribution of particle volumes between 490 and 8580 \( \mu^3 \) only.

Splicing the two particle size distributions together is made possible through the use of calibration equations [B-1] through [B-3]. Four sets of calibration spheres, two per orifice, are used to determine the actual window width for each aperture tube as described. The appropriate fraction of the population size distribution generated by each of the two orifices can then be matched at the common volume of 490 \( \mu^3 \), thus creating a seamless, accurate population size distribution of particle volumes from 2.55 to 8580 \( \mu^3 \) without diminishing the ability to discern single platelets from small aggregates.

It seems clear that the accuracy of electronically determined population size distribution measurements is a direct function of the window width value employed in the calibration. As was shown, window width determined from the orientation of a mechanical dial can reflect errors of 15% as compared to the actual, electronic window width. Careful determination of the window width parameter, such as can be performed with the dual calibration particle volume technique described here, is indicated.