THE ROLE OF PLATELET-DERIVED MATERIALS IN MURAL THROMBOGENESIS

By

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ABSTRACT

Washed human platelet suspensions were used to study platelet-surface interactions in tubes at wall shear rates between 80 s^{-1} and 640 s^{-1} and exposure times up to 900 s. Radiolabeled platelets were used to measure platelet accumulation and release from accumulated platelets as a function of time, shear rate, distance from the inlet of a tube, surface composition and drug treatments of platelets. These empirical data were used in a calculation procedure based upon diffusion and convection, designed to yield the maximum interfacial fluid concentration (IFC) for each of the materials which are liberated from platelets during platelet accumulation upon surfaces. Substances such as AMP, ATP, serotonin, pyrophosphate, PGG_2 and PGH_2 were found not to be present in sufficient quantity to produce IFC's which could affect platelet aggregation. A second set of materials, von Willebrand factor, fibronectin, and calcium had IFC's less than the concentrations normally found in plasma. A third group, ADP, PGD_2, and TA_2 had IFC's close to those known to affect platelet aggregation. These last materials, along with materials formed by a vessel wall or in plasma are most likely to determine the rate of thrombus growth on subendothelium or on a blood-contacting biomaterial.

As well, epi-fluorescent video microscopy was used to monitor platelet-surface interactions with different surfaces giving different spectrums of results.

Surface	Adhesion	Aggregate formation	Non-adhesive encounters	Thrombo-emboli	Distribution
collagen	+++	+++	0	0	patchy
fibrinogen	+++	0	++	0	even
fibronectin	+++	0	++	0	even
albumin	0	0	+++	0	0
glass	+	++	+	+++	patchy

Aspirin, sulfinpyrazone, indomethacin and PGE_1 treatment of platelets inhibited aggregate formation on a collagen surface but not platelet adhesion. Heparin, hirudin, imipramine, mepacrine, adenosine triphosphate, creatine phosphate/creatine phosphokinase treatment of platelets had no or little effect on adhesion, release or aggregate formation. Modification of platelet functions using pharmacological and suspension modification techniques demonstrated mural aggregate formation was independent of release of adenosine diphosphate by adherent platelets but dependent on prostaglandin and thromboxane formation.

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CHAPTER 1

INTRODUCTION

1.1 Aims and Structure of This Thesis

The primary function of blood platelets is the formation of a hemostatic plug during hemorrhage due to partial or complete severing of a blood vessel. The arresting of blood loss is accomplished by the formation of a platelet mass at the site of injury. The process of mural thrombogenesis is a similar one, wherein platelets adhere to the side of a vessel, but in this case the platelet mass ceases growing prior to occlusion of the vessel. Mural thrombogenesis also occurs on the blood-contacting biomaterials used in artifical organs and implantable vascular prostheses. The formation of thrombi and their subsequent embolization represents one of the principle problems limiting the use of such devices. A knowledge of the mechanisms involved in surface-induced thrombogenesis is required for the logical development of biocompatible materials, as well as for a greater comprehension of the pathological states associated with thrombosis.

The work presented in this thesis was conducted to probe one aspect of this problem, namely, the role of those materials stored in platelets and liberated during the process of platelet accumulation on a surface. Since both physical and biological conditions are important in determining the availability of materials close to a surface, a general approach has been taken to use engineering principles and biological

data to predict the materials most likely to be controlling platelet accumulation on the surfaces. These predictions were tested using experimental procedures.

The thesis is compiled into nine chapters. The first chapter gives background information about basic platelet physiology for the reader unfamiliar with platelet functions. This chapter also contains a review of previous studies, conducted by other researchers, on the response of blood to surfaces. The second chapter catalogues the experimental procedures used in obtaining the data reported in subsequent chapters. The third chapter presents a calculation procedure that yields estimates of the maximum concentration possible near a surface for any material liberated from platelets and predicts those materials most likely to be influencing platelet accumulation on surfaces. The fourth chapter uses empirically obtained data on the extent of liberation of materials stored in the dense granules of platelets to estimate the actual concentrations of liberated materials (not the maximum concentrations as in Chapter 3). One of the principal conclusions from Chapter 4 that adenosine diphosphate (ADP) may be in sufficient concentration to affect platelet accumulation on a surface is tested experimentally in Chapter 5. The use of pharmacological modification of platelet functions to isolate platelet activation pathways and the association of these pathways with platelet-surface phenomena is presented in Chapter 6. The use of surfaces with different compositions in Chapters 7 and 8 allows the extent of release and the platelet accumulation to vary independently. The use of different

surfaces was necessary as no drug treatment used in chapter 6 was found to inhibit release from platelets accumulated on a collagen-coated glass tube. A general discussion of the experimental and calculated results is given in Chapter 9 along with some ideas for possible extensions of the present work.

1.2 Platelet Physiology

1.2.1 The Structure of Platelets

Platelets contain at least 3 types of storage granules that can release their contents upon stimulation (reviews, 1-4). Dense granules contain a variety of materials including serotonin, ADP, ATP, AMP, calcium, magnesium and perhaps epinephrine and dopamine(5). The number of dense granules varies from platelet to platelet and the average number of dense granules in platelets varies with different species(6). Human platelets contain 7-8 dense granules of average diameter 160 nm(1-4). Alpha granules contain proteins such as fibrinogen, fibronectin, platelet factor 4 (PF4), beta-thromboglobulin (βTG), factor VIII and a factor that is mitogenic for smooth muscle cells(1-4). A third class of granules is lysosmal granules that contain acid-hydrolase enzymes(7).

1.2.2 Pathways of Platelet Stimulation

Platelets change shape from their normal disc shape to a more spherical form with spiny protrusions and aggregate when stimulated by adenosine diphosphate (ADP), collagen, thrombin, serotonin, epinephrine,

calcium ionophores, arachidonic acid metabolites and other materials(8). The device normally used to measure platelet aggregation is based on the decrease in turbidity of the platelet suspension as aggregates form(9). Using such a device, researchers have demonstrated that a limited number of pathways exist through which these stimulatory agents act.

Stimulation of platelets by ADP results in platelet shape change and aggregation (review 10). In citrated plasma, ADP also induces release of granule contents(8,10,11). The mechanism of platelet stimulation by ADP has not been fully defined but hypotheses include an ecto-dinucleotide kinase that uses platelet ATP as a phosphate donor(12), inhibition of adenylate cyclase leading to decreased intracellular cyclic adenosine monophosphate (AMP) levels(13) and mobilization of intracellular calcium(14). ADP-induced platelet aggregation can be inhibited specifically by the removal of ADP using enzymes such as apyrase and creatine phosphate/creatine phosphokinase(15) or by addition of exogenous ATP(12,16) or other triphosphate nucleotides(12). Since platelet granules contain ADP and are released during platelet activation, a positive feedback amplification loop is possible and ADP released from platelets has been shown to be one of the pathways whereby stimulants activate platelets(8,15).

The liberation of arachidonic acid from platelet membrane phospholipids and its subsequent metabolism results in the formation of materials that constitute a second pathway for platelet activation (Figure 1.1) (reviews 17-21). The arachidonate metabolites,

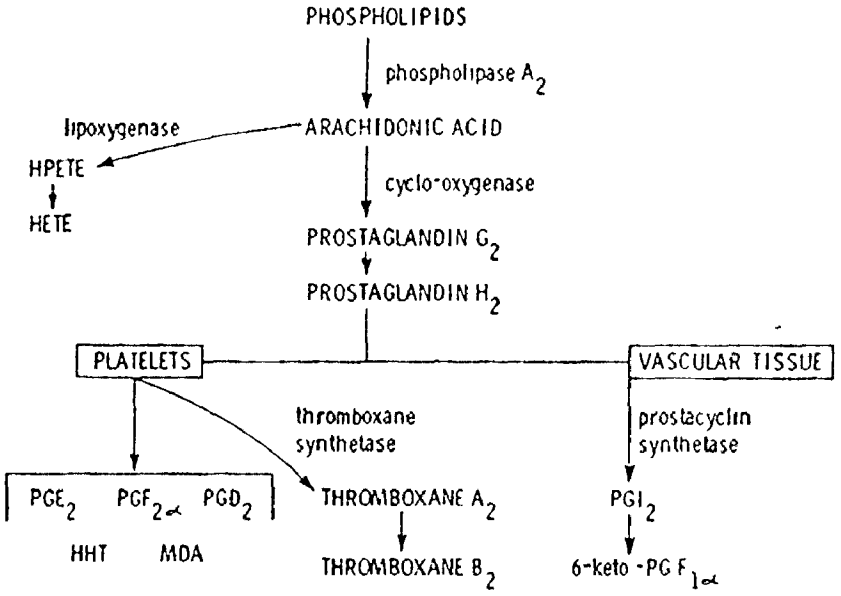


Figure 1.1: Metabolism of arachidonic acid and biosynthesis of prostaglandins (PG) and thromboxanes in platelets and vascular tissues. HPETE = 12-L-hydroperoxy-5,8,10,14-eicosatetraenoic acid; HETE = 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid, HHT = 12-L-hydroxy-5,8,10-heptadecatrienoic acid; MDA = malondialdehyde, from reference 18.

prostaglandins (PG) G_2 and H_2 and thromboxane A_2, can all induce platelet aggregation, while prostaglandin D_2 inhibits platelet responses. Other cells have similar capabilities of forming prostaglandins from internal stores of arachidonic acid; notably endothelial cells produce prostaglandin I_2, an inhibitor of platelet functions(17-21). A number of materials have been reported to inhibit the arachidonate pathway at various levels(17-21). Mepacrine(18) (cf. 1.3.2.6) and perhaps sulfinpyrazone(20) inhibit arachidonic acid liberation from membrane phospholipids. Non-steroidal antiinflammatory drugs such as aspirin, sulfinpyrazone and indomethacin all inhibit the cyclo-oxygenase enzyme that converts arachidonate to PGG_2 and PGH_2. Imidazole and 9-11, azoprosta, 4-13, dienoic acid, have been reported to inhibit thromboxane synthetase which converts PGH_2 into thromboxane A_2(20).

The use of inhibitors of the pathways of platelet activation has enabled the mechanisms of action of some stimuli, such as low concentrations of collagen, to be defined. The removal of the ADP that is released by stimulated platelets or the inhibition of thromboxane formation by stimulated platelets, both partially inhibit platelet aggregation induced by low concentrations of collagen(22). The combined removal of ADP and inhibition of thromboxane formation completely inhibits platelet aggregation induced by low concentrations of collagen. Accordingly both release of ADP from platelet granules and the arachidonate pathway are thought to be activation pathways for platelet aggregation in response to collagen.

Collagen, thrombin, ionophore A23187, and in citrated plasma, ADP all activate the arachidonic acid and the ADP pathways(8). One or more other pathways exist to activate platelets, as thrombin will aggregate platelets when the ADP and arachidonate pathways are inhibited(8,20). Since thrombin is formed on platelet surface and is a powerful stimulator of platelet aggregation and release, some researchers feel trace thrombin mediates platelet responses(20). To this end, high concentrations of heparin or hirudin have been reported to inhibit collagen-induced platelet aggregation(20). However other researchers have shown thrombin-degranulated platelets that do not respond to re-addition of thrombin do aggregate to collagen(23). The exact intracellular biochemical and physiological processes involved in platelet stimulation are unknown.

Emerging concepts in platelet activation suggest that all the stimulatory agents may act through a common intracellular messenger, such as calcium. Greater stimulation leads to more extensive responses going from platelet shape change through platelet aggregation, release of dense granules, release of alpha granules and finally release of lysosomal granules for maximum stimulation(24). If such a hypothesis were true than different stimulating agents should have at least additive effects, which they do(24). However some conditions such as exposure of platelets to low concentrations of thrombin lead to preferential release of alpha-granule contents suggesting that dense-granule release and alpha-granule release need not parallel each other nor be successive to each other(25). The role of cyclic AMP is also

being explored and present concepts suggest that cyclic AMP may control calcium concentrations by stimulating the removal of calcium from the cytosol(14). The mechanism of how increased cytosolic calcium leads to platelet activation is not known. Thus, while there are at least three pathways whereby stimulants can activate platelets, different stimuli act through different pathways. The concentration of the stimulus and the concentration of calcium in the medium are important parameters in determining the pathway or pathways activated. In addition for platelet aggregation to proceed, the medium must contain certain cofactors.

1.2.3 Cofactors in Platelet Stimulation

A number of cofactors are required for platelet aggregation to occur. Stirring to facilitate collisions between platelets is necessary. Also, washed human or porcine platelets will not aggregate to ADP or low concentrations of collagen without the addition of fibrinogen(26). The fibrinogen binds to the platelet membrane during platelet shape change and dissociates from the membrane when the platelets deaggregate(27,28). However platelets do aggregate to thrombin or high concentrations of collagen in the absence of added fibrinogen probably because sufficient platelet fibrinogen (stored in the alpha granules) to support platelet aggregation is released from stimulated platelets(1,8,26). The aggregation of platelets in plasma can be blocked using monovalent antifibrinogen fragments indicating that fibrinogen is a cofactor(29).

Platelets can also aggregate in response to ristocetin, in the

presence of factor VIII(26). This reaction does not require fibrinogen. Whether material is available in plasma or within platelet granules that can act like ristocetin is unknown. Platelet retention in glass bead columns has been shown to require factor VIII(30). Other possible mechanisms for platelet cohesion then, may be through bridges involving factor VIII or other materials released from platelets.

Extracellular calcium is required for platelet aggregation. Platelet aggregation and release of granule contents occurs in citrated platelet-rich plasma(8). In calcium containing suspensions, platelets aggregate but do not undergo the release reaction upon stimulation with ADP. Modification of the calcium containing suspensions by addition of citrate results in release from platelets upon stimulation by ADP as occurs in citrated platelet-rich plasma(8). It is believed that the citrate promotes activation of the arachidonate pathway that leads to the release reaction(8). Chelating agents such as ethylene-diamine-tetra acetate (EDTA) that have high affinities for calcium block platelet aggregation but not release from platelets(26).

Platelets have coagulation factors in close association with their surface (review 31). Binding sites for factors V, X and fibrinogen have been identified. The complex of [Factors Va, Xa, calcium and platelet factor 3] leads to a rapid conversion of prothrombin to thrombin. By analogy the conversion of factor X to Xa by factor VIII and factor IX also is enhanced by platelet factor 3 suggesting the platelet has binding sites for factors VIII and IX. Lipscomb and Walsh(32) have presented evidence that platelets and factor

XI can activate the intrinsic coagulation cascade. Platelets have receptors for many other materials but the role of these materials in platelet activation has not been determined (review 10).

Thus, platelets require plasma cofactors to complete the aggregation response to certain stimuli such as ADP. Platelets can also release fibrinogen to participate in platelet aggregation when plasma is not present. Even when the release reaction takes place, some calcium is required for platelet aggregation to result as demonstrated by the inhibition of thrombin-induced aggregation by EDTA.

1.2.4 Platelet Aggregometry and Mural Thrombogenesis

Most of the studies on platelet functions have been conducted using platelet aggregometry techniques. The physical conditions of platelet interactions during aggregometry studies are greatly different from the physical conditions during platelet accumulation on a surface. In an aggregometer test, a platelet suspension with no red blood cells present is added to a cuvette. The suspension is stirred vigorously by means of a magnetic stir bar in the bottom of the cuvette while the platelet stimulant is added and platelet aggregation occurs(9). Stirring is required to rapidly disperse the stimulant as well as to facilitate platelet-platelet collisions and aggregate growth. The number of platelets associated with the collagen fibrils that are added as the stimulant in a collagen aggregation test is small. However, it is the materials released from the few platelets that are in contact with the collagen fibrils that activate the bulk of the platelets

resulting in aggregation of the entire sample. The two known mediators that are liberated from adherent platelets are ADP and thromboxane-A_2(8). The stirring also rapidly disperses the materials released by platelets. The final supernatant concentrations of materials released by platelets in the aggregation cuvette may be quite high as all of the platelets are stimulated to release.

In contrast during the contact between a surface and flowing blood (red blood cells present), the stirring required in aggregometry studies is replaced by the tumbling motions of the red blood cells. These tumbling motions induce movement of platelets through the blood facilitating platelet collisions with the surface and mural aggregate growth. The surface is effectively a stimulant so adherent platelets release their granule contents into the flowing blood. The dispersal mechanism of red blood cell tumbling has little effect on the materials released from adherent platelets as these materials are molecules with a much smaller size than platelets. The dispersal mechanisms for materials released from platelets during mural thrombogenesis are not mixing but diffusion away from the surface and convection along the surface by the flowing blood. These dispersal mechanisms of diffusion and convection are slow relative to the stirring used in aggregometry studies. Also in mural thrombogenesis only a few of the total platelets in the blood adhere to the surface and release their granule contents. The generation of a region of high concentration of materials released from platelets near the surface is a distinct possibility, despite the few platelets releasing materials, as the dispersal mechanisms are

slower.

Thus, while the conditions in aggregometry results in a rapid dispersal of materials released from all the platelets to give a high concentration of released materials, the same concentration can develop near a surface due to the release of materials from a few platelets and slower dispersal mechanisms. The mechanisms and pathways of platelet stimulation by surfaces will probably be similar to those already determined and enumerated in section 1.2.2. The controlling pathways and extent of platelet stimulation by surfaces remains to be determined.

1.3.1 Protein Adsorption to Surfaces

Proteins adsorb to synthetic materials (reviews, 33-37) within a few seconds of exposure to blood and form a coating that may influence subsequent platelet deposition. The composition of the adsorbed layer varies between different surfaces and with different mixtures of proteins. In addition, the functional states of the adsorbed proteins, the rates of adsorption and exchange between proteins and the change in adsorbed protein coating with incubation time are all parameters that need further elucidation.

Albumin, fibrinogen alpha-, beta- and gamma-globulins, transferrin, caeruloplasmin, thrombin, factors XII, XI, V, VIII, hemoglobin and fibronectin(38,39) have all been shown to adsorb to surfaces(33-39). Adsorbtion from single protein solutions results in a monolayer coating provided the protein concentration of the solution is high enough (33-37). Conformational changes occur in factor XII and

fibronectin(38) when they adsorb to a surface whereas other proteins undergo little distortion (review 40). Adsorbed thrombin is still enzymatically active (41) and may represent a portion of the adsorbed plasma components. Lipoproteins have also been reported to be adsorbed to artificial surfaces (34). The adsorbed proteins are in dynamic exchange with solution proteins. The biological status of proteins that adsorb to a surface and then come off has not been determined(33).

In mixtures of proteins, preferential adsorbtion of one protein species may occur. Fibrinogen adsorbs strongly to a number of surfaces(33-34). Fibrinogen adsorbtion is unaffected by binary mixtures of albumin/fibrinogen and gamma-globulin/fibrinogen(42). However, the extent of fibrinogen adsorption from plasma is much less than that observed with fibrinogen alone or in binary solutions. Hemoglobin has been suggested to be an important adsorbed protein as it has been shown to displace fibrinogen from polyethylene(42). Hemoglobin would be present in plasma at low but significant concentrations(43). Exposure of glass to plasma and subsequent in situ radioiodination, elution of the adsorbed proteins and analysis of the eludates with electrophoresis has demonstrated adsorption of other, as yet unidentified, plasma components(42). In adsorbtion from mixtures of proteins or plasma, the initially deposited material changes with time presumably due to exchange of proteins and the preferential adsorbtion of the proteins with the highest affinity for the surface. Different surfaces exhibit different affinities for the different plasma proteins. In addition fluid motion and the presence of red blood cells also affect protein deposition(33).

The exact nature of the glycoprotein coat that forms on synthetic materials, its change with time and the plasma components responsible for its thrombogenic properties remains obscure.

Since proteins rapidly adsorb to surfaces plasma proteins have been studied as possible bridging molecules between artificial surfaces and platelets. Fibrinogen, gamma-globulin and fibronectin have all been shown to support platelet adhesion while albumin prevents platelet deposition. Kim and Lee(44) have advanced a hypothesis suggesting that it is the lack of carbohydrate in albumin that gives it its nonthrombogenic properties. This is consistent with a general theory of cellular adhesion advanced by Roseman(45) of glycosyltransferase reactions linking glycoproteins and cells. However, other reseachers dispute such a mechanism (review 46,47).

Characteristics of surface charge (ionic), surface charge (polar), conductivity, zeta potential, surface energy, work of adhesion, interfacial energy, critical surface tension, hydrophilicity, hydrophobicity and surface texture have all been implicated in thrombogenicity of surfaces (reveiws, 33-37,48,49). Despite the study of a large variety of different surfaces no one characteristic or group of characteristics that controls platelet deposition and thrombogenesis, have been identified. Part of the reason for this lack of knowledge may be due to inability to accurately define surface characteristics and an equally poor characterization of platelet responses to the surfaces.

1.3.2 Platelet Accumulation on Surfaces

Platelets adhere to a great variety of both biological and synthetic surfaces (Table 1.1, references (51-150). While numerous studies have been conducted to probe the mechanisms of platelet accumulation little is known about the process of mural thrombogenesis or the conditions that impart normal endothelium with its nonthrombogenic properties (reviews 8,17,33-38,50). Differences in experimental variables such as hematocrit, anticoagulants, platelet preparation procedures, hemodynamic conditions, surface composition, surface roughness, species differences and methods of evaluation of deposition of platelets, make it difficult to compare the studies on platelet-surface interactions in detail. In general, the response of blood to surfaces varies greatly and has been observed in roughly four categories (see Figure 1.2).

1. Some conditions allow surfaces to continue to accumulate platelets until occlusion of the vessel or repair of the injury site occurs such as in normal hemostasis.
2. Some conditions allow surfaces to accumulate platelets forming thrombi but embolization occurs leading to a cyclic accumulation/ embolization process.
3. Some conditions allow surfaces to accumulate platelets initially but then some platelets are removed or lost leaving a passive surface that may be a platelet monolayer.
4. Finally, some conditions allow surfaces to accumulate platelets approaching a limit where no further accumulation occurs. Such a

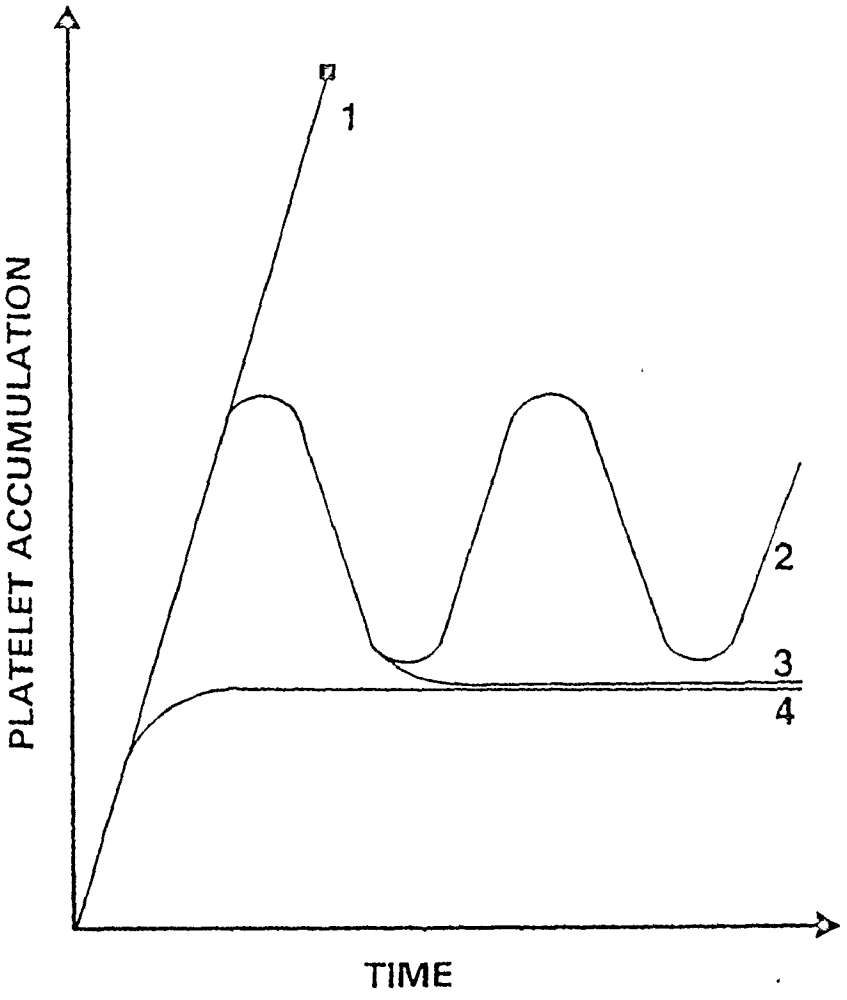


Figure 1.2: Patterns of change in platelet accumulation with time that have been reported 1) occlusion, 2) cyclic thrombus formation and embolization, 3) initial platelet deposition and subsequent embolization , 4) saturation (cf. also 1.3.2 and Chapter 8).

TABLE 1.1

LITERATURE SURVEY OF PREVIOUS WORK DONE BY OTHERS ON PLATELET ACCUMULATION ON SURFACES

FLOW GEOMETRY	PRINCIPAL INVESTIGATOR	REFERENCE NUMBER	SURFACE SHEAR RATE (s^{-1})	EXPOSURE* TIME (MIN.)	SPECIES	ANTICOAGULANT	PRINCIPAL PARAMETERS
"T" chamber	Dutton	(51)	60	0-30	dog	N	Sh, T, dist.
(ex vivo)	Dutton	(52)	60	2, 8	dog	N	Su, dist
stagnation point	Petschek	(53)	0-600	0-24	dog	N	Su, T
(ex vivo)	Madras	(54)	0-500	0-180	dog	HB, N	Sh
	Nyilas	(55)	0-900	0-45	dog	N	Su, Sh
	Morton	(56)	0-30	0-180	dog	N	Sh, T
	Morton	(57)	0-900	0-180	dog	N	review
	Lederman	(58)	0, 150	0-10	dog	?	Su, Sh, Temp
parallel plates	Grabowski	(59)	150-800	0-7	dog	HB	Sh, T
(in vitro)	Grabowski	(60)	394, 989	10,20	dog, human	HB, CB	Sh, T, Sp, M
	Grabowski	(61)	99-936	0-5	dog	CB	T, dist.
	Grabowski	(62)	986	5-180	rabbit, dog, human	HB	T, Su, Sp
	Didisheim	(63)	740	10	baboon, mocaque, pig, calf, sheep	HB, CB, N	Sp, M, Su
	Muggli	(64)	1006	10-40	rabbit	CB	T, dist., D
	Richardson	(65)	?	0-5	human	?	dist.

FLOW GEOMETRY	PRINCIPAL INVESTIGATOR	REFERENCE NUMBER	SURFACE SHEAR RATE (s^{-1})	EXPOSURE* TIME (MIN.)	SPECIES	ANTICOAGULANT	PRINCIPAL PARAMETERS
rotating rod with	Schultz	(66)	150	0-225	dog	N	T, Po
axial flow	Schultz	(67)	150	0-225	dog	N	T, Su, Sh
(ex vivo)	Schultz	(68)	?	10	dog	N	Su
Elliptic cell	Lyman	(69)	5	1	human	HB	Su
(ex vivo)	Lyman	(70)	5	1	human	N	Su, Pc
	Lyman	(71)	5	1	human	N	Su
	Lyman	(72)	5	1,3,4,5	dog	N	Su
	Lyman	(73)	5	1, 5	dog	N	Su
	Day	(74)	5	-	-	-	Flow study
rotating disc	Turitto	(75)	0-10,000	1-4	dog	HB	Sh, T
(in vitro)	Butruille	(76)	0-2,300	4	dog	HB	Sh, Dist
	Kochwa	(77)	0-1,400	1-4	dog	HB	T, Su
	Leonard	(78)	0-1,400	2	dog	HB	dist.
	Butruille	(79)	0-1400	0.5-2	dog	HB	T
U-tube	Friedman	(80)	20-80	2-45	dog	HB	PC, T, Sh, Hct, Su, Po
(ex vivo)	Grabowski	(81)	20-80	2-45	dog	HB	T, Sh,
	Friedman	(82)	10-1000	2-60	dog	HB, N	T, Sh, Su

FLOW GEOMETRY	PRINCIPAL INVESTIGATOR	REFERENCE NUMBER	SURFACE SHEAR RATE (s^{-1})	EXPOSURE* TIME (MIN.)	SPECIES	ANTICOAGULANT	PRINCIPAL PARAMETERS
rotating rod	Feuerstein	(83)	10-30	1-8	pig	S, CB	Sh, Hct, PC, T, Su M
stationary blood	Brash	(84)	19	1-8	pig	S, CB	Hct, Su, M
(in vitro)	Whicher	(85)	19	8	pig	S	M, PC, Hct, Su
	Whicher	(86)	19	8	pig	S	D, Hct
	Cazenave	(87-93)	19	10	pig, rabbit, human	S	Su, D, nc, RBC
	Cazenave	(94)	38	10	rabbit	S	M, Hct, Su, D
	Cazenave	(95)	38	5, 10	human, rabbit	S	D, Sp, Su
	Cazenave	(96)	38	1-60	rabbit	S	T, Su, D
	Dejana	(97)	38	10	rabbit	S	D, Su
	Kinlough-Rathbone	(98)	38	10	rabbit	S	D, Su
	Cazenave	(99)	38	10	rabbit	S	D, Hct
	Cazenave	(100)	38	2,5,10,30	rabbit	S	D, Hct, t
	Cazenave	(101)	38	10	rabbit	S	D, Su
stationary rod	Baumgartner	(102)	50-840	1-40	rabbit	CB	T, Sh, dist.
with axial flow	Turitto	(103)	840	10	rabbit	CB	Temp.
(in vitro)	Turitto	(104)	6-247	10	rabbit	CB	Sh
	Baumgartner	(105)	840	1-40	rabbit	CB	dist.
	Tschopp	(106)	12-840	1-20	rabbit	CB	Sh, Po
	Weiss	(107)	840	10	human	CB	BD.
	Baumgartner	(108)	840	10	rabbit	CB	D, dist.
	Turitto	(109)	12-840	1-20	rabbit	CB	Sh, Po
	Tschopp	(110)	840	10	human	CB	BD
	Turitto	(111)	840	1-100	rabbit	CB	Hct, T
	Weiss	(112)	840	10	human	CB	BD
	Tschopp	(113)	840	10	human, rabbit	CB	D, Su

FLOW GEOMETRY	PRINCIPAL INVESTIGATOR	REFERENCE NUMBER	SURFACE SHEAR RATE (s^{-1})	EXPOSURE* TIME (MIN.)	SPECIES	ANTICOAGULANT	PRINCIPAL PARAMETERS
	Baumgartner	(114)	6-247	10	rabbit, Human	CB	Sh, Het, T, Dist, su, BD
	Baumgartner	(115)	840	1-40	human, rat, rabbit	CB	Su, T, Po, D
	Tschopp	(116)	840	10	rabbit	CB	Pc, D
	Baugmgartner	(117)	840	2-40	human	CB	T
	Baumgartner	(118)	840	10	human	CB	BD, D, Su
	Tschopp	(119)	840	2-15	rat	CB	T, Su
	Turitto	(120)	11-10,000	2-40	rabbit	CB	Sh, T
	Weiss	(121)	840	10	human	CB	BD, M, Su
	Turitto	(122)	840	10	rabbit	CB	Su, T, Dist.
	Turitto	(123)	10-10,000	1-40	rabbit	CB	T, Po, Sn
ex vivo	Weiss	(124)	1300, 3300	2-3	human	CB, N	Sh, BD, M
	Weiss	(125)	840	10	human	CB	BD
	Baumgartner	(126)	840	10	human	CB, N	M
	Turitto	(127)	80-10000	1-40	rabbit	CB	Sh, T
	Weiss	(128)	800, 2600	10, 5	human	CB	D, Sh, Su
	Turitto	(129)	840	1-40	rabbit	CB	Su, T
ex vivo	Baumgartner	(130)	1300	3	rabbit	HB, CB, N	M, D
	Tschopp	(131)	840	10	human	CB	Su
ex vivo	Baumgartner	(132)	650-3300	2-3	human	CB, N	M, Sh
	Turitto	(133)	50-10000	1-40	human	CB	Sh, T, M
	Turitto	(134)	50-10000	5-10	human	CB	Sh, BD
	Baumgartner	(135)	200-5200	2-6	human	CB	Sh, BD
	McGill	(136)	840	10	human	CB	storage
	Sakariassen	(137)	805	5	human	CB - ASA	BD, D
	Jaeger	(138)	840	10	human	CB	D
	Dosne	(139)	840	10	human	CB	PC, M
	Bolhuis	(140)	840	10	human	CB - ASA	BD, D, T, PC

FLOW GEOMETRY	PRINCIPAL INVESTIGATOR	REFERENCE NUMBER	SURFACE SHEAR RATE (s^{-1})	EXPOSURE* TIME (MIN.)	SPECIES	ANTICOAGULANT	PRINCIPAL PARAMETERS
A-v shunt (in vivo)	Rembaum	(141)	?	60	dog	N	
	Harker	(142)	?	chronic	human	?	D
	Harker	(143)	500-2000	chronic	baboon	N	Su, T, D, Ps
	Hanson	(144)	500-2000	chronic	baboon	HB, N	Su, Ps
	Ihlenfeld	(145)	?	1-120	dog	?	Su, T
	Olson	(146)	?	60	dogs	N	histology
	Harker	(147)	?	chronic	human, baboon	N	Ps, D, Sp
	Van Kampen	(148)	?	chronic	baboon	N	Su, T
	Van Kampen	(149)	?	chronic	baboon	N	Su
	Harker	(150)	?	chronic	baboon	N	PC, Area, Ps
Denuded aortae (in vivo)	Baumgartner	(102)	?	1-40	rabbit	N	T, dist.
	Groves	(151)	?	10, chronic		rabbit	N T, dist., Ps
	Baumgartner	(108)	?	10	rabbit	N	D, dist.

TABLE 1.1: FOOTNOTES

Pc	platelet concentration	S	Suspensions
T	time	HB	heparinized blood
Sh	shear rate	CB	citrated blood
Su	surfaces	N	no anticoagulant
Hct	hematocrit	ASA	aspirin-treated platelets
Po	axial position	BD	bleeding disorders
M	medium composition including anticoagulants	D	drugs
Ps	platelet survival	Temp.	temperature
Sp	Species		

* Exposure times beginning at 0 demonstrate systems that continuously monitor platelet accumulation.

surface is said to be saturated.

While all these responses of blood to surfaces have been documented, the reasons for the differences remain obscure. Some insight into the problem of evaluation of platelet-surface interactions can be obtained by reviewing the experimental variables enumerated above.

1.3.2.1 Effects of Medium Composition

Blood must be anticoagulated to be used in experimentation. The nature of the anticoagulant used greatly affects the platelet responses to a surface. Chelating agents impede thrombus formation at low concentrations, prevent thrombus formation at moderate concentrations and decrease platelet adhesion at high concentrations (124,126,131). This effect of chelating agents may be species dependent. No platelet adhesion studies have been done using hirudin as an anticoagulant possibly due to its unavailability and expense, both of which have decreased in recent years and probably is well within the range of systems that use small quantities of blood. Heparin has been used as an anticoagulant in studies, ex vivo, and does not appear to alter platelet accumulation as compared with unanticoagulated blood(54,63,82,129,143). However studies, in vitro, using heparin are difficult as spontaneous platelet clumping occurs when blood is anitcoagulated with heparin. As more purified subfractions become available, a heparin should be available that anticoagulates but does not induce platelet clumping. Thus, it should be possible to find an anticoagulant that does not

activate platelets directly in the near future.

A second approach to conduct platelet-surface studies, in vitro, has been to remove the plasma. Washing procedures have been employed to remove plasma and resuspension in artificial medium(83-101). When done carefully platelets appear to be intact with normal, life spans and hemostatic function, in vivo, as well as normal, platelet functions, in vitro(26). The procedure has the advantage of manipulation of medium composition focusing on one component or combinations of components. The removal of the plasma removes not only the coagulation components but complement, fibrinolytic and kinin generation components as well; all of which could be important in thrombogenesis(49,50). Both approaches to obtaining platelets for studies in vitro, anticoagulation and washing, have their advantages and disadvantages which must be born in mind when interpreting the literature.

1.3.2.2 Hemodynamic Considerations

It was recognized that fluid mechanical conditions greatly affect platelet deposition on all surfaces. The result was the development of different perfusion apparatus each with its own operating characteristics (51-151) (Table 1.1). The studies conducted using these apparatus will be discussed in detail in the following sections, 1.3.2.3, 1.3.2.4 and 1.3.2.5.

Fortunately, the rate of platelet accumulation correlates well with the surface shear rate in studies using blood at normal hematocrits and for surfaces that readily bind platelets. Increasing platelet

deposition rate with increasing shear rate suggests that the rate controlling stage is the delivery of platelets to the surface. This has led researchers to model platelet adhesion as a diffusion limited process with analysis using classical mass transport theory(61,67,80,81, 83,84,109,132). Empirically determined diffusion coefficients are two to three orders of magnitude above those expected based on Stokes-Einstein diffusion theory. Microrheological investigations have shown that augmentation of platelet diffusion occurs due to red blood cell motions during shear flow(152). Reducing the concentration of red blood cells greatly reduces the deposition of platelets further supporting this concept(80,85,86,94,99,100,111,114). High hematocrits and high shear rates lead to increased hemolysis and enhancement of platelet mural thrombogenesis(133). Further, drugs thought to reduce hemolysis by stabilization of red cell membranes increase bleeding times, in vitro(153). Whether the effects of red blood cells on platelets is purely physical or both physical and hormonal is still controversial(84,133,152,153). More direct methods are needed to dissociate the physical and hormonal roles of red blood cells in platelet-surface interactions.

In the next three sections, a review of the major finding of each group with respect to platelet-surface interaction will be presented.

1.3.2.3 Continuous Monitoring of Platelet Deposition

Almost all experiments in platelet-surface phemonena have been conducted in a manner where the surface is exposed to a platelet

containing fluid for a defined period of time and then rinsed and platelet accumulation measured. Such experiments have intrinsic limits in that only the net accumulation is measured. Any dynamic events such as non-adhesive encounters, translocation of platelets, thromboembolism, platelet removal by rinsing procedures or detailed mechanisms of aggregate growth are undetectable. A few systems have been developed to watch platelet thrombus growth, white cell adhesion and embolization of thrombi.

Indirect methods to monitor platelet accumulation on surfaces have employed densitometry and radiolabels. Grabowski(59,61) used video densitometry to observe the growth of platelet aggregates on a semi-permeable membrane through which ADP was diffusing into the blood. Mural aggregates only formed provided a layer of platelets was present. He was able to calculate the concentration of ADP near the surface required to induce thrombogenesis to be 1.5 μM. The ADP released from the adherent platelets was not considered in Grabowski's calculations. Shultz et. al.(66-68), using radiolabeled platelets and monitoring platelet accumulations with a gamma counter documented embolization of thrombi from a steel rod rotating in an annular perfusion chamber. These experiments are interesting as the rates of accumulation of platelets in such a system are an order of magnitude higher than that in other systems at similar shear rate(110). A large surface area was monitored and deposition of platelets had to be extensive to overcome background radioactivity. Ihlenfeld et. al.(144), using radiolabeled platelets and fibrinogen have monitored platelet and fibrin deposition

in arterial-venous shunts. Background radioactivity was avoided by rinsing the test segment prior to radioactive measurements at each time point and then re-establishing flow. Inlenfeld et al. found a transient accumulation of platelets and fibrinogen on a number of different shunt surfaces.

Direct observation has also been employed to monitor platelet deposition on surfaces. Application of exogenous ADP or laser injury to small blood vessels results in cyclic thrombus formation, embolization and thrombus regrowth. These events have been monitored using microscopic techniques(154,155). Such studies can be criticized as the nature of the thrombotic stimulus (the concentration of ADP or the laser damage) was not quantified and was unphysiological. Richardson et. al.(65), have documented embolization of single cells from a glass surface using microscopic techniques but it is unclear if red blood cells were present in the platelet suspensions. The stagnation flow apparatus has been used(53-58) to study the accumulation of blood components on synthetic materials using dog blood, ex vivo. Leukocyte adhesion was inhibited at surface shear rates above 5 per second. Many surfaces saturated with platelets in this device. Thrombi formed in characteristic wedge shapes initiating at sites of local imperfections in the surface and propagating downstream.

All of the above experimental systems have tried to monitor platelet deposition continually but have only had the resolution to observe thrombi either directly or indirectly. No system has the resolving power to watch individual platelets adhering from blood with a

hematocrit of 40%. To be able to discern between different hypothesized mechanisms of platelet deposition, mural aggregate growth and thromboembolism more precise techniques to measure the growth and development of these structures are required.

1.3.2.4 In vitro and ex vivo Studies

Drs. Baumgartner, Turitto, Weiss, Tschopp and Muggli and many others(102-139) have used an annular perfusion chamber and segments of subendothelium or the fibillar collagen matrix of the subendothelium to examine platelet accumulation on these surfaces (Table 1.1). A morphometric evaluation system permitted platelets in contact with the surface, spread on the surface, or large platelet aggregates (thrombi) to be distinguished. Platelet adhesion was defined as the total of contact and spread platelets and was found to increase with time until 100% coverage of the subendothelium existed. Numbers of platelet thrombi similarly increased with time, but after 10-20 minutes the thrombi dislodged leaving only adherent platelets. Platelet adhesion increased with increasing shear rate, increasing citrate concentration, aspirin (ASA), or sulfinpyrazone; but was unchanged with CP/CPK, or in thrombasthenia or hemophilia; and decreased with PGI_2, dipyridamole, EDTA, low temperatures, high shear rates, or in von Willebrand's, Bernard-Soulier, or storage pool diseases. Platelet thrombus formation increased with increasing shear rate; but was unaffected in von Willebrand's disease and Bernard-Soulier disease at low shear rates, < 1300 s^{-1}; and decreased with EDTA, CP/CPK, ASA, PGI_2, PGE_1,

dipyridamole, sulfinpyrazone, increasing citrate concentrations, low temperatures, or in storage pool disease, thrombasthenia or von Willebrand's and Bernard-Soulier diseases, at high shear rates, > 2000 s^{-1}.

Recent reports from Baumgartner et. al.(124,126,129,131) have studied native blood, ex vivo. Platelet adhesion was similar to the citrated blood studies summarized above. Thrombus formation was more pronounced with larger aggregates that were less easily dislodged with time and higher shear rates. Also, ASA, sulfinpyrazone, and dipyridamole no longer inhibited thrombus growth nor increased platelet adhesion in the non-citrated blood. The latter results highlight the difficulties in studying thrombogenesis where conditions can work in synchrony to change results.

Drs. Feuerstein, Brash, Cazenave et. al.(83-101), have employed a couette flow system that operates at low shear rates but platelet deposition is not diffusion-limited. Whicher and Brash(85) have studied the effects of medium composition on porcine platelet accumulation and release of serotonin from adherent platelets on a number of surfaces using radioisotopic techniques. Surfaces were found to fall into three groups having high adhesion and release, moderate adhesion and release and low adhesion and release. Changes in platelet concentration, hematocrit, fibrinogen and albumin all had effects on platelet-surface interactions that were surface specific(85). No steps were taken to prevent reuptake of serotonin by adherent platelets. However, subsequent study showed only a slight increase in measured release from

adherent cells by preventing reuptake of serotonin by adherent platelets(86). Cazenave et al.(94-101) have used this device to study the effects of drugs on rabbit-platelet adhesion to collagen and subendothelium. Platelet adhesion only and release of serotonin from adherent platelets were measured in this system. Platelet adhesion was decreased by PGI_2, PGE_1, indomethacin, EDTA, citrate, EGTA, sulfinpyrazone, dipyridamole methylprednisolone, penicillin G, cephalothin, albumin and pre-exposure of platelets to thrombin, plasmin, chymotrypsin, periodate or ADP. Platelet adhesion was not affected by aspirin or prior exposure to neuraminidase. Release of serotonin from adherent rabbit platelets was inhibited by EDTA, EGTA, PGE_1, PGI_2 and combinations of ASA + CP/CPK or indomethacin + CP/CPK. Release of serotonin from adherent human platelets was not inhibited by any of the materials shown to inhibit release from rabbit platelets. Release was increased slightly by ASA, indomethacin and sulfinpyrazone but was unaffected by CP/CPK.

Didisheim and Grabowski(60,62,63) have studied species differences in platelet accumulation on different surfaces and found great differences between species and surfaces. In a new two stage procedure where the surface is first exposed to the plasma of one species and then the blood of a different species they were able to show that the plasma component mediating adhesion in one species does not support the accumulation of platelets from another species(63). These authors have also shown that aspirin treatment does not inhibit human platelet adhesion or release of granules on a collagen surface(156).

1.3.2.5 In vivo Studies

In vivo studies using rabbits which have had the endothelium removed from their aorta showed an initial rapid accumulation platelets with formation of a saturated surface consisting of a continuous monolayer of spread platelets(102,108,151). This surface was in dynamic equilibrium with continual turnover of platelets on the surface(151). After a few days a neointima was formed. Reinjury of the aorta brought a new sequence of similar events(151). Despite the relatively large surface area of damage (the entire aorta) no reduction in platelet survival was detected(151). An arterial-venous shunt placed in baboons has been demonstrated to reduce platelet survival despite the relatively unthrombosed surface of the shunt suggesting that continual thrombembolism may be occurring on the surface(142,143,146). Alternately, since proteins such as Hageman factor are activated by surfaces, it may be the activation of plasma protein cascades with formation of enzymes such as plasmin that led to the reduced platelet life span. A test system for screening biomaterials is needed and some estimate of dynamic events albeit indirect is possible through platelet survival and protein turnover measurements.

1.3.2.6 Effects of Mepacrine on Human Platelets

Mepacrine (quinacrine dihydrochloride) (Figure 1.3) is rapidly taken up by platelets and stored in the dense granules (157). This uptake was not inhibited by serotonin or metabolic inhibitors and resulted in the displacement of a portion of the serotonin stored in the

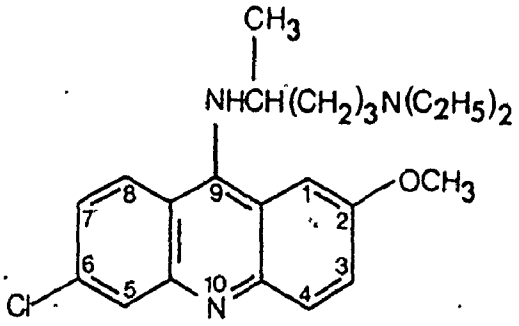


Figure 1.3: Chemical structure of mepacrine (quinacrine dihydrochloride).

dense granules(1). Mepacrine has an excitation peak at 440 nm and an emission peak at 505 nm(158). This fluorescent behaviour has been used to specifically count dense granule in normal(5,159-160) and abnormal platelets of various species(6). Platelet activation by thrombin resulted in 50% release of dense granule bound mepacrine(159) with the remaining mepacrine associated with the membranes of the granules(161). Mepacrine inhibited platelet aggregation induced by ADP and collagen and partially inhibited platelet aggregation induced by low concentrations of thrombin(162,163). The mechanism of action of mepacrine are at least three fold. The first pathway is through the inhibition of phospholipase A_2 at a mepacrine concentration of 50 µM(162,163). The second pathway is through the inhibition of cyclo-oxygenose at a mepacrine concentration of 500 µM(162,163). The third pathway is through the inhibition of fibrinogen binding to platelets during platelet aggregation at a mepacrine concentration of 50 µm(164). Mepacrine is also a chelating agent and it may be through the combined effects of the concentration of the mepacrine in membranes and this chelating activity that enables it to be such an effective inhibitor of platelet aggregation.

1.4 Discussion and Rationale for Present Work

The foregoing review of platelet functions and platelet-surface interactions is meant to identify the previous work and to point out their limits. The exposure of blood that contains different anticoagulents to surfaces under different dynamic conditions seems to have

gained us some feel for the depth of the problem of platelet-surface interactions. Both the physical parameters and the biological parameters influence platelet-surface interactions and the consideration of both is mandatory. A more detailed understanding of platelet-surface interactions is required to enable a logical approach to the biomaterials synthesis and testing, as well as the comprehension of vascular diseases and their complications.

Those studies that have attempted to look at dynamic events have been restricted to low resolution and/or indirect methods of observation. New procedures need to be employed to allow direct observation of events at surfaces during platelet accumulation. The epi-fluorescent video microscopic technique employed in this work fulfills these criterion completely.

From section 1.2 on platelet physiology it is obvious that platelet functions are well enough defined to apply this knowledge to platelet-surface interactions permitting detailed correlations of platelet phenomena and functions. To date the study of platelets and artificial surfaces has been restricted to the testing of new biomaterials with little research on the mechanism of platelet accumulation on foreign surfaces. The two major research groups(83-101,101-139) have begun to apply the knowledge of platelet physiology to platelet accumulation on subendothelium. However, the use of the annular perfusion chamber which gives detailed morphometric information on platelet accumulation is confounded by the use of citrated blood and limited by the lack of dynamic information(102-138).

The use of the cuvette flow device which gives platelet adhesion and release of granules from adherent platelets is confounded by the species differences as most of this work was done using rabbit platelets and limited by the lack of dynamic information, the low shear rates and the measurement of adhesion only(83-101).

The work presented in this thesis has been done in washed human platelet suspensions that contain physiological levels of calcuim, magnesium and red blood cells and no anticoagulants. Microscopic observations of individual platelets during the processes of platelet accumulation on surfaces yielded detailed dynamic information. Simultaneous measurement of the release of materials from platelets and the accumulation of platelets over a large surface area augmented the microscopic data. Analysis of the biological data using well defined engineering principles enabled estimates of the concentrations near a surface of materials released from platelets to be made for the first time. A greater understanding of mural thrombogenesis on a mechanistic level ensued from the engineering analysis and experimental procedures.

CHAPTER 2

METHODS AND MATERIALS

2.1 Preparation of Platelet Suspensions

2.1.1 Human Platelet Suspensions with Apyrase

Washed human platelets were prepared (Figure 2.1) according to the technique of Mustard et al.(1). Platelets were labeled with $Na_2{}^{51}Cr\ O_4$ (New England Nuclear, Boston, specific activity = 100 mCi/Mg Cr, $2\mu Ci/10^{12}$ platelets) and ^{3}H-serotonin (New England Nuclear, Boston, specific activity = 25.7 Ci/mmole, $4\mu Ci/10^{12}$ platelets). The final suspension contained 3.5 gm/l of bovine albumin (ICN, Canada Ltd., Montreal, fraction V), apyrase, (cf 2.1.4), 1.0 gm/l of dextrose(BDH Chemicals, Toronto), red blood cells to produce a hematocrit of 40% (cf 2.2.1) and a platelet concentration of $3x10^8$ cells/ml in a magnesium and calcium containing Tyrode's solution. The properties of platelets prepared in this manner have been extensively studied(2).

2.1.2 Human Platelet Suspensions with CP/CPK

Human platelet suspensions were prepared with the apyrase replaced by creatine phosphate/creatine phosphokinase (CP/CPK) at a concentration sufficient to prevent spontaneous aggregation but maintain responses to ADP. The platelets were labeled in the first wash with disodium chromate and ^{3}H-seotonin (cf 2.1.1). The final suspensions contained 3×10^8 platelet/ml, red blood cells at a hematocrit of 40%,

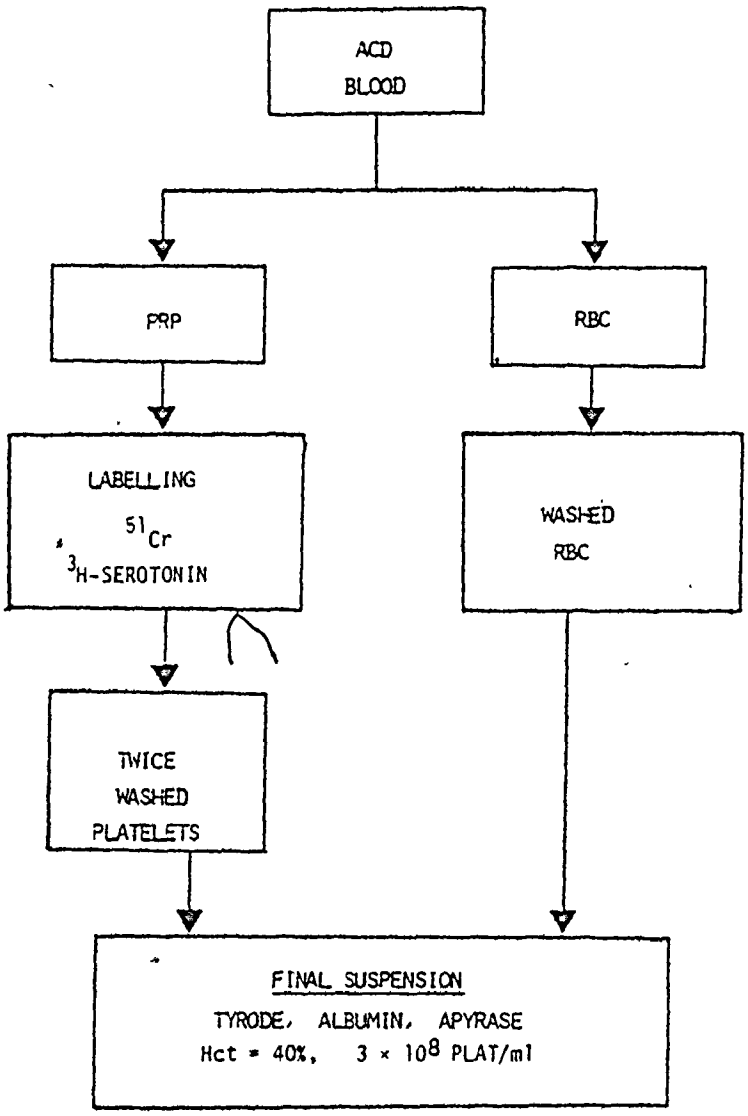


Figure 2.1: Washed human platelets were prepared according to the technique of Mustard et al. . Platelets were labelled with ^{51}Cr (New England Nuclear, $2\mu Ci/10^{12}$ platelets) and ^{3}H serotonin (New England Nuclear, $4\mu Ci/10^{12}$ platelets) in the first wash. The red cells were washed twice in a Sorensen's phosphate buffered saline and twice in platelet-suspending solution. They were further incubated with apyrase, 1 µl/ml, for 15 minutes in the final wash to remove any residual ADP. The final suspension had a hematocrit of 40%, 3×10^8 platelets/ml, 1 µl/ml apyrase, 3.5 gm/l albumin, 1 gm/l dextrose in Tyrode's solution (which contains 2mM $CaCl_2$ and 1 mM $MgCl_2$). No anticoagulant was present.

3.5 gm/l albumin (ICN), 1.0 gm/ml dextrose (BDH), 5 mM creatine phsophate (Sigma) and 1 unit/ml creatine phosphokinase (Sigma) in a Tyrode's buffer, pH 7.35. These suspensions were used in Chapter 5 only, in conjunction with ATP and CP/CPK experiments.

2.1.3 Apyrase Characteristics

The apyrase used in these experiments was isolated from potatoes using the method of Molnar and Lorand(3). Apyrase enzymatically degrades adenosine triphosphate (ATP) and ADP into adenosine monophosphate(AMP)(3). The activity of the preparation was determined using the firefly assay for ATP (cf 2.6.2). At the enzyme concentration used for the platelet suspensions (1 µl/ml) and over the range of substrate concentrations, 10^{-6} to 10^{-9} molar ADP (Sigma Chemical Co., St. Louis, Missouri), the apyrase exhibited first order kinetics with a rate constant of 1.8×10^{-3} s^{-1} at 37°C (cf 2.6.2). This rate of ADP degradation is sufficient to prevent the platelet suspensions from spontaneously aggregating or becoming refractory to aggregation due to leakage of adenine nucleotides but does not affect ADP-induced aggregation as measured in a turbidimetric device(2).

2.2 Preparation of Red Blood Cells

2.2.1 Red Blood Cells Used With Platelet Suspensions

The red cells were washed twice in a Sorenson's phosphate buffered saline, pH 8.0 and twice in platelet-suspending solution, pH 7.35. They were further incubated with apyrase, (cf 2.1.3) 1 µl/ml, for

15 minutes in the final wash to remove any residual ADP.

2.2.2 Determination of ADP Derived From Red Blood Cells

Red blood cells prepared from 3.8% trisodium citrate or acid-citrate-dextrose anticoagulated blood(4) were washed as outlined in preparation of suspensions. The final platelet-free, red-cell suspension, 40% hematocrit, 0.2 µl/ml apyrase, (cf 2.1.3) was allowed to incubate for 45 minutes prior to being passed through a 120 cm long, 0.1 cm diameter collagen coated tube at 2 ml/min (320 s^{-1}). Since red cell damage is known to increase with flow rate (5,6), the highest flow rate examined in the platelet flow studies was used. The combined amount of free ATP and ADP in the supernatant of the red blood cell suspension prior to exposure of the suspension to the tube and after exposure to the tube was determined using the method described (cf 2.6.1). The difference in nucleotide concentration between the inlet and outlet was statistically evaluated using a paired-t test.

2.3 Preparation of Protein-Coated Glass Tubes

Glass tubes (1.3 mm internal diameter and 10 cm long) were cleaned in Chromerge (Manostat, New York, NY) overnight, rinsed with distilled water and dried in an oven. Plasma proteins were adsorbed to the internal surface by filling a tube that was prewetted using Tyrode's solution, with protein solution. After one hour the protein solution was flushed out with Tyrode's buffer and the tube exposed to cell suspension. A collagen coating was applied by filling a dry tube with

solution of collagen (Sigma, Type I, 2.0 mg/ml) soluble in acid and then rinsing with Tyrode's buffer after two minutes. The tube was kept in a moist atmosphere for 15 minutes prior to exposure to cell suspension. The proteins used were human fibrinogen (A.B. Kabi, Stockholm, Sweden, Grade L, 1 mg/ml), human fibronectin (Colloborative Research, Waltham, Mass., 200 µg/ml), human albumin (A.G. Behringwerke, Marburg-Lahn, Germany, electrophoretically pure, 3.5 mg/ml) and bovine albumin (ICN, fraction V, 3.5 mg/ml). Fibrinogen was first treated with diisopropylfluorophosphate, DFP, as described by Mustard et al(2). Fibronectin - depleted fibrinogen was prepared according to Lawrie et al.(7) by DEAE chromatography of the DFP-treated material. Bovine tendon collagen was prepared according to Cazenave et al.(8). All other proteins were dissolved in calcium and magnesium-free Tyrode's buffer.

2.4 Perfusion Apparatus

A flow device was constructed consisting of a glass tube positioned vertically with one end placed in a beaker of suspension (cf 2.1) which was maintained at 37°C, and the other end connected to a syringe pump (Harvard Apparatus, Millis, Mass.) (Figure 2.2). The advantage of this apparatus is its simplicity, making it inexpensive to construct. The conditions of drawing a fluid from a stagnant pool into a tube has been analysed in detail and represents a well-defined laminar flow. Added advantages for biological experiment are the absence of feeder tubing that may activate platelets prior to exposure to the test section and the maintenance of the blood at 37°C by suspending the

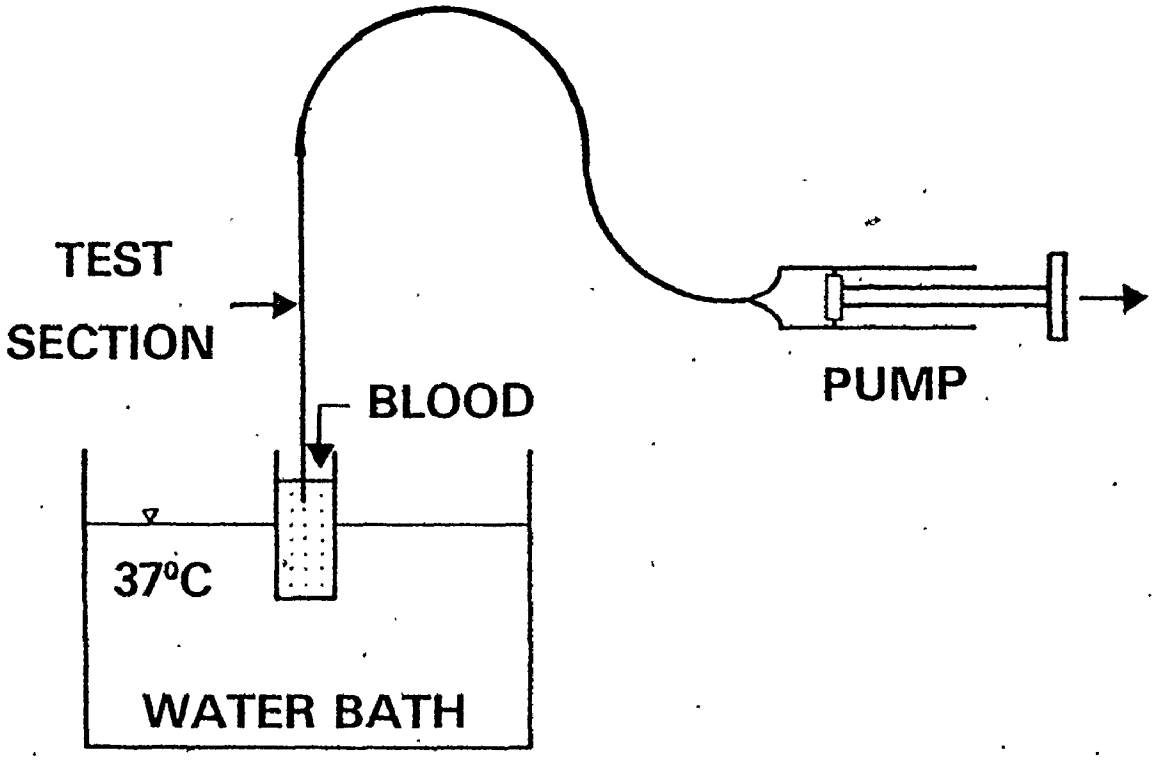


Figure 2.2: Platelet/red blood cell suspension is drawn from the beaker which is sitting in a water bath which is maintained at 37°C. The glass tube is placed with one end in the beaker and the other end attached to the syringe pump. The rate of withdrawal of blood through the tube is controlled by a syringe pump and was steady at all times.

beaker in a water bath.

2.5 Procedure For Perfusion Experiments

The tubes were primed with calcium and magnesium-free Tyrode solution before the platelet suspension (cf 2.1) was allowed to flow, to prevent the passage of a blood-air interface which could influence adhesion. The suspension was drawn through the protein-coated tube (cf 2.3) by a syringe pump for various lengths of time and shear rates and discarded. After exposure to the cell suspension, the tubes were rinsed with Tyrode solution for 5 minutes at a flow rate of 1 ml/min to remove loosely adherent cells.

The tube was cut into segments (Figure 2.3). For the tubes with 1.3 mm internal diameter and 10 cm long the first four segments were 1 cm long and the last 3 were 2 cm long. Only one segment was required per counting vial to maintain radioactive counts. Each segment was counted in a gamma counter to determine the ^{51}Cr associated with the tube's surface; the platelet surface concentration (platelets/1000 μm^2) was then computed(9). The adherent platelets were then removed from the tube's surface using a tissue solubilizer (NCS, Amersham, Oakville) and counted in a liquid scintillation counter. The scintillation fluid contained 5 gm of 2, 5-diphenyloxazole (BDH) and 0.3 gm of 1, 4-Di[2-(5-phenyloxazolyl)]-benzene puriss. (Canadian Scientific Products Ltd., London) in one litre of toluene (Fisher Scientific Ltd., FairLawn, N.J.). 0.4 ml of NCS, 3.5 ml scintillation fluid were combined with the segment of the glass tube in a mini-vial (Wheaton Scientific,

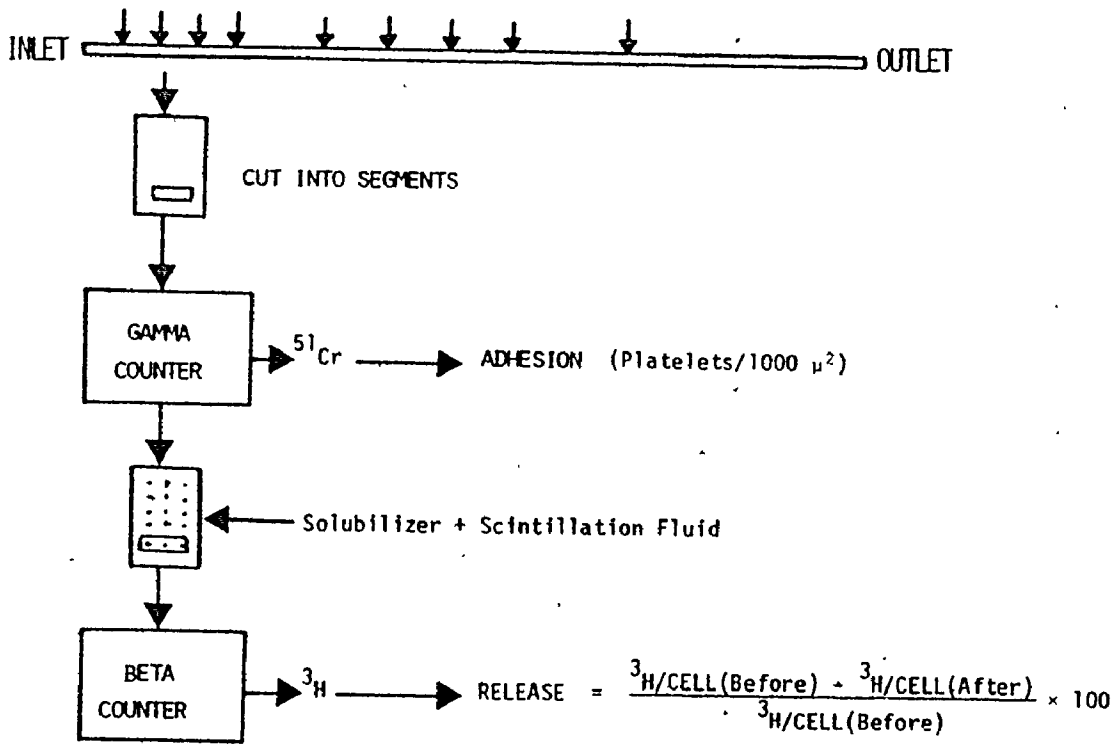


Figure 2.3: Calculation of platelet accumulation and release from adherent platelets. The tube was cut into segments and each segment counted in a gamma counter to determine the ^{51}Cr activity associated with the tube's surface; the platelet surface concentration (platelets/1000 μm^2) was then computed. The adherent platelets were then removed from the tube's surface using a tissue solubilizer (NCS, Amersham) and counted in a liquid scintillation counter. A standard ^{51}Cr sample was also evaluated in the liquid scintillation counter. The radioactivity counts from the scintillation counter for the solubilized platelets were adjusted by subtraction of the ^{51}Cr activity as determined in the gamma counter where ^{3}H-serotonin is not detectable. The net ^{3}H-serotonin counts were then used to calculate the percent release of serotonin from adherent cells.

Milleville, NJ). A standard ^{51}Cr sample was also evaluated in the liquid scintillation counter. The radioactivity counts from the scintillation counter for the solubilized platelets were adjusted by subtraction of the ^{51}Cr activity as determined in the gamma counter where ^{3}H-serotonin is not detectable. The net ^{3}H-serotonin counts were then used to calculate the percent release of serotonin from adherent cells using Equation 1.1.

$$\text{Percent Release} = \frac{\left(\begin{array}{c}{}^{3}\text{H-serotonin}\\ \text{per cell}\\ \text{before adhesion}\end{array}\right) - \left(\begin{array}{c}{}^{3}\text{H-serotonin}\\ \text{per cell}\\ \text{after adhesion}\end{array}\right)}{{}^{3}\text{H-serotonin per cell before adhesion}} \times 100 \qquad \ldots\ldots\ldots 1.1$$

2.6 ATP and ADP Assay

2.6.1 Red Blood Cell Studies

Supernatants of the platelet-free red blood cell suspensions (cf 2.2)were obtained by centrifugation at 9000 g for 2 minutes. The ADP that had leaked from the red blood cells was converted to adenosine triphosphate (ATP) using the PEP/PK (Phosphoenolpyruvate/Pyruvate Kinase, Boehringer-Manheim GmbH, Germany) enzymatic system described by Holmsen(10). The combined concentration of ADP and ATP was determined within 15 minutes of the end of the flow experiment using the luciferin-luciferase (firefly) assay(10, Sigma). Although there was apyrase (cf 2.1.4) (0.2 µl/ml) in the red cell suspension it did not significantly degrade liberated ADP or ATP in the 15 minutes between the flow experiment and the assay. This was checked by doing two standard

curves, one with apyrase (0.2 µl/ml) and one without. No difference was noted after the 15 minute incubation time between the two standard curves. Longer incubation times showed apyrase was degrading ADP.

2.6.2 Apyrase Kinetics

The rate of degradation of ADP by apyrase was determined by adding 1 µl/ml (the concentration used in the platelet suspending solution) of apyrase to a solution containing 10^{-6} M ADP. The concentration of ADP remaining after 5, 10, 20, 40 and 80 minutes was determined by enzymatic conversion (PEP/PK) of ADP to ATP and measurement of ATP concentration using the firefly assay. The rate constant (1.8×10^{-3} s^{-1}) was determined as the slope of the straight line obtained by linear regression between the natural logarithm of the residual concentration of ADP and the time of incubation. All work was performed in the platelet suspending solution at 37°C.

2.7 Drugs

Mepacrine (BDH), imipramine (Sigma) hirudin (Sigma), heparin (Sigma) were dissolved in Tyrode's buffer, frozen and thawed prior to use. Dipyridamole (Persantine, Boehinger), dipyridamole placebo (Boehinger), sulfinpyrazone (Anturan, Ciba-Geigy, Dorval, PQ), sulfinpyrazone placebo (Ciba-Geigy) were used as supplied. Ac etylsalicyclic acid (Sigma) was prepared fresh daily by dissolving equimolar $NaHCO_3$ and ASA in water and adjusting the pH to 7.35 and the osmolarity to 300 mosm using NaOH and 300 gm/l NaCl, respectively.

Prostaglandin E_1 (Upjohn Co., Kalamazoo, MI) was prepared by dissolving 1 mg in 0.1 ml of 95% ethanol and adding 0.9 ml of 0.02% Na_2CO_3 and frozen. Creatine phosphokinase (Sigma) and creatine phosphate (Sigma) were prepared in Tyrode's buffer fresh daily. Indomethacin (Merck, Sharp and Dohme, Penn.) was prepared in ethanol at a concentration of 10 mM and diluted in Tyrode's. Adenosine diphosphate (ADP, Sigma) and CP/CPK were dissolved in cold Tyrode's buffer. Adenosine triphosphate (Sigma) was dissolved in CP/CPK-Tyrode's at 1 unit/ml CPK and 5 mM CP. All treatments were administered to platelet suspensions 15 minutes prior to mixing with red blood cells and perfusion.

2.8 Epi-fluorescent Video Microscopy

A Nikon microscope with blue excitation epi-fluorescent attachment and UV-F100 glycerol immersion objective was mounted horizontally (Figure 2.4). The protein-coated tube to be examined was mounted on the now vertical stage. A television camera (Sony, Japan, 3210) coupled to an image intensifier (RCA, USA, 4550 P20) was used to monitor fluorescently-labeled platelets interacting with the internal surface of the tube. A permanent record was obtained on 1/2" video tape. The rate of platelet accumulation was determined by recording the time of arrival of every platelet in 2500 μm^2 of surface area. To determine the residence times of individual cells upon surfaces, the video tape was advanced one frame at a time and records were made of the presence or absence of cells on the 2500 μm^2 viewing area. This process permitted residence times greater than 33 ms (time for a single T.V.

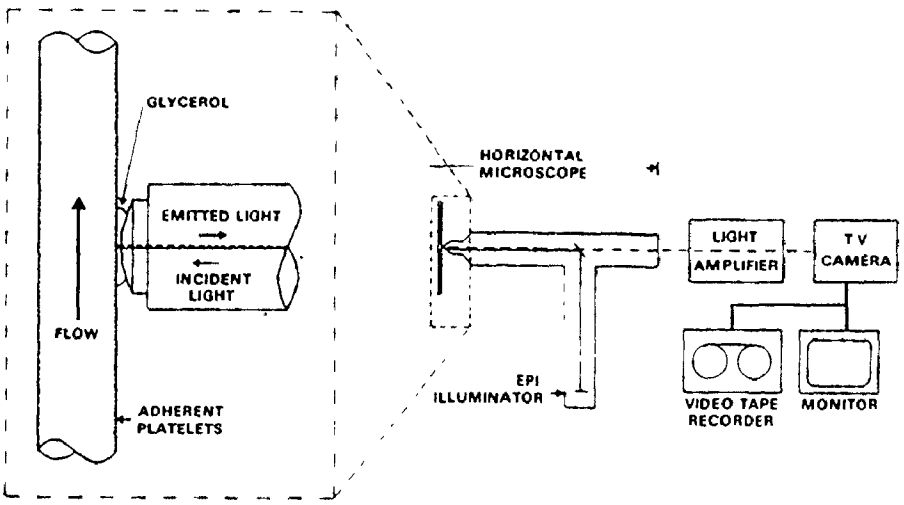


Figure 2.4: Epi-fluorescent video microscopy. A Nikon microscopy with blue excitation epi-fluorescent attachment and UV-F100 glycerol immersion objective was mounted horizontally. The collagen-coated tube was mounted on the now vertical stage. A television camera (Sony 3210) coupled to an image intensifier (RCA 4550 P20) was used to monitor fluorescently-labeled platelets interacting with the internal surface of the tube. A permanent record as obtained on 1/2" video tape. The rate of platelet accumulation was determined by recording the time of arrival of every platelet in 2500 μm^2 of surface area. In order to determine the residence times of individual cells upon surface, the video tape was advanced one frame at a time and records were made of the presence or absence of cells on the 2500 μm^2 viewing area. This process permitted residence times greater than 33 ms (time for a single T.V. frame) to be recorded.

frame) to be recorded.

When epi-fluorescent microscopy was performed the platelets were incubated in 50 µM mepacrine (BDH) (cf 1.3.2.6) for 20 minutes rendering the dense granules fluorescent. No further washing was necessary as the mepacrine was taken up by the platelets and background fluorescence was not a problem. Photomicrographs were taken off the screen of an image intensifier (RCA 4450) coupled to a Nikon (Mississaga) microscope with a blue excitation epi-fluorescent attachment. The platelets were made fluorescent by adding mepacrine (50 µM) to the rinsing solution rendering not only the dense granules but also the cytoplasm of the platelet fluorescent (cf 1.3.2.6).

2.9 Aggregation Studies

Platelet suspensions (red cells absent) at a concentration of 5 x 10^8 platelets/ml were stimulated by ADP (Sigma), acid-soluble collagen (type I, Sigma), or ADP plus collagen, in an aggregometer (Payton Instruments). One hundred percent aggregation was set at the level of transmitted light present with only the suspending medium in the light path. Decreasing concentrations of stimulus were used until no response was defined as that concentration of stimulus giving 10% increase in light transmittance (aggregation) or 10% release or last detectable shape change (increased light absorbance). DFP-fibrinogen (cf 2.3) was added to the suspension at a final concentration of 0.5 mg/ml as washed human platelets will not aggregate to ADP or collagen without it.

CHAPTER 3

PLATELET ADHESION AND RELEASE: MAXIMUM INTERFACIAL FLUID CONCENTRATIONS OF MATERIALS LIBERATED FROM PLATELETS

3.1 Introduction

Mural thrombosis can be divided into three stages: thrombogenesis, stabilization of the thrombus and dissolution of the thrombus. The first stage of thrombogenesis entails the accumulation of blood platelets on a surface initially through platelet-surface adhesion and later through platelet-platelet cohesion. The process of platelet accumulation on a surface is similar for intravascular mural thrombogenesis and the early stages of hemostasis and thrombogenesis on the biomaterials used in artifical organs or implanted prostheses. The extent and rate of thrombogenesis is governed by: (a) the surface, (b) the local hemodynamic condition, (c) plasma components, and (d) the functional state of the platelets(1,2). While it is difficult to know how all four of these parameters interact to produce a thrombus, a knowledge of the concentration of those materials which are liberated from platelets during platelet accumulation on a surface would enhance an understanding of the mechanisms for thrombogenesis.

The materials which are liberated by platelets and have pro-thrombogenic properties are adenosine diphosphate (ADP) (1,2), serotonin (1,2), prostglandins G_2, and H_2, thromboxane A_2 (3), von Willebrand factor (4) and fibrinogen (1,2). The materials which are

liberated by platelets and have anti-thrombogenic properties are adenosine triphosphate (ATP) (5), prostaglandin D_2 (3) and heparin neutralizing materials (6). Although presence of all these materials within platelets and their liberation has been documented no quantitative information on the concentration of these materials in the medium adjacent to the surface during the process of mural thrombus formation has been presented. Holmsen and Weiss (6) have reviewed the empirically obtained biological information available to discern which of the materials released from platelets are most likely to be affecting mural thrombogenesis. A list of the amounts of secretable materials stored in the dense granules of platelets was prepared by Holmsen and Weiss (6) and used to calculate for each substance the average fluid concentration that would develop in an aggregation cuvette due to maximal release by platelets. The relationship between these average concentrations of releasable materials as determined in a well-mixed cuvette and the concentrations at a site of thrombogenesis remains obscure.

Another approach is possible. The action of platelet accumulation and liberation generates a region of high concentration of liberated materials at a site of accumulation. The rate of liberation and the rate of transport from the surface determine the magnitude of the local concentration. For each substance, the maximum concentration locally produced may then be estimated and compared with the range of concentration which is known to affect platelets and information upon the probable effect that substance can have on thrombosis then becomes

available. Using this method one may have most confidence in saying that a substance will or will not affect platelet aggregation when the calculated value is well above or below those known to stimulate platelets. Two pieces of information are necessary to calculate the maximum possible concentration of liberated materials near an injury site in the vasculature or on a blood-contacting biomaterial, these are the rate of platelet accumulation and the amount of the material in the platelet. For this study, the latter quantitites were gathered from the literature and the former information was obtained from experiments that measured the rate of platelet accumulation on a collagen-coated glass tube. The effect of flow rate or more precisely shear rate at the surface, upon the local concentration of liberated substances was investigated.

3.2 Theory

Materials liberated at the interface between a moving fluid and an impermeable surface undergo diffusion away from the site of liberation and simultaneous transport downstream by the fluid. In a circular tube, the fluid velocity varies in the radial direction and is maximal along the centerline and zero at the tube's surface. The resultant concentration of liberated material then depends on the rate of the liberation and the interconnected dilution processes of diffusion and convection. For a flow in a tube, the mathematical solution for the fluid concentration of any material liberated at a constant rate along the tube at the fluid-solid interface is available in the literature

(8). For the case of liberation over short lengths of the tube, the parabolic velocity profile can be replaced with a linear relationship where the velocity at any radial position, y - measured from the surface, is equal to Gy where G is shear rate at the surface evaluated from a parabolic velocity profile (9). This linearization of the velocity profile allows an analytical solution to be obtained which is reproduced here from reference (9). The shear rate at the surface, G, will be referred to as shear rate for the remainder of this presentation.

$$IFC = \frac{NR}{Df} \frac{\sqrt[3]{9\lambda}}{\Gamma(2/3)} \tag{3.1}$$

$$\lambda = \frac{z\,D}{GR^3} \tag{3.2}$$

$$N = (PAR) \times S \times 10^5 \tag{3.3}$$

where

IFC = concentration of liberated material in the fluid at the interface, units/cm^3

N = flux of the liberated material at the interface, amount/cm^2/s

f = fraction of blood that is non-cellular, for 40% hematocrit, f = 0.6

R = tube radius, cm

$\Gamma(2/3)$ = gamma function

z = dimensional axial position measured from the tube's inlet, cm

G = surface-shear rate, s^{-1}

D = diffusion coefficient for liberated material in red blood

cell containing suspension, cm^2/s

λ = non-dimensional axial position measured from the tube's entrance

PAR = platelet accumulation rate, platelets/1000 $\mu m^2/s$

S = total amount of material stored in a platelet that is liberated upon accumulation on the surface.

This solution is valid for $\lambda < 5x10^{-4}$ (9). In the tubes used here for up to 1 cm of length and for the shear rate range and diffusion coefficient range studied, the solution remains valid. Since we are concerned with the concentration of materials liberated from platelets for a small injury site, this solution will suffice.

The flux, N, of the liberated materials is proportional to the product of PAR and S, Equation 3.3. The PAR's were determined empirically at a number of shear rates, 80, 160, 320, and 640 s^{-1}. A power law correlation was determined for the platelet accumulation rate, PAR, with shear rate.

$$PAR = KG^n \tag{3.4}$$

where K and N are parameters to be found by regression (see Results).

Combining Equations 3.1, 3.2, 3.3 and 3.4, for z = 1.0 cm, and R = 0.065 the concentration in the fluid adjacent to the surface due to liberation of platelet materials can be calculated as follows:

$$IFC = 2.56 \times 10^5 \, KSG^{(n-1/3)}/D^{2/3} \tag{3.5}$$

This equation is restricted to platelet/red blood cell suspensions of 40% hematocrit, f = 0.6, and may be altered for other hematocrits according to Equation 3.1. The factor f in Equation 3.1 is necessary to convert the average concentration of a substance for the suspension,

fluid plus cells, to the concentration within the fluid. For those materials which are stored in the dense and alpha granules of platelets and are released all at once, the parameter S is simply the amount of secretable material in the platelet which we have obtained from the literature. For those materials progressively liberated by platelets, such as prostaglandins and thromboxanes, there will exist a period of time of increasing flux from onset of flow until the first platelet to adhere is exhausted and ceases to emit material. For this analysis, the total amount of material liberated by a platelet over this time period can then be treated as an effective total amount of liberatable material in a platelet, S. For prostaglandins and thromboxanes, platelets become exhausted approximately four minutes after stimulation (10,11). Since new platelets continually arrive to replace exhausted platelets (see results), the IFC of materials progressively liberated from platelets would reach a constant value some time after four minutes of perfusion. It is this steady-state IFC that is reported in the results section.

Since we are interested in the highest possible concentrations that could be developed and any metabolism or inactivation of liberated materials would decrease the concentration at the surface, no correction for inactivation was included in the analysis.

One final parameter must be estimated for each substance; that is its diffusion coefficient. The molecular weights of the releasable materials considered here are known allowing the diffusion coefficient to be calculated using the Stokes-Einstein equation (12), provided the specific volume of the material in solution can be estimated. Proteins

typically have a specific volume of 0.7 to 0.75 (12). Since we are principally concerned with order of magnitude estimates and not exact concentrations, a specific volume of 0.73 was used for calculations of diffusion coefficients for proteins. A specific volume of 1.0 was used for substances with a molecular weight less than 600 Daltons. The effect of red blood cell mixing upon the effective diffusion coefficient for the molecular size and shear rate range considered here would be small and was not included (13). However, the presence of red cells has been shown to impede diffusion and thus reduce the diffusion coefficient of dissolved species. This has been accounted for by a method described in reference (14).

3.3 Results

Platelet accumulation on the internal surface of a collagen-coated glass tube (cf 2.3) was determined at shear rates of 80, 160, 320 and 640 s^{-1} using suspensions of washed human platelets (cf 2.1.1). For each shear rate the platelet accumulation for the first centimeter of tubing was evaluated for four or five durations of exposure (cf 2.4, 2.5). A platelet accumulation rate, PAR, was then evaluated by linear regression of these data for each shear rate studied. Table 3.1 gives the estimated regression parameters and their upper and lower 95% confidence values. Figure 3.1 shows plots of the best-fit line for each shear rate. The parameters n and K of Equation 3.4 were evaluated by non-linear regression of the equation

$$\text{Platelet Accumulation} = K\, G^{n}\, t + C \qquad (3.6)$$

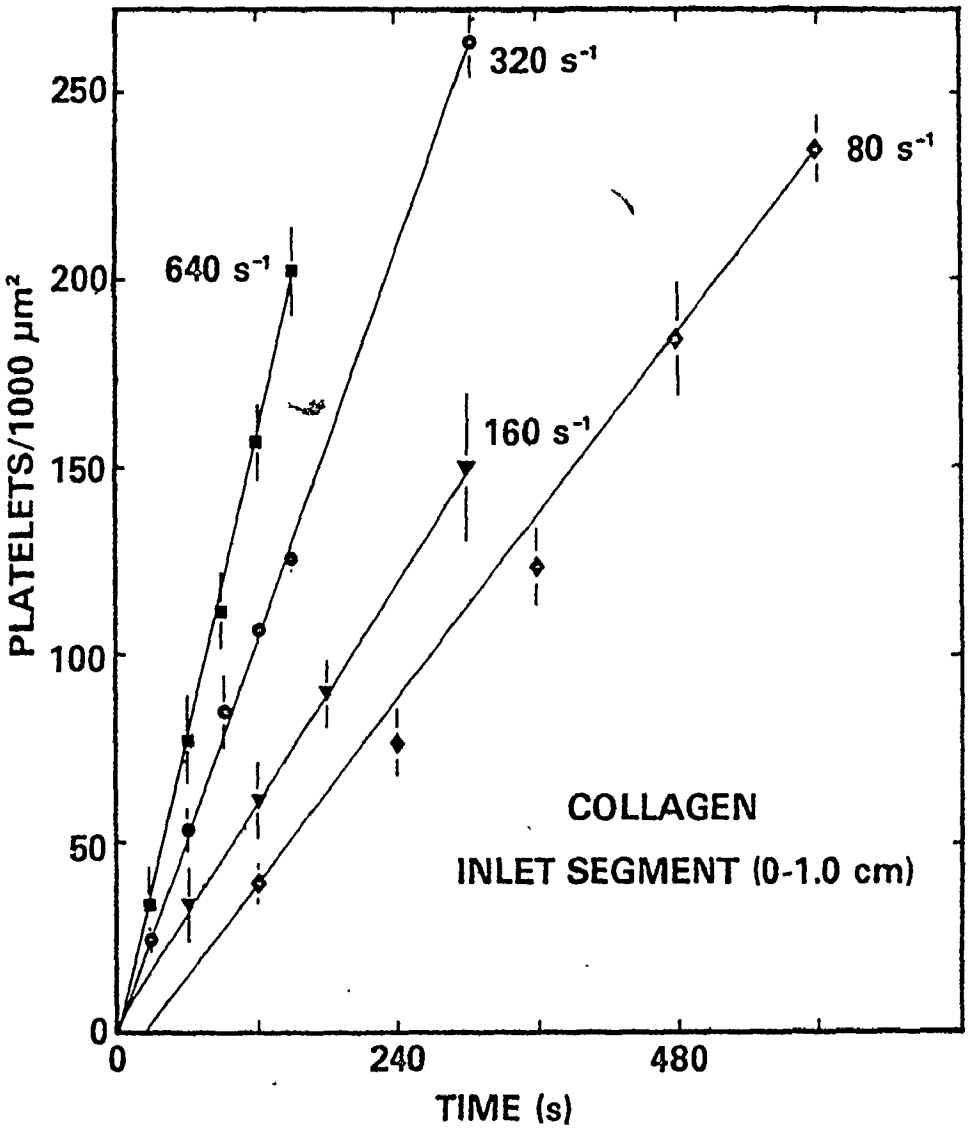


Figure 3.1: Human platelet accumulation on a collagen-coated glass tube using washed platelet/RBC suspensions as a function of time and surface shear rate. Each data point represents the mean of the average platelet accumulation over the fist centimeter of length along a 1.3 mm internal diameter tube. The vertical bars denote standard errors about the means. The solid lines were determined by linear regression of the relationship, accumulation = K_1 (time) + K_2, for each shear rate. The regression coefficients, K_1, K_2 and their 95% confidence intervals are reported in Table 3.1.

where t was the time of exposure. The input data for the regression were the measured platelet accumulation values with their corresponding exposure-times and shear rates. Equation 3.6 is the integral of Equation 3.5 over the time of perfusion, where C is the constant of integration corresponding to the platelet accumulation level at zero time. The parameter C then should be zero as the tubes were platelet-free at the beginning of each experimental run. The regression parameters and their upper and lower 95% confidence values are: K = 0.029 (0.020 to 0.038); n = 0.60 (0.53 to 0.66); C = -8.0 (-24.0 to 8.6). It can be demonstrated that C is not significantly different from zero. The units for PAR in Equation 3.4 which are consistent with these values of K and n are platelets/1000 $\mu m^2/s$. In Figure 3.2 are plotted Log (PAR - Platelet Accumulation Rate) versus Log (G - Shear Rate) with values determined from Equation 3.4 using the above estimated values for K and n. This plot is a straight line with slope n. Also appearing in Figure 3.2 are the platelet accumulation rate values and 95% confidence ranges resulting from regression of separate accumulation and exposure-time data for each shear rate studied, Table 3.1 and Figure 3.1. The values of K and n can then be used in Equation 3.6 to calculate the IFC for materials liberated by platelets. For each substance, the estimated value for D, and IFC at 80 and 640 s^{-1} are given as well as a number of quantities obained from the literature which are: the amount of material released or progressively liberated from platelets, S, the plasma concentration and the concentration required to affect platelets, as determined in an aggregometer, see

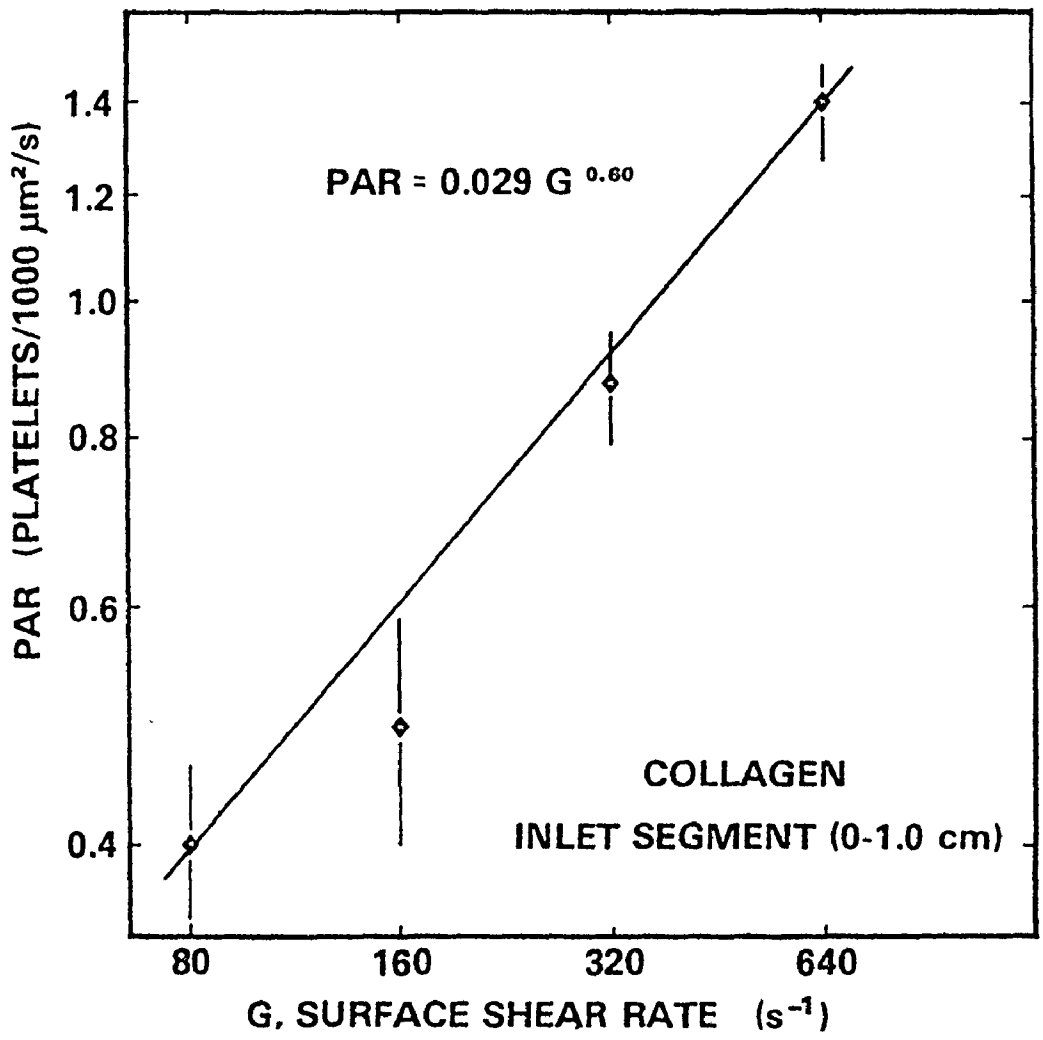


Figure 3.2: The rate of platelet accumulation (PAR) as a function of surface shear rate (G) on a collagen-coated glass tube using washed human platelet/RBC suspensions. The data points on the rates of platelet accumulation as determined by linear regression of the platelet accumulation versus time curves presented in Figure 3.1 and Table 3.1 for the four shear rates studied. Vertical bars denote the 95% confidence intervals. The solid line was obtained by non-linear regression of all the data with the equation, platelet accumulation = $K G^n t + C$, where K, n and C are regression parameters the values of which were K = 0.029 (0.020 to 0.028); n = 0.60 (0.53 to 0.66); C = -8.0 (-24.0 to 8.6) where the error values in parentheses are 95% confidence intervals for 166 data points used in the regression.

Table 3.1: Estimated Parameters for the Equation: Platelet Accumulation = $K_1 t + K_2$

Shear Rate s^{-1}	n	K_1 Platelets/1000 $\mu m^2/s$	K_2 Platelets/1000 μm^2
80	94	0.41 (0.35 to 0.46)	-10.6 (-39 to 18.1)
160	20	0.49 (0.40 to 0.59)	2.1 (-13.6 to 17.8)
320	26	0.88 (0.79 to 0.96)	0.8 (-11.1 to 12.8)
640	25	1.39 (1.27 to 1.51)	-5.0 (-15.6 to 5.5)

The values presented are the estimated parameters with their upper and lower 95% confidence values. K_1 is the platelet accumulation rate. An independent regression was done for each shear rate studied with platelet accumulation and exposure time as input data; n is the number of data points for each regression.

Table 3.2: Dense-granule Materials Released from Stimulated Human Platelets

Materials	Diff. Coeff. (D)	Plasma Conc.	S	Effect on Platelets	Conc. for Effect	IFC at $80\ s^{-1}$	IFC at $640\ s^{-1}$
ATP	1.05	0(6)	2.0(6)	inhibitor	20(5)	4.7	8.2
ADP	1.10	0(6)	3.0(6)	inducer	1-5(5)	6.8	12.0
AMP	1.35	0(6)	0.4(39)	inhibitor	1000(5)	0.8	1.4
Serotonin	1.49	0.2(21)	0.3(6,27)	inducer	5-250(5,20)	0.6	1.0
Calcium	2.44	2500(6)	10.0(6)	co-factor	100(19)	13.0	23.0
Pyrophosphate	1.27	0(6)	1.5(6)	co-factor	1000(19)	3.1	5.4
Units	cm^2/s x 10^6	μM	Moles/ Platelet x 10^{17}	-	μM	μM	μM

( ) Reference-source for the information.

Table 3.3: Alpha-Granule Materials Released from Stimulated Human Platelets

Material	Diff. Coeff. (D)	Plasma Conc.	S	Effect on Platelets	Conc. for Effect	IFC at $80\ s^{-1}$	IFC at $640\ s^{-1}$
LA-PF4	0.3	-	24 µg (29) .06 U (29)	heparin inhibitor	?	13 µg 0.03 U	23 µg 0.05 U
PF4	0.3	0.01 µg (40)	18 µg (27) .21 U (29)	heparin inhibitor	?	10 µg 0.11 U	18 µg 0.19 U
βTG	0.28	0.03 µg (40)	18 µg (27) .03 U (29)	heparin inhibitor	?	10 µg 0.02 U	18 µg 0.04 U
HNA	0.3	-	1.41 U (27)	heparin inhibitor	?	0.8 U	1.4 U
PDGF	0.4	-	12 U (27)	mitogen	?	5.4 U	9.5 U
VWF	0.1	1.0 U (31,32)	0.2 U (27)	adhesion co-factor	0.5 U (4)	0.23 U	0.40 U
Fibrinogen	0.13	3000 µg (6)	93 µg (27,46)	aggregation co-factor	20 µg (25)	88 µg	154 µg
Fibronectin	0.12	300 µg (30)	3.4 µg (30)	?	?	3.4 µg	6.0 µg
Units	cm^2/s x 10^6	Amount/ Platelet x 10^9	Amount/ml	-	Amount/ ml	Amount/ ml	Amount/ ml

( ) Reference-source for the information.

Table 3.4: Materials Progressively Liberated From Stimulated Human Platelets

Materials	Diff. Coeff.	Plasma Conc.	S	Effect on Platelets	Conc. for Effect	IFC at 80 s^{-1}	IFC at 640 s^{-1}
PGG_2	1.26	0	0.1*	inducer	200 (36)	0.2	0.4
PGH_2	1.26	0	0.1*	inducer	200 (36)	0.2	0.4
PGE_2	1.26	0	1.5 (41)	inducer	3000 (36)	3.1	5.4
PGD_2	1.26	0	8.9 (34)	inhibitor	12-21(34,35)	19	33
TA_2	1.26	0	89 (34)	inducer	?	186	326
Units	cm^2/s x 10^6	nM	Moles/ Platelet x 10^{20} in 4 min.	-	nM	nM	nM

* Estimated as 1000 times less than the amount of liberated TA_2 based on ratios of radioactive counts from reference (11) and an amount of TA_2 from reference (34).

() Reference-Source for the information.

Tables 3.2, 3.3 and 3.4.

In the preparation of this thesis numerous literature articles, which reported the amounts of the materials stored in platelet granules and amounts of materials which are liberated through the arachidonate pathway, were obtained. There was excellent agreement amongst research groups suggesting that the amounts of materials secreted by human platelets are constant and reproducable quantities. I have chosen only to cite reviews or selected articles which quote specific values for the contents of platelets in the interests of brevity.

3.4 Discussion

The rate of platelet accumulation on the internal surface of a collagen-coated tube was found to increase with increasing surface-shear rate. The rate of platelet accumulation was found to be proportional to the shear rate raised to the 0.60 power, $G^{0.60}$. This calculated experiment is in agreement with Turitto and Baumgartner (18) who obtained a value 0.61. The flux at the tube's surface, N, amount/cm^2/s, of materials secreted or continuously liberated from platelets, for this analysis, is proportional to the product of the platelet accumulation rate, PAR and the amount of material per platelet secreted or continuously liberated, S, Equation 3.3. Since PAR is directly proportional to $G^{0.60}$, N will also be directly proportional to $G^{0.60}$. Equation 3.6 indicates that the IFC is dependent upon the shear rate, G. This dependence arises from two sources: (a) The factor N which is proportional to $G^{0.60}$ and related to PAR and (b) a factor $G^{-1/3}$ which

accounts for the effect of shear rate upon the transport of materials away from the surface and which indicates that removal is enhanced by increased shear rate (IFC is reduced with increased shear rate by this factor). The net effect of these two factors is that IFC is proportional to $G^{0.27}$. This means that an 8 fold increase in shear rate (for example 80 s^{-1} to 640 s^{-1}) will increase IFC by 1.75 times. The dependence of the concentration of liberated substances from platelets on shear rate may be a factor in the experimental evaluation of platelet adhesion in von Willebrand's disease. Decreased platelet deposition on subendothelium using platelets from patients with von Willebrand's disease, has been reported at high shear rates but not at low shear rate (17). It may be that some material, lacking in von Willebrand's platelets, which was liberated from normal platelets is controlling platelet deposition at higher shear rates.

Each liberated agent will be discussed separately with respect to the possible enhancement of the concentration in plasma of the agent and its ability to affect platelets. For each substance discussed, a range of IFC will be presented. The smaller value was computed for a shear rate of 80 s^{-1} and the larger was computed for 640 s^{-1}. The combined effect of low concentrations of liberated agents will then be discussed.

3.4.1 Platelet Dense-Granule-Bound Materials

Calcium: The role of extracellular calcium in platelet aggregation is unclear. Since the plasma concentration of calcium is 2.5 mM (6) and 100 μM will support aggregation (19) and the expected

increase due to platelet release is 13 to 23 μM, it seems unlikely that calcium release from platelets will be important in normal thrombus formation. The transfer of granule-bound calcium into the cytoplasm of platelets is believed to be an important phase in platelet aggregation and release and it may be in this capacity that granule-bound calcium is contributing to platelet activation (5).

Pyrophosphate: An IFC of 3 to 5 μM can be expected for pyrophosphate due to platelet release with none normally in plasma. A pyrophosphate concentration of 1 mM is required to potentiate ADP induced platelet aggregation (19). It seems unlikely that this material is important in thrombogenesis.

Serotonin: The concentration of serotonin required to induce aggregation of platelets in an aggregometer varies with the platelet isolation procedure used (5,20) from 5.0 μM to 250 μM. However the degree of aggregation over this range of concentration is small, less than 10%. The maximum IFC would be 0.6 to 1.0 μM, with 0.2 μM present in normal plasma (21). Serotonin would not be present in high enough concentration to induce aggregation by itself but may interact with other relased agents synergistically to activate platelets.

ADP: Platelets contain at least two pools of ADP, a metabolic pool and a granule-bound pool (22). When platelets adhere to a surface, release occurs from the granule pool. The calculated IFC of ADP that would occur is 6.8 to 12.0 μM. This is in the same range as the concentration required to induce platelet aggregation in an aggregometer (20). Specific removal of ADP by enzymes decreases platelet retention

in glass bead columns (23), and reduces aggregate formation on subendothelium (24), indicating the importance of ADP in these systems. It therefore seems possible that ADP is present in sufficient quantities to aggregate platelets by itself but may require other agents to produce full aggregation.

ATP and AMP: Both ATP and AMP inhibit platelet aggregation induced by ADP but not by other stimuli (5). ATP is effective at a concentration of 20 μM (5) and AMP requires a concentration of 3 mM (5). The calculated IFC for ATP is 4.7 to 8.2 μM and for AMP 0.8 to 1.4 μM. The net effect of released adenine nucleotides then would be a small inhibitory effect on platelet aggregation by ATP and AMP but a larger aggregation inducing effect by ADP. It is interesting to note that some storage-pool-disease platelets have near normal granule-bound ATP levels but low ADP levels resulting in a net inhibitory action on platelet aggregation (25).

3.4.2 Platelet Alpha-Granule-Bound Materials

Fibrinogen: Fibrinogen is required to stabilize the platelet aggregate in hemostasis as demonstrated by the fragility of clots formed by patients with coagulation factor deficiencies (1). Thrombin will aggregate washed human platelets without exogeneous fibrinogen suggesting that granule-bound fibrinogen can be sufficient to support aggregation (20). The platelet-derived-fibrinogen concentration that would develop in an aggregation cuvette, with 5×10^8 platelets/ml after complete release by platelets, would be 47 μg/ml. This calculation is

based on a releasable fibrinogen content of 93 $\mu g/10^9$ platelets (25). A fibrinogen concentration of 20 µg/ml is sufficient to support ADP-induced aggregation of washed platelets (26). Thus, there is sufficient fibrinogen released by platelets to support platelet aggregation in an aggregometer cuvette using a non-plasma medium. In a flowing system, the IFC would be 88 to 154 µg/ml as compared with 3000 µg/ml in normal plasma (6). It is therefore unlikely that platelet fibrinogen affects thrombus formation, in vivo, unless the release of fibrinogen into the interstices of a platelet thrombus enhances thrombus stability.

Platelet Derived Growth Factor (PDGF): Platelets have a smooth muscle cell mitogen stored in their alpha granules (6). The release of this mitogen close to the de-endothelized vessel wall could be the initial stimulus for development of atherosclerosis (6). The platelet has 12×10^{-9} units of PDGF (27). The maximum concentration of PDGF at the interface of the tube, IFC, would be 5.4 to 9.5 units/ml. One unit/ml is defined as the amount of ^3H-thymidine incorporated by smooth muscle cells cultured in 1% fetal calf serum (27). Should this material have a high affinity for the vessel wall, it is possible that a portion of it would be sequestered by the smooth muscle cells and hence not be carried away by the flowing blood as this analysis assumes. It recently has been shown that another alpha-granule bound substance, platelet factor 4, can enter the de-endothelialized vessel wall during platelet accumulation at the surface of the vessel (28). Some estimation of the binding constant of PDGF for smooth muscle cells is required to

determine if the rate of adsorption to the wall is significant. It is not known if a concentration of 5.4 units/ml is important in atherogenesis.

Anti-heparins: Platelet factor 4 (PF4), low affinity platelet factor 4 (LA-PF4) and beta thromboglobulin (βTG) have all been shown to prevent heparin from acting as a co-factor for antithrombin III inactivation of serine proteases (29). The calculated value of IFC for the sum of the heparin neutralizing activities related to PF4, LA-PF4 and βTG is 0.16 to 0.28 units/ml. These three materials account for a fraction of the total heparin neutralizing activity (HNA) released from platelets in an aggregometer-type experiment (29). A value for the total platelet-derived heparin neutralizing activity has been reported (27) and based on it the IFC for total heparin neutralizing activity is 0.8 to 1.4 units/ml. It is difficult to say how important anti-heparin materials are in normal thrombogenesis as the heparin level, in vivo, is unknown.

Fibronectin: Fibronectin does not support aggregation of washed human platelets (30). The calculated IFC was 3.4 to 6.0 μg/ml. This value is much less than the plasma concentration of 300 μg/ml (30). Thus it is unlikely that the release from platelet granules naturally increases the fibronectin concentration near a thrombus.

von Willebrand Factor (VWF): The high molecular weight component of the factor VIII complex is present in the alpha granules of platelets (31,32). Patients deficient in this factor have von Willebrand's disease, which is characterized by prolonged bleeding and abnormal

platelet aggregation induced by ristocetin (4,33). Recent studies have shown that blood from these patients has defective platelet adhesion to collagen and subendothelium at high shear rates (17,33). Addition of 0.5 to 1.5 units/ml of factor VIII complex restores normal adhesion and aggregation functions (4,33). The calculated IFC for VWF from platelet granules was 0.23 to 0.40 units/ml. The normal plasma concentration is 1.0 unit/ml. It therefore seems unlikely that the contribution of VWF from platelets would elevate the normal plasma concentration significantly. This is further suggested by the restoration of normal platelet adhesion by addition of VWF to deficient blood where the platelets are still deficient in granule-bound VWF (4,33). It is unknown whether or not washed normal platelets (VWF present within alpha granules) suspended in VWF deficient plasma would have normal adhesion.

3.4.3 Substances Synthesized from Arachidonic Acid (Prostaglandins and Thromboxanes)

Thrombin or collagen stimulation of platelets causes arachidonic acid metabolism with the formation of prostaglandins and thromboxanes. The functions of these metabolites are not fully agreed upon (3). An inhibitor of platelet aggregation, PGD_2, is formed and liberated by the platelet and has IFC values of 19 to 33 nM which are capable of inhibiting platelet aggregation, as 12 to 21 nM is required for inhibition (3,34,35). PGH_2, PGG_2 and thromboxane A_2(TA_2) are formed by platelets and are all inducers of platelet aggregation (3,36). Only TA_2 appears to be present in sufficient quantity, IFC values are 186 to 326

nM. Although the exact concentration needed for TA_2 to induce aggregation is not known due to its labile nature, the level required to induce aggregation is probably close to that for PGG_2 and PGH_2 which is 200 nM (36). Aspirin treatment of platelets permanently acetylates the cyclo-oxygenase enzyme which is responsible for arachidonic acid metabolism leading to TA_2 formation (3). Since PGD_2 is an inhibitor and TA_2 is an inducer of platelet aggregation, one may question what the net effect of these substances may be. It may be that the time sequence of liberation is important. Should TA_2 be produced first and then PGD_2, the result would be an initial stimulus for platelet thrombus formation followed by inhibition.

Synergisms

Many of the released or continuously liberated agents have been shown to have a more than additive effect on human platelet aggregation when used simultaneously (34,37). Similar data are available for rabbit platelets (38). To date, studies have focused on combinations of two agents. No two groups use the same platelet isolation procedures or the same two stimuli in combination. Also, some workers are studying potentiation of second phase aggregation in a turbidimetric device while others are studying potentiation of initial or primary aggregation. All together, the data shows that low concentrations of stimuli in combination can elicit strong platelet responses. Synergism between platelet inhibitors that could be formed at a vascular injury site such as PGD_2 from platelets and PGI_2 from endothelial cells may also exist.

TA_2 and ADP are present in sufficient concentrations to stimulate platelet aggregation individually and PGD_2 is present in sufficient concentration to inhibit platelet aggregation alone. In addition, potentiation of aggregation by combinations of aggregation inducers with substances just below the necessary concentration to induce platelet aggregation may be possible, e.g. ADP plus serotonin; as is potentiation of inhibition of aggregation with substances just below the necessary concentration to inhibit aggregation, e.g. PGD_2 plus ATP. Detailed experiments to determine the potential interactions between combinations of these materials are required to learn which substances would provide the dominant effect and which combination of materials are important in mural thrombogenesis.

3.5 Summary

This paper deals with the estimation of local concentrations of materials which are liberated from platelets during platelet accumulation upon surfaces. Empirical data were used in a calculation procedure, based upon diffusion and convection, designed to yield an upper bound on the interfacial fluid concentration (IFC) for each substance considered. The necessary empirical data are the rate of platelet accumulation and the maximum amount of material in the platelet. It was found that the IFC is dependent upon the shear rate at the surface, G, $IFC \propto G^{0.27}$. Substances such as, serotonin, pyrophosphate, PGG_2 and PGH_2 were found not to be present in sufficient quantity to produce IFC's which could induce platelet aggregation. A

second set of materials, von Willebrand factor, fibronectin and calcium had IFC's less than the concentrations normally found in plasma. A third group, ADP, ATP, PGD_2, and TA_2 had IFC's close to those known to affect platelet aggregation. These last materials, along with any materials formed in plasma such as thrombin, are most likely to determine the rate of thrombus growth on a blood-contacting biomaterial and, along with any vessel-wall-derived substances such as PGI_2, will control the rate of thrombus growth on subendothelium.

CHAPTER 4

INTERFACIAL FLUID CONCENTRATIONS OF ADP NEAR A COLLAGEN-COATED SURFACE

4.1 Introduction

In Chapter 3, the concentration of ADP at the surface of a vessel developed due to 100% release of ADP from the granules of adherent platelets was estimated to be 6.8 to 12.0 μM. These concentrations were based on the rate of platelet accumulation obtained on the first centimeter of a collagen-coated glass tube. In Chapter 4, the release of serotonin from the dense granules of accumulated platelets was measured. Since serotonin is a marker of dense-granule release, the actual amount of ADP released by platelets can be estimated. The interfacial fluid concentration (IFC) of ADP can also be more precisely estimated than in Chapter 3 where 100% release was assumed. In addition the change in IFC along the tube (not just at the inlet) and into the lumen of the tube (not just at the surface) was estimated by expanding the theoretical model of mass transfer to include a parabolic velocity profile yielding estimates of IFC for larger distance along the tube. The expanded theory is given in Appendix A.

4.2 Results

The original experiments conducted to probe platelet accumulation and release from platelets adherent to a collagen-coated glass tube employed porcine platelet suspensions. Some initial probing of the tubular perfusion system, such as the effects of a hydrodynamic entrance region, heterogeneous labeling of platelets with ^{51}Cr, priming of the tube before perfusion and the contribution of ADP liberated from red blood cells were conducted and are presented in Appendix B. Experiments on the rate of platelet accumulation, the degree of release of dense-granule materials from adherent platelets and the IFC of ADP are presented in Appendix B for porcine platelets and in this chapter for human platelets.

Radiolabeled platelet suspensions (cf 2.1.1) containing red blood cells (cf 2.2.1) were used to study platelet accumulation and release of serotonin from platelets (cf 2.4, 2.5) deposited on a collagen-coated glass tube (cf. 2.3). Four flow rates were chosen corresponding to surface shear rates of 80, 160, 320 and 640 s^{-1} and the duration of exposure was varied from 30 to 600 s.

4.2.1 Platelet Accumulation:

The change in platelet accumulation with increasing distance from the inlet of a collagen-coated glass tube at a shear rate 80, 320 and 640 s^{-1} and perfusion times of 600 s, 300 s, and 120 s are presented in Figures 4.1, 4.2, and 4.3 respectively. Also presented in these figures are the effects of the addition of imipramine (cf 2.7) to a final

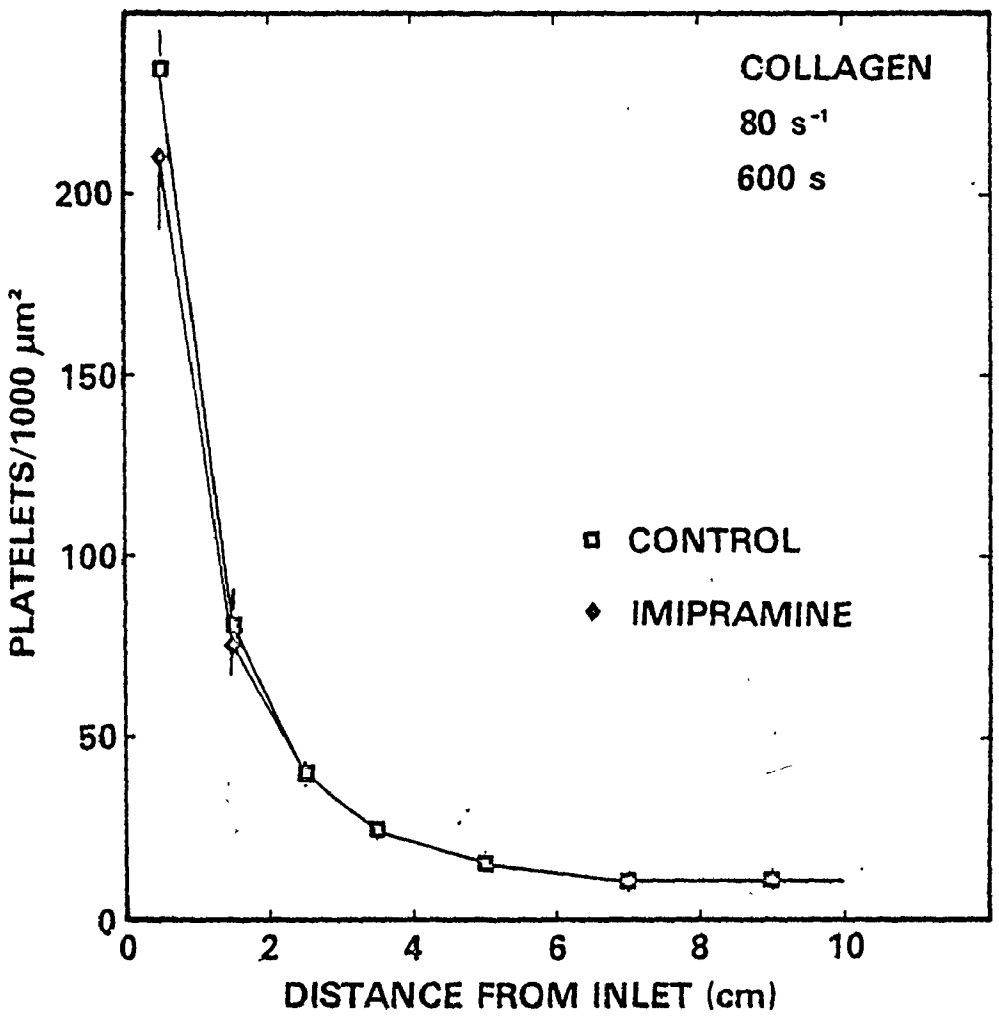


Figure 4.1: Human platelet accumulation as a function of distance from the inlet of a collagen-coated glass tube at a surface shear rate of 80 s^{-1} after 600 s of perfusion. Values are means of 51 determinations for the control experiments and 10 determinations for the imipramine experiments. Vertical bars denote standard errors. There was no difference between platelet accumulations using control and imipramine-treated platelets and the points were coincident at positions greater than 2 cm from the inlet. Similar data for surface shear rates of 320 and 640 s^{-1} are presented in Figure 4.2 and 4.3.

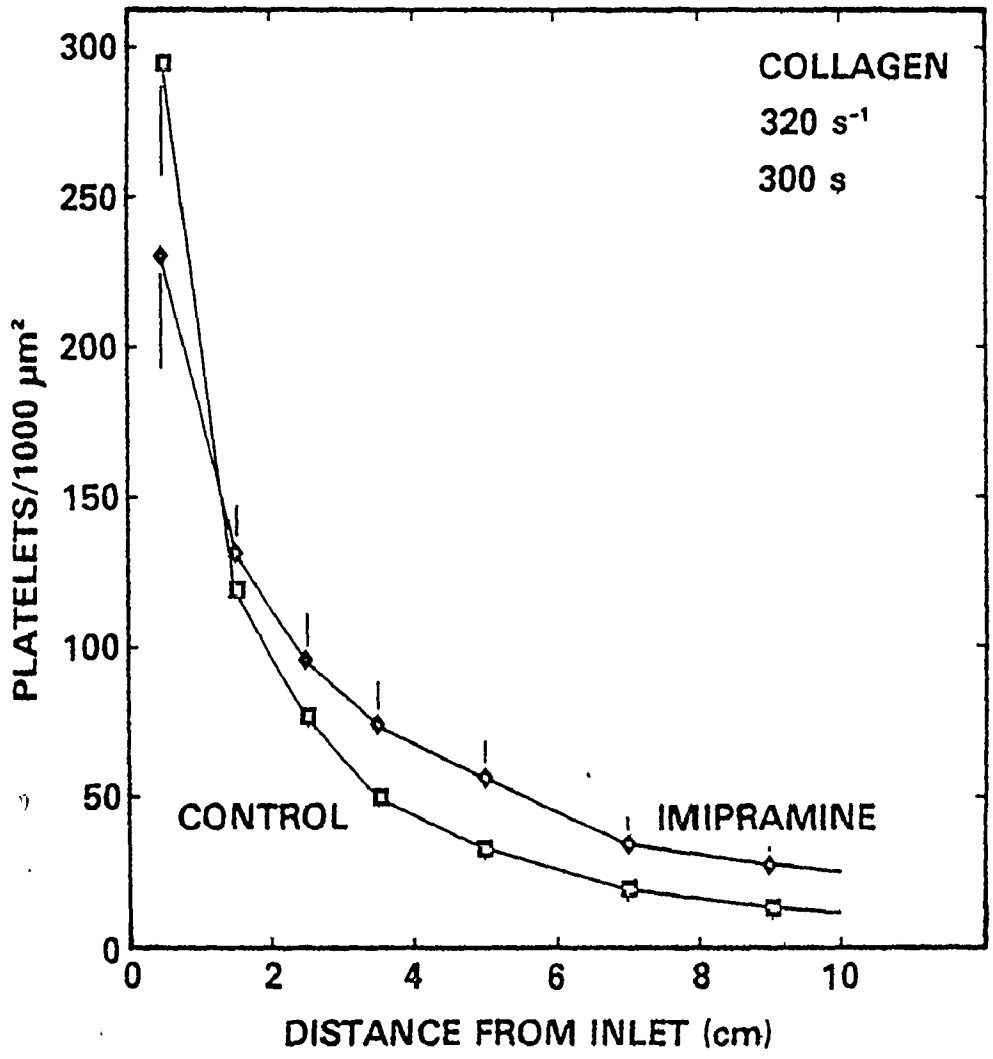


Figure 4.2: Human platelet accumulation as a function of distance from the inlet of a collagen-coated glass tube at a surface shear rate of 320 s^{-1} after 300 s of perfusion. Values are means of 2 determinations. Vertical bars denote standard errors. Platelet accumulation using imipramine-treated platelets was not significantly different from untreated controls. Similar data for surface shear rates of 80 s^{-1} and 640 s^{-1} are presented in Figures 4.1 to 4.3.

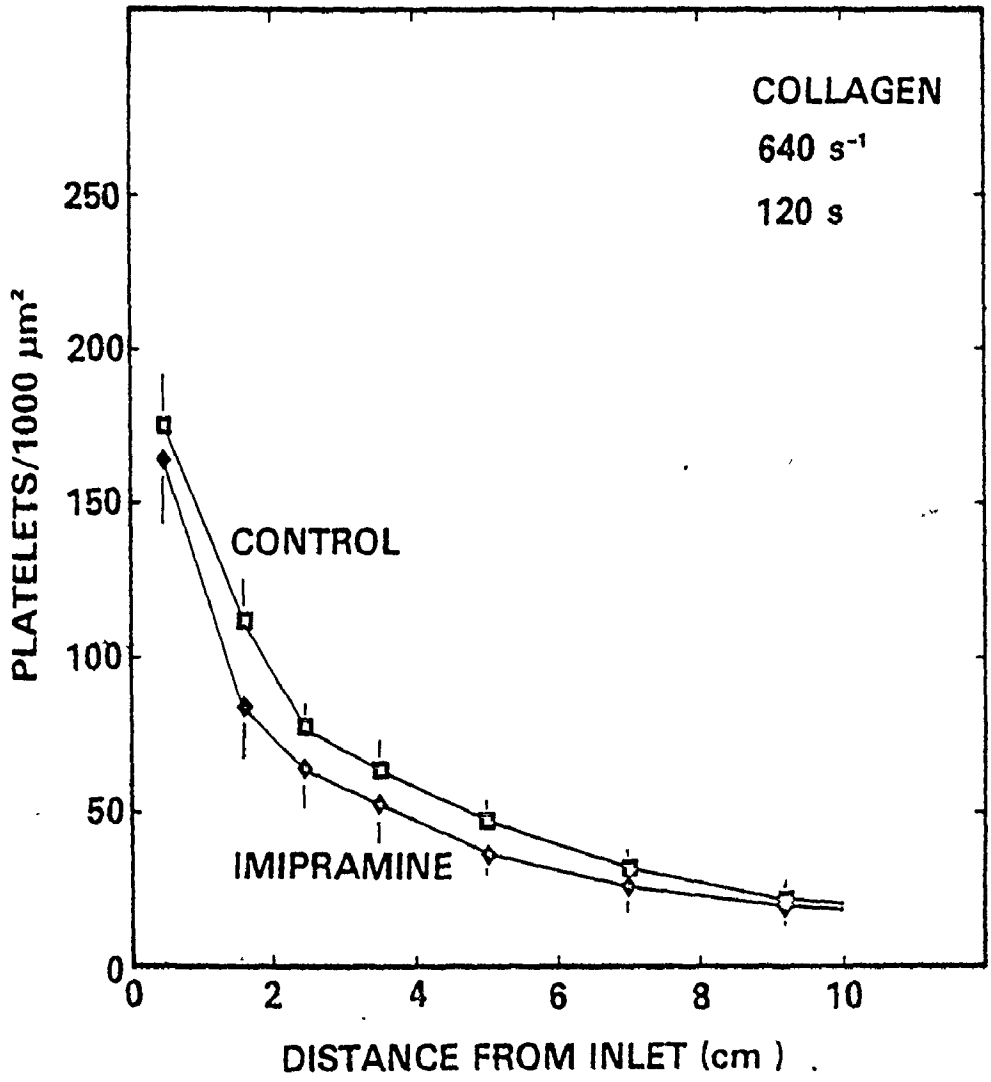


Figure 4.3: Human platelet accumulation as a function of distance from the inlet of a collagen-coated glass tube at a surface shear rate of 640 s^{-1} after 120 seconds of perfusion. Values are means of 2 determinations for the control experiments and 3 determinations for the imipramine-treated experiments. Vertical bars denote standard errors. There was no significant difference between the platelet accumulation using imipramine-treated or control platelets. Similar data for surface shear rates of 80 s^{-1} and 320 s^{-1} are presented in Figures 4.1 to 4.2.

TABLE 4.1: Power Law Exponents Between Platelet Acummulation and Distance from the Inlet of a Collagen-coated Tube.

Time (s)	Drug	Surface Shear Rate(s^{-1}) 80	160	320	640
30	-			-0.59(.04)/ 2/	
	I			-0.59(-)/ 1/	-0.50(.04)/ 5/
60	-			-0.82(.01)/ 2/	
	I		-0.64(.04)/ 4/	-0.62(-)/ 1/	-0.64(.03)/ 4/
90	-			-0.90(.01)/ 2/	
	I			-0.70(.05)/ 2/	-0.63(.10)/ 4/
120	-	-0.75(.04)/ 6/		-0.98(.01)/ 2/	-0.70(.02)/ 2/
	I	-0.74(.18)/ 2/	-0.80(.07)/ 4/	-0.78(.02)/ 2/	-0.75(.06)/ 3/
150	-			-1.01(.01)/ 2/	
	I			-0.76(.01)/ 2/	-0.71(.01)/ 3/
180	-				
	I		-0.85(.09)/ 4/		
240	-	-0.95(.05)/ 5/			
	I	-0.88(.21)/ 2/			
300	-			-1.04(.04)/ 2/	
	I		-0.80(.09)/ 4/	-0.73(.06)/ 2/	
360	-	-1.00(.11)/ 3/			
	I	-1.00(.26)/ 2/			
480	-	-1.30(.10)/ 4/			
	I	-0.94(.35)/ 2/			
600	-	-1.17(.03)/51/			
	I	-1.11(.08)/10/			

* mean(S.E.M.)/number of tubes each having 7 data points/

I= Imipramine added to suspension; final concentration 2μM

concentration of 2 μM on platelet accumulation at the same shear rates and times of perfusion. The imipramine treatment had no effect on platelet accumulation at any of the shear rates or times studied. In all cases there was a rapid decline in platelet accumulation with increasing distance from the inlet. This pattern is in agreement with a mass transport theory(1,2,3) which predicts a logarithmic relationship between platelet accumulation and distance along the tube (z), platelet accumulation = constant z^m, where m is the power-law exponent. The value of m for the conditions studied were determined by regression of the seven measurements of platelet accumulation (Figures 4.1 to 4.3) and are presented in Table 4.1. The correlation coefficient was consistently greater than 0.98 indicating the excellent fit of the data by the theoretical relationship (example of the data points and the regressions are given in Figures A.2 and 8.1).

4.2.2 Release From Accumulated Platelets

The release of serotonin from platelets deposited on a collagen-coated glass tube was measured radioisotopically (cf 2.5) as a function of distance from the inlet and shear rate (Figures 4.4, 4.5, 4.6). The effect of the addition of imipramine at a final concentration of 2 μM on the release of serotonin was also studied and is presented in Figures 4.4, 4.5 and 4.6. The percent release from deposited platelets increased with increasing distance from the inlet, in both control and imipramine-containing experiments. The addition of imipramine, however, shifted the percent release to higher values all the way along the tube.

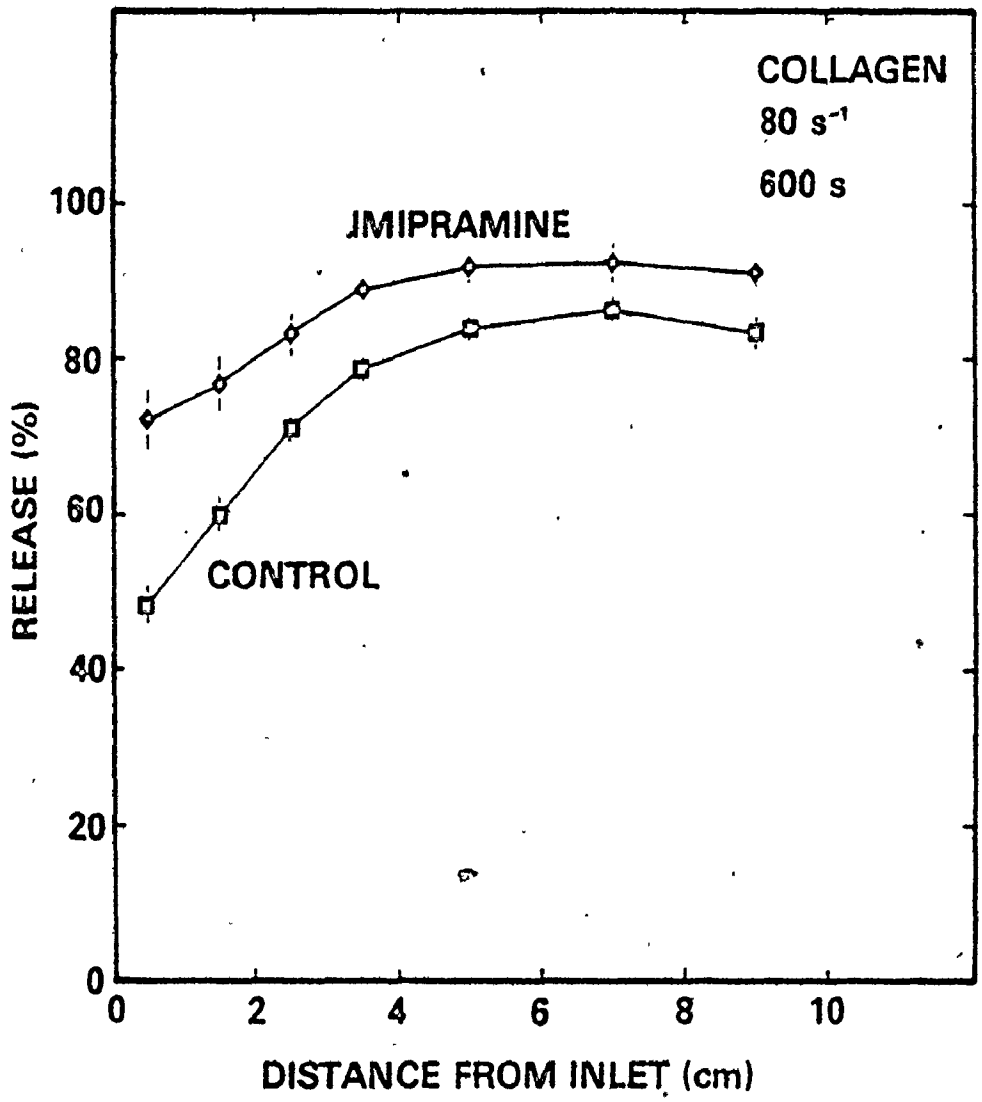


Figure 4.4: Percent release of serotonin from the dense granules of human platelets adherent to a collagen-coated glass tube as a function of distance from the inlet at a surface shear rate of 80 s^{-1} after 600 s of perfusion. Values are means of 51 determinations in the control experiments and 10 determinations in the imipramine-treated experiments. Vertical lines denote standard errors. Imipramine-treatment increased the measured percent release by blocking reuptake of released serotonin. Similar data for surface shear rates of 320 s^{-1} and 640 s^{-1} are presented in Figures 4.4 and 4.5.

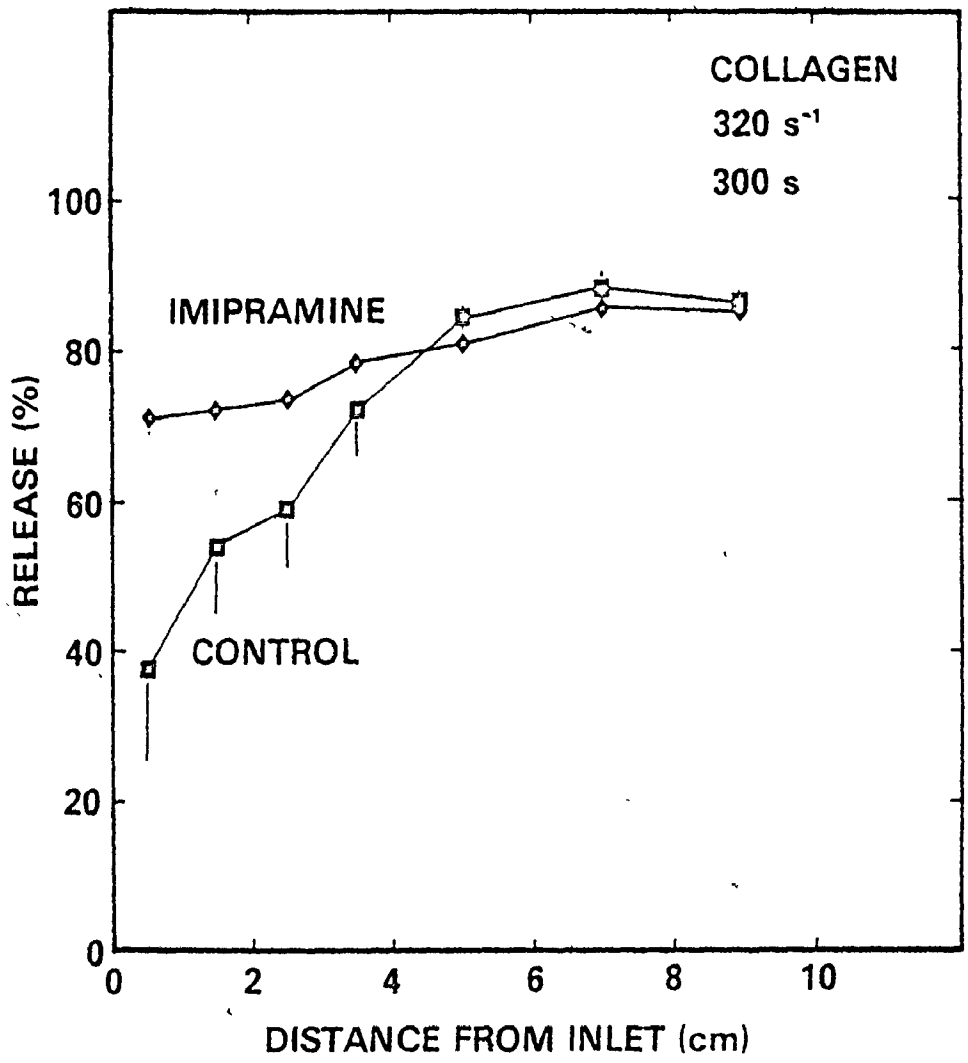


Figure 4.5: Percent release of serotonin from the dense granules of human platelets adherent to a collagen-coated glass tube as a function of distance from the inlet at a surface shear rate of 320 s^{-1} after 300 s of perfusion. Values are means of two determinations. Vertical bars denote standard errors. Imipramine treatment of platelets increased measured percent release by blocking reuptake of released serotonin. Similar data for surface shear rates of 80 s^{-1} and 640 s^{-1} are presented in Figures 4.4 and 4.6.

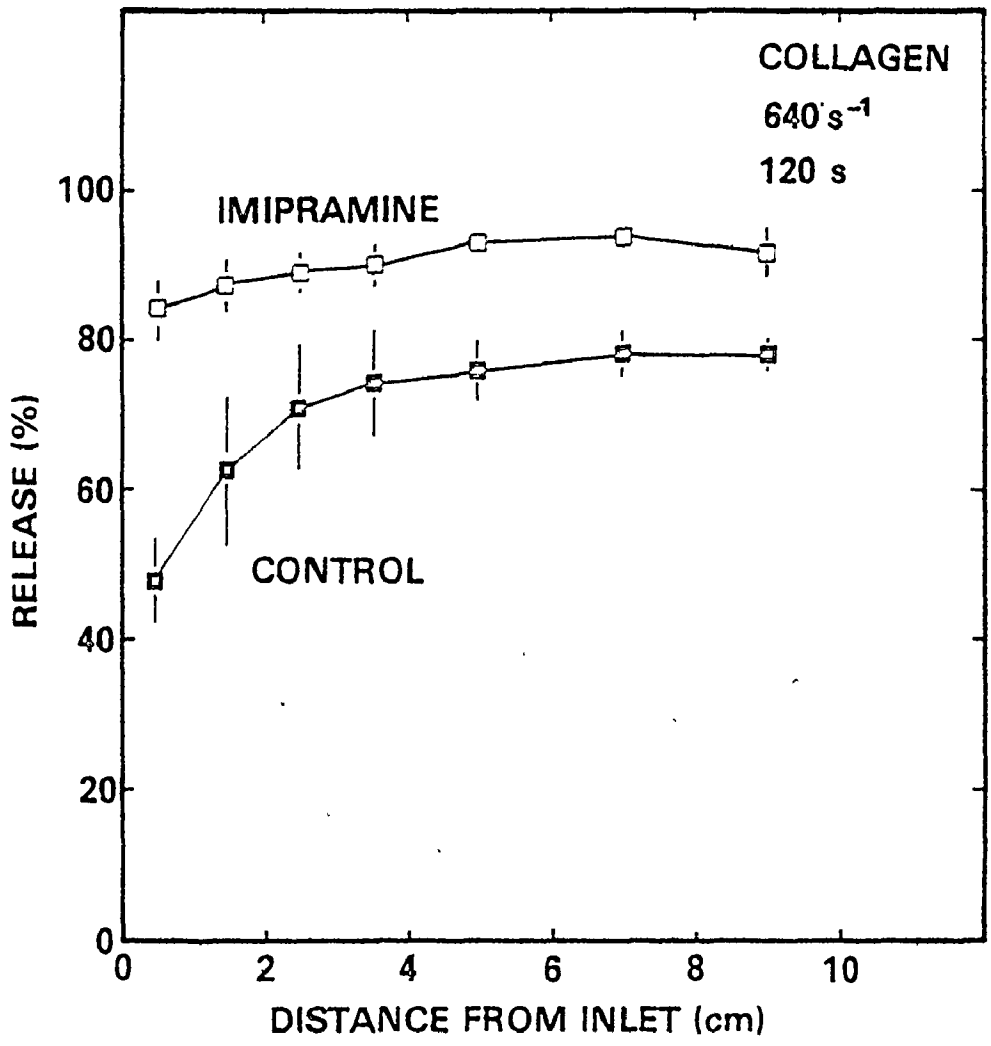


Figure 4.6: Percent release of serotonin from the dense granules of human platelets adherent to a collagen-coated glass tube as a function of distance from the inlet at a surface shear rate of 640 s^{-1} after 120 s of perfusion. Values are means of two determinations for the control experiments and the three determinations for the imipramine-treated experiments. Vertical bars denote standard errors. Imipramine treatment increased measured percent release by preventing reuptake of released serotonin. Similar data for surface shear rate of 80 s^{-1} and 320 s^{-1} are presented in Figures 4.4 and 4.5.

The increase was greatest at the inlet and least at the outlet of the tube resulting in a less pronounced change in release with distance from the inlet in the imipramine-containing experiments as compared with the control experiments. A study of the change in percent release of serotonin with duration of perfusion is presented in Chapter 8.

4.2.3 Flux of ADP at the Interface

The rate of release of ADP at the interface can be computed as the platelet accumulation and the degree of release of serotonin have been measured (cf Appendix A, equation A.5) and is called the flux. The basic assumption was that the degree of release of serotonin from deposited platelets was the same as the degree of release of all dense granule materials including ADP. Both serotonin and ADP have been documented to be stored in the dense granules of platelets (cf 1.2.1) and to be released simultaneously(4,5). The flux estimated here differs from the flux, N, used in Chapter 3, equation 3, in that the degree of release, since it was measured was no longer assumed to be 100%. The change in flux of ADP is present in Figure 4.7 as a function of distance from the inlet, surface shear rate and imipramine treatment. The flux was highest at the inlet and lowest at the outlet of the tube. The flux increased with higher shear rates and imipramine addition.

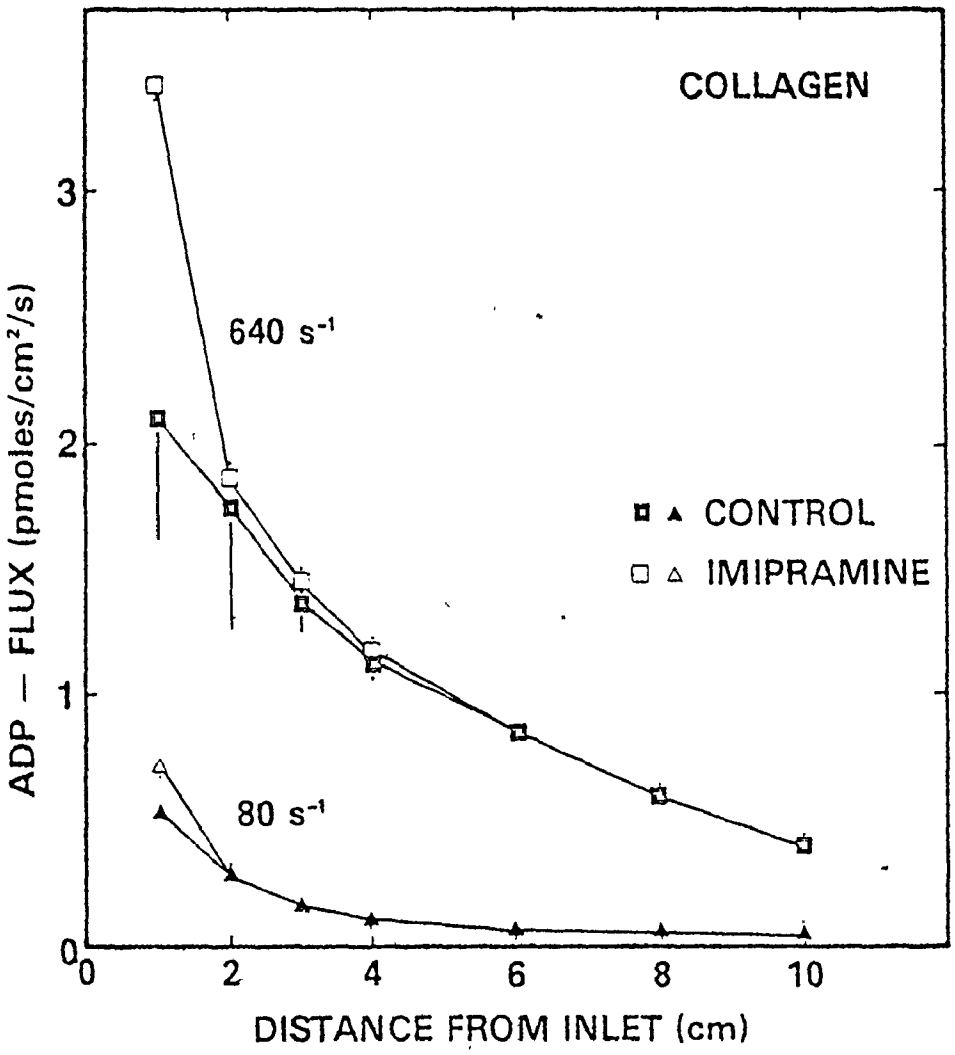


Figure 4.7: The amount of adenosine diphosphate (ADP) released by human platelets per unit surface area per unit time, flux, as a function of distance from the inlet of a collagen-coated glass tube. At a surface shear rate of 640 s^{-1}, the flux values for the control and imip ramine-containing experiments were coincident at distances greater than 4 cm from the inlet. At surface shear rates of 80 s^{-1}, the flux values for the control and imipramine-containing experiments were coincident at distances greater than 2 cm from the inlet. Values are means of 51 determinations for the 80 s^{-1}/control, 10 determinations for the 80 s^{-1}/imipramine, 2 determinations for the 640 s^{-1}/control and 3 determinations for the 640 s^{-1}/imipramine cases. Vertical bars denote standard errors of the means.

4.2.4 Interfacial Fluid Concentration of ADP

The interfacial fluid concentration, IFC, of ADP was computed using the calculation procedure in Appendix A and the data presented in Figures 4.1 to 4.7. The IFC of ADP changed relatively little along the length of the tube but ranged from 1.5 μM at a shear rate of 80 s^{-1} to 10.3 μM at a shear rate of 640 s^{-1}. The values of the IFC in Figure 4.8 are steady-state concentrations that would not change with time of perfusion as platelets are constantly arriving (cf Figure 3.1) and releasing their contents. The IFC of ADP for the first segment of tubing, corresponding to the position 1 cm from the inlet in Figure 4.8 and for comparison with the IFC calculated in Chapter 3 are tabulated in Table 4.2 as a function of shear rate time, and imipramine treatment.

The IFC of ADP did not change with increasing time in the imipramine-containing experiments but did decrease with time for the control experiments. This was due to the decrease in measured release of serotonin with time due to re-uptake of serotonin by adherent platelets. The release values with imipramine present are then the correct values to be used to calculate the IFC for ADP and the release values without imipramine are the correct values to be used to calculate the IFC for serotonin.

While the concentration of materials released from platelets varied slightly along the table, it would be useful to know how the concentration would decrease normal to the tube's surface. Two asymptotic solutions are available that allow us to see how the concentration would change in the radial direction at the inlet and the

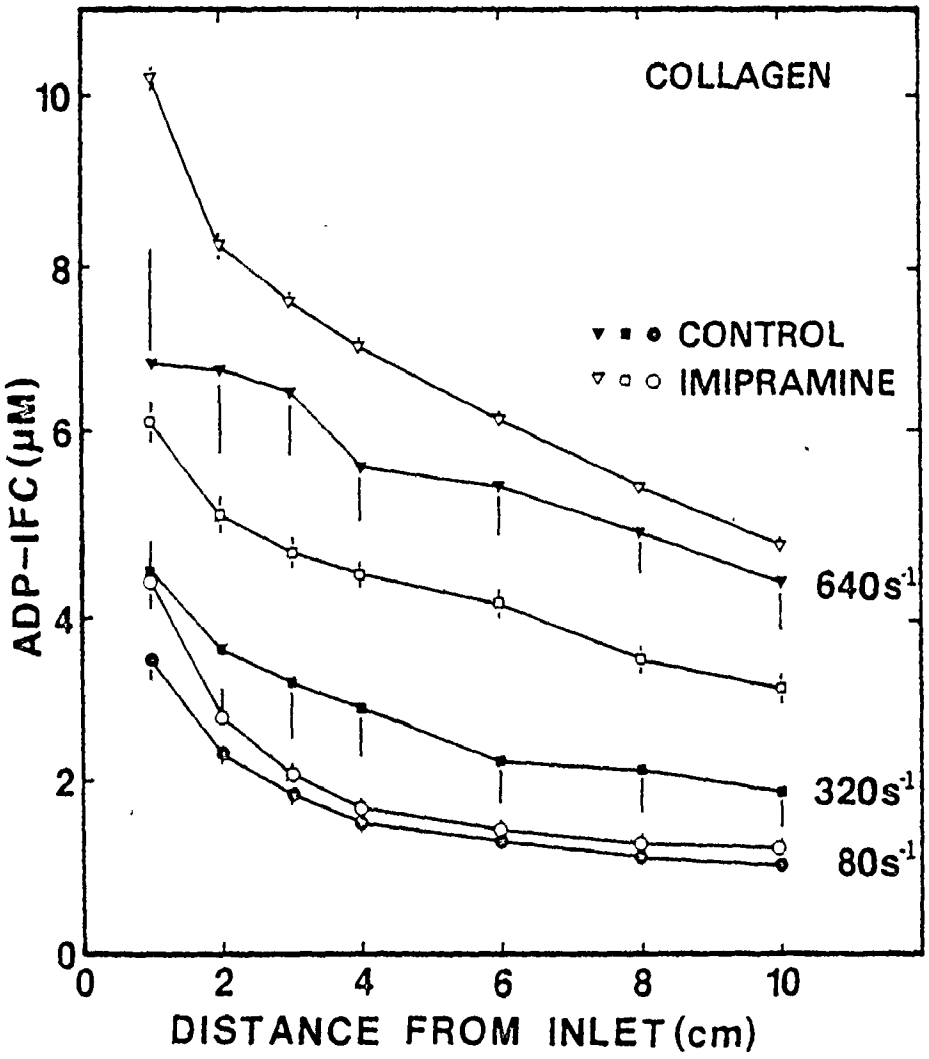


Figure 4.8: The interfacial fluid concentration, IFC, of adenosine diphosphate, ADP, as a function of distance from the inlet of a collagen-coated glass tube and surface shear rate. Values are means of n determinations where 80 s^{-1}/control, n = 51; imipramine, n = 10/; 320 s^{-1}/control, n = 2; imipramine, n = 2/; 640 s^{-1}/control, n = 2; imipramine, n = 3/. Vertical bars denote standard errors about the mean. See Appendix A for procedures to calculate the IFC of ADP from the data in Figures 4.1 to 4.6.

TABLE 4.2: IFC of ADP(uM) Developed at the Inlet of a Collagen-Coated Tube due to Release from Adherent Platelets.

Time (s)	Drug	Surface Shear Rate (s^{-1})			
		80	160	320	640
30	-			7.8(0.4)/ 2/	
	I			7.9(-)/ 1/	9.0(1.0)/ 5/
60	-			8.3(0.9)/ 2/	
	I		6.9(0.8)/ 4/	7.6(-)/ 1/	9.9(0.8)/ 4/
90	-			5.0(1.8)/ 2/	
	I			7.1(0.8)/ 2/	9.5(1.2)/ 4/
120	-	5.5(1.1)/ 8/		5.7(2.2)/ 2/	6.3(1.2)/ 2/
	I	4.3(0.2)/ 2/	5.8(0.6)/ 4/	6.3(0.2)/ 2/	10.3(0.1)/ 3/
150	-			4.2(1.9)/ 2/	
	I			6.5(0.1)/ 2/	9.8(0.7)/ 3/
180	-				
	I		5.6(1.1)/ 4/		
240	-	4.2(0.8)/ 5/			
	I	4.2(0.1)/ 2/			
300	-			4.5(3.2)/ 2/	
	I			6.2(0.2)/ 2/	
360	-	3.1(0.8)/ 3/			
	I	4.6(0.7)/ 2/			
480	-	3.2(0.8)/ 4/			
	I	4.7(1.0)/ 2/			
600	-	3.4(0.2)/51/			
	I	4.3(0.4)/10/			

* mean(S.E.M.)/ number of tubes /.

I= Imipramine added to the suspension; final concentration 2 μM.

outlet (cf Appendix A). Close to the inlet, the parabolic velocity profile can be replaced with a linear one with a slope equal to the surface velocity gradient (cf 3.2, Appendix A). The solution yields, for a shear rate of 80 s^{-1}, an 8-fold reduction in concentration of ADP, 69 μM away from the interface at a position of 1 cm from the tube's entrance(6). The same reduction in concentration would occur 43 μm from the interface at a shear rate of 320 s^{-1}. This rapid decline in ADP concentration in the radial direction emphasizes the local nature of the increased concentration of released substances. Only close to the surface would the concentration of released materials be sufficiently high to affect platelets.

The second asymptotic solution deals with the fully-developed radial concentration profile which would be attained in the 80 s^{-1} shear rate case, 40 cm (400 diameters) from the tube's entrance. The solution shows that the concentration of released agents would be 8 times higher at the interface than at the center line of the tube (6). These calculated results indicate that even if extensive damage to the arterial vasculature occurred the high concentrations of released agents would still only be present close to their site of liberation.

4.3 Discussion

4.3.1 Platelet Accumulation

The accumulation of platelets on the internal surface of a collagen-coated tube was found to decrease with distance from the inlet more rapidly than predicted by classical, mass, transport theory(1,2,3).

The platelet accumulation was found to be proportional to the distance downstream from the inlet raised to an exponent, m, which ranged from -0.50 to -1.16 (Table 4.1) as compared to -0.33 which was predicted by theory(1,2,3). Baumgartner et. al.(3) found an exponent of -0.17 and Freidman and Leonard(1) reported a value of -0.41 for the power law exponent. Some of the possible reasons for the more rapid decline in platelet accumulation with distance from the inlet were investigated.

The effects of priming the tube, heterogenous labeling of platelets with ^{51}Cr and the possibility of disturbed flow patterns at the inlet were investigated using porcine platelets and are presented in Appendix B. None of these three possibilities were found to explain the difference between empirical and theoretical exponents. Other possible mechanisms are aggregate formation at the inlet, embolization of cells at the outlet, preferential accumulation of a subpopulation of platelets at the inlet or the release of a platelet-derived inhibitor of platelet accumulation that has a different pattern of liberation than dense-granule materials. More experimentation would be required to differentiate between these other possibilities or a new theoretical model constructed to explain the empirical power law exponents.

4.3.2 Release From Deposited Platelets

The degree of release of serotonin from deposited platelets was found to increase with distance from the inlet in the control experiments (Figures 4.4 to 4.6). The percent release of serotonin also increased with increasing distance from the inlet in the experiments

with imipramine added. The measured release from the control platelets was lower than the measured release from the imipramine-treated platelets probably due to the reuptake of serotonin by the control platelets. The question of reuptake of serotonin is discussed in detail in Chapter 8.

The release data (Figures 4.4 to 4.6) show that the degree of release of granule contents from platelets deposited at the inlet of a tube is lower than that of platelets deposited at the outlet. This suggests that some material is released by the platelets, and convected downstream where it induces platelets adhering that are downstream to release more extensively. The degree of release being dependent on the local concentration of released material. It does not appear that the change in release with distance from the inlet can be directly related to the IFC of ADP, or any other dense granule material as the IFC changes little with distance from the inlet (Figure 4.8). It may be that other factors are working in parallel with released substances to produce the observed release pattern. Another possibility to explain the increased release at the outlet could be the sensitization of platelets by the released ADP. The platelets that adhere at the exit of the tube would have had longer periods of incubation and may have had time to become more sensitive to the collagen stimulus while those platelets that adhere at the inlet of the tube would have incubated for a short time and may not have been fully sensitized. The degree of release could then be dependent on the time from entry into the tube. This scheme is consistent with the observed pattern of release from

platelets along the tube. Platelets aggregate and release more extensively in response to collagen when small concentrations of ADP are present than in the absence of ADP (Appendix A, cf 3.4.4). The time required for sensitization by ADP incubation has not been studied in detail.

A third possibility for the increase in measured release with distance from the inlet may be that released agents from other granules or progressively secreted agents such as thromboxane A_2 could modulate release (cf 2.4, 5.4, 9.1). The concentration of such a material may vary with distance from the inlet in some manner other than that shown for dense-granule materials.

4.3.3 Interfacial Concentration of ADP

The interfacial concentration of ADP can be estimated using the computation procedure in Appendix A and the experimental data in this chapter and are presented in Section 4.2.4. As predicted in chapter 3, the IFC increased with increasing shear rate. The actual percent release of dense-granule serotonin from deposited platelets was not much less than the 100% assumed in Chapter 3 and the resultant IFCs (Figure 4.8) are not much less than the values predicted in Chapter 3 of 6.8 μM at 80 s^{-1} and 12.0 μM at 640 s^{-1}. The ADP-IFC did not change with increasing duration of perfusion when imipramine was present but ADP-IFC did decrease with duration of perfusion in the control experiments (Table 4.2). Imipramine blocks reuptake of serotonin and it is this reuptake of serotonin that lowers the measured release in the control

experiments. This topic is dealt with in greater detail in Chapter 8.

We may surmise that there is sufficient ADP released by deposited platelets to develop concentrations of ADP in the 1 to 10 μM range near the interface of a collagen-coated tube and probably a de-endothelialized vessel. This amount of ADP is sufficient to induce platelets to change shape and aggregate. However, the concentration of ADP decreases quickly away from the interface and would not cause platelet aggregation in the suspension. It is not possible to measure the concentration of ADP developed at the interface to validate the calculations but it is possible to use biological procedures to alter the response of platelets to ADP. Chapter 5 uses such conditions to probe if ADP is important in platelet-surface interactions.

4.4 Summary

These results are pertinent to the process of thrombosis in blood vessels and on biomaterials, and to hemostasis. Calculations based on experimental data show that ADP released from platelets produces sufficiently high concentrations at a potential site of injury to induce platelet shape change and platelet aggregation. The concentration of ADP does not change quickly along the surface but drops off rapidly away from the surface. Higher shear rates result in enhanced arrival of platelets at the surface and higher concentrations of released materials. The amount of ADP released from red blood cells was found to be negligible relative to that released from platelets (cf Appendix B). The results suggest that the contribution of ADP to thrombosis is quite

possibly through platelet activation or synergistic action of combinations of agents acting close to the site of thrombus formation.

CHAPTER 5

THE ROLE OF ADP IN MURAL THROMBOGENESIS

5.1 Introduction

In Chapter 3 the maximum concentration of ADP that could develop due to complete release of ADP from platelets attached to a collagen-coated surface was estimated. In Chapter 4, the concentration of ADP that could develop due to partial release of ADP from platelets was estimated. The conclusions of these chapters suggest ADP may be present in high enough concentrations close to the surface to promote thrombus formation by inducing platelets to change shape prior to collision with the surface. In Chapter 5, a biological approach has been taken to determine if the ADP released from platelets is significantly influencing platelet-surface interactions.

Platelet storage granules contain adenosine diphosphate (ADP) which is released upon induction by many stimuli(1,2). Since ADP will also induce platelets to aggregate, an autocatalytic role for released ADP has been proposed in platelet aggregation, mural thrombogenesis and hemostasis(1,2). This concept has been further supported by studies showing that the rapid removal of ADP by enzymatic conversion reduces the response of platelets in an aggregometer to stimuli such as thrombin, collagen, serotonin and epinephrine(3,4). Similar enzymatic techniques have identified platelet-derived ADP to be important to normal platelet retention in glass bead columns(5) and in mural

thrombogenesis on subendothelium(6). Begent and Born(7), with flow experiments in vivo and Grabowski(8) with in vitro flow experiments have induced thrombus formation by controlled administration of ADP. While the above experiments suggest ADP may be important in platelet responses, the effect of platelet ADP at a surface under flowing conditions remains obscure.

The experiments presented here were designed to evaluate to what extent platelet ADP is important in determining the amount of platelet adhesion, total accumulation and release from adherent platelets upon a collagen-coated surface under flowing conditions. Two experimental approaches that specifically inhibit platelet activation by ADP are: the creatine phosphate/creatine phosphokinase system, which has been shown by others to be effective in reducing platelet aggregation induced by ADP in an aggregometer(3,4), was used. Second, exogenous adenosine triphosphate (ATP), an agent used by others to block ADP-induced platelet responses (9,10), was used. A lack of change in our results in the presence of these materials when compared with a control would indicate that platelet ADP was not a controlling factor in mural thrombogenesis.

5.2 Results

Human platelet suspensions were prepared using creatine phosphate/creatine phosphokinase in place of apyrase in all procedures (cf 2.1.2). Radioisotopic measurements of platelet accumulation and release of serotonin from adherent cells on a collagen-coated glass tube, after 600 s of exposure to flowing platelet/red blood cell

suspension, given in Table 5.1. The accumulation is given as the average number of platelets per unit surface area for the first centimeter, inlet, and last two centimeters of tubing, outlet. The average release of serotonin from adherent platelets for these tube segments is also given. Epi-fluorescent microscopic evaluation (cf 2.8) of the inlet section of a control tube showed large aggregates surrounded by platelet-free areas (Figure 5.1). At the inlet, some adhesion (surface-platelet bonding) existed but cohesion (platelet-platelet bonding) dominated. The accumulation of platelets decreased with distance from the inlet as has been previously shown (cf 4.2). Microscopy revealed the outlet segment to be predominantly platelet-surface bonding with individual platelets (Fig. 5.1). There was no difference in platelet accumulation or release from adherent platelets at the inlet or the outlet for either CP/CPK at 45 U/ml and 90 U/ml or ATP at 10^{-5}M, 10^{-4}M and 10^{-3}M when compared to controls. However, both CP/CPK at 45 U/ml and ATP at 10^{-3}M prevented the platelets from aggregating to 20 μM ADP as measured in an aggregometer (Figure 5.2). There was also generally more release at the outlet than at the inlet.

5.3 Discussion

Adenosine diphosphate has been suggested as a modulating material in mural thrombogenesis due to its release from platelets during adhesion and its platelet stimulatory properties(1,2). It is difficult to determine the concentration of ADP that could develop in the vicinity of a surface and so indirect methods have been employed to ascertain the

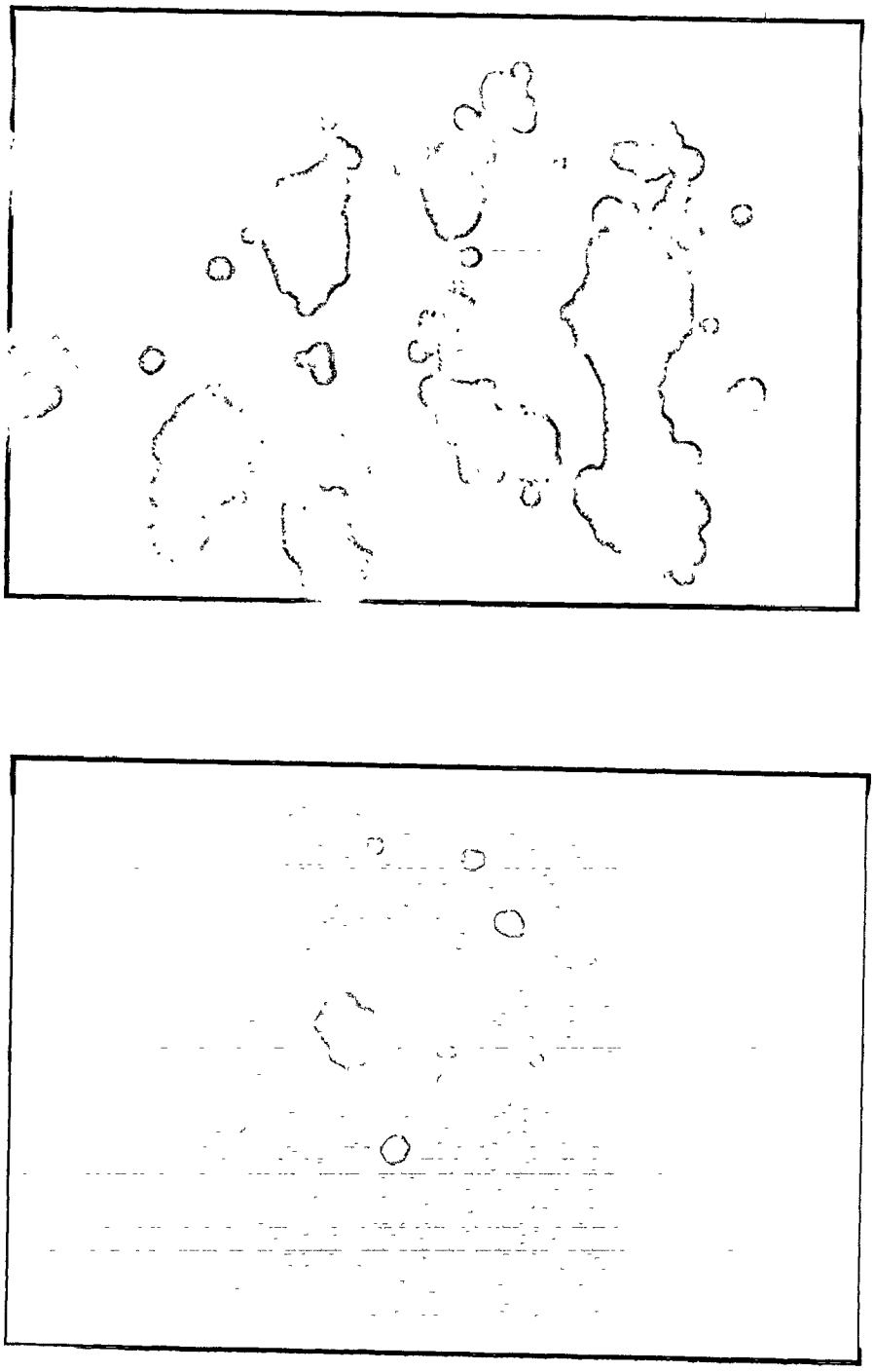


Figure 5.1: Photomicrographs of fluorescently labeled human platelets upon collagen-coated glass after 600 s of perfusion, platelets stained with mepacrine. On top, tube inlet; on bottom, tube outlet. The longer side of centrally located dotted crosshair is 7.5 μm long.

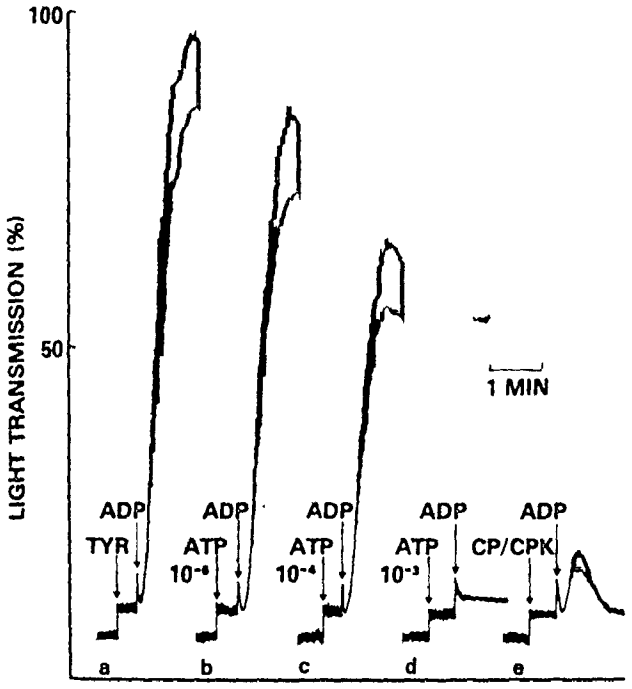


Figure 5.2: Inhibition of platelet aggregation by ATP and CP/CPK. The change in light transmission with time through a washed human platelet suspension prepared using CP/CPK at a final concentration of 1 unit/ml of CPK and 5 mM CP. The suspension contained 5 x 10^8 platelets/ml, no red blood cells and 50 µg/ml of human, DFP-treated fibrinogen. a) tyrodes (TYR), b) ATP to make a final concentration of 10^{-5} M, c) 10^{-4} M, d) 10^{-3} M, e) CP/CPK to make a final concentration of 45 units/ml of CPK and 5 mM of CP were added prior to the addition of ADP to make a final concentration of 20 µM. ATP inhibited platelet aggregation in a dose-dependent manner but not shape change. CP/CPK inhibited aggregation but not shape change.

TABLE 5.1: Human Platelet Accumulation on Collagen-Coated Glass Tubes

Treatment	Inlet[a] Platelets/ 1000 μm^2	Inlet[a] Release (%)	Outlet[b] Platelets/ 1000 μm^2	Outlet[b] Release (%)
ATP				
0 M	245.4 (34.9)	55.2 (5.6)	8.5 (2.0)	90.7 (1.9)
10^{-5} M	217.0 (29.5)[<.50]	47.2 (9.2)[<.50]	8.9 (1.1)[<.50]	91.2 (3.7)[<.50]
10^{-4} M	216.5 (16.0)[<.45]	50.0 (10.1)[<.50]	8.6 (2.1)[<.50]	90.2 (2.7)[<.50]
10^{-3} M	237.2 (33.1)[<.50]	64.1 (8.9)[<.45]	10.6 (3.0)[<.50]	91.5 (4.9)[<.50]
CP/CPK[c]				
1 U/ml	181.7 (12.6)	53.1 (3.4)	16.2 (4.0)	83.3 (3.2)
45 U/ml	186.8 (15.6)[<.50]	56.9 (3.8)[<.50]	20.7 (4.3)[<.50]	85.2 (4.0)[<.50]
90 U/ml	163.6 (14.7)[<.40]	61.2 (4.8)[<.25]	14.3 (3.7)[<.50]	93.2 (4.0)[<.10]

[a] Average for first centimeter of tube.

[b] Average for last two centimeters of tube.

[c] Creatine phosphate concentration 5.0 mM.

[d] mean (S.E.M.) [2P] t-test between means of control and treatment groups. n = 4 for ATP experiments, n = 3 for CP/CPK experiments.

role of ADP in platelet-surface interactions(7,8). In the present study, two procedures, designed to specifically prevent platelet aggregation by ADP, were used; the enzymatic conversion of ADP to ATP, (3,4,5) and competitive inhibition by exogenous ATP(9,10). The former treatment has been used by others to show the importance of released ADP in platelet aggregation by different stimuli (3,4,5). However the contribution of ADP in thrombin-induced platelet aggregation has recently been challenged(11).

The administration of CP/CPK did not alter platelet accumulation at the tube's inlet where aggregate formation predominates nor did it alter platelet accumulation at the outlet where platelet adhesion predominates. No significant difference in release of serotonin from deposited platelets was observed at either the inlet or outlet positions between control tubes and tubes exposed to CP/CPK-treated suspensions. The release values presented were determined for serotonin and are indicative of the release of other materials stored in the dense granules such as ADP(1,2). While CP/CPK removes ADP fast enough to prevent ADP-induced aggregation in a violently stirred cuvette such as used in aggregrometry studies, it may not be sufficiently fast to prevent released ADP from affecting platelets close to the surface in tube flow where a potentially slower convective-diffusion process for dispersal of released substances predominates (cf 1.2.4).

A second approach was to inhibit platelet aggregation to ADP stimulation by high concentrations of exogenous ATP(9,10). Platelets will become refractory to ADP-induced aggregation if exposed to low

concentrations of ADP. To remove trace amounts of ADP normally present in commercial ATP preparations, the ATP was dissolved in a CP/CPK-Tyrode buffer to allow any ADP present to be converted to ATP before addition to the platelet suspension. The exact mechanism of ADP-induced aggregation is not known (cf 1.2.2), but has been postulated to be through a membrane receptor that induces transport of calcium(12), or inhibits adenylate cyclase(13) or through an ecto-dinucleotide kinase enzyme that uses intracellular ATP for a phosphate donor(10). All of these proposed mechansims are consistant with the observed competitive inhibition of exogenous ATP on ADP-induced platelet aggregation(9). Exogenous ATP acts instantaneously and does not prevent aggregation to epinephrine, collagen or thrombin(9). Since the platelets were incubated with ATP prior to exposure to the tube, the platelets would not have responded to ADP released from platelets throughout the perfusion. ATP administration failed to alter platelet adhesion, platelet accumulation or release from control levels.

There were generally lower release values at the tube's inlet than at the outlet for all conditions suggesting that platelet-collagen adhesion stimulates more release than platelet-platelet cohesion. Since no procedures were undertaken to prevent reuptake of serotonin by deposited platelets, the measured releases are net values representing the initial release plus the amount taken back up (cf 4.2). It could be then that platelet-platelet cohesion allows for more reuptake than platelet-surface adhesion. It may also be that the serotonin released from platelets is trapped in the aggregate and is unable to be carried

away by the flowing blood.

The role of red blood cells in mural thrombogenesis has been suggested to be only physical(14,15), or both physical and chemical through enhanced transport of platelets to the tube surface and liberated ADP, due to hemolysis(16,17). Recent evidence suggests that high shear rates and high hematocrits are required to demonstrate red blood cell derived ADP effects(16). While red blood cells were present in the current study the shear rate was low, 80 s^{-1}. In a previous experiment at a higher shear rate, 320 s^{-1}, no significant ADP liberated from red blood cells could be detected in this system (Appendix B).

Tschopp and Baumgartner(12) added CP/CPK to citrated blood and concluded that removal of ADP strongly inhibited platelet thrombus formation on subendothelium but CP/CPK was only moderately inhibitory on collagen fibrils. (For human blood, a 95% reduction on subendothelium and a 37% reduction on collagen fibrils were reported.). While the differences between that study and the present study are numerous, it should be pointed out that the addition of citrate alone to human blood reduced thrombus volume and thrombus height on subendothelium(18) such that the joint stimuli of collagen on the surface and ADP released from platelets may be necessary for thrombus growth with citrate-containing blood. Although, thrombus growth has been induced, on surfaces not containing collagen, by controlled administration of ADP (7,8) the concentration levels attained may have been much above those provided by release from platelets. The present study found no reduction in platelet accumulation or release on a collagen surface when released ADP

was removed with CP/CPK or when the platelets were rendered unable to aggregate to ADP by exogenous ATP administration.

It should be noted that neither ATP nor CP/CPK prevented the shape change response of platelets even at 10^{-3} M ATP. Should the mechanism of promotion of thrombus formation by ADP be through the induction of platelet shape change, the procedures employed in this study would not demonstrate this component. It can be concluded that using a washed human platelet/red blood cell suspension, containing physiological levels of calcium and magnesium ions with no anticoagulant present, ADP does not modulate mural thrombogenesis by inducing aggregate formation prior to platelet accumulation. However ADP released from platelets may still contribute to mural thrombogenesis by inducing shape change prior to platelet collision with the adherent platelets on the surface.

Since the degree of release of serotonin and the platelet accumulation in the experiments in this chapter are the same as for the experiments in Chapter 4, the interfacial fluid concentration that would develop near the surface would be the same as previously calculated of 3.4 μM at the inlet. Procedures that block platelet shape change would determine if shape change is required prior to platelet collision with the surface for mural aggregates to form. Chapter 6 uses pharmacological procedures to probe the pathways of platelet activation other than ADP and the role of shape change in mural thrombogenesis.

5.4 Summary

Inhibition of the aggregation response of platelets to ADP did not prevent mural aggregate formation on a collagen-coated surface. The platelet shape change response to ADP released by adherent platelets may still be important but formation of platelet aggregates in the blood prior to deposition is not important in mural thrombogenesis.

CHAPTER 6

PLATELET ADHESION AND RELEASE: PHARMACOLOGICAL STUDIES

6.1 Introduction

In Chapter 3, the concentration of materials liberated by platelets at a site of deposition was estimated with ADP, thromboxane A_2 and prostaglandin D_2 being in sufficient concentration to affect platelet aggregation. In Chapters 4 and 5 the role of ADP in mural thrombogenesis was investigated. In this chapter, platelet functions were modified pharmacologically to study the contribution of the arachidonate pathway (cf 1.2.2), platelet shape change and thrombin formation to mural thrombogenesis.

Numerous inhibitory materials both pharmacological and naturally occuring have been documented to specifically inhibit one or other of the platelet activation pathways (cf 1.2.2) as measured in an aggregometer. While the basic activation pathways and biochemical modification of those pathways should not change in surface-induced thrombogenesis from those operating in an aggregometer, the dominant mechanisms may be different (cf 1.2.4).

Under flowing conditions, drugs that inhibit platelet functions such as aspirin and sulfinpyrazone, which prevent the formation of thromboxanes and prostaglandins, or prostaglandins I_2 and E_1 that raise intracellular cyclic adenosine monophosphate levels preventing platelet shape change and aggregation, or creatine phosphate/creatine

phosphokinase (CP/CPK) that converts ADP to adenosine triphosphate, have all been reported to prevent thrombus formation on subendothelium (1,2,3). All these results which demonstrate the effects of drugs on thrombogenesis were obtained with the annular perfusion system of Dr. Baumgartner's group using blood anticoagulated with sodium citrate(1,2,3). Baumgartner(1) recently reported that aspirin does not inhibit thrombus formation on subendothelium when heparinized or unanticoagulated rabbit blood is used, sheding some doubt on the earlier work.

In the present study, washed human platelet/red blood cell suspensions that contain physiological levels of calcium and magnesium have been used to study the effects of drugs that alter platelet function on platelet-collagen interactions under flowing conditions. Platelets were treated with imipramine, dipyridamole, prostaglandin E_1, hirudin, heparin, sulfinpyrazone, indomethacin, aspirin, mepacrine and CP/CPK. The platelet adhesion, total platelet accumulation and release of serotonin from the dense granules of platelets adherent to a collagen-coated tube were measured. The results were interpreted so as to establish the role of materials released and liberated by platelets in mural thrombogenesis.

6.2 Results

The number of platelets associated with the surface of a collagen-coated glass tube (cf 2.3) after 600 s of perfusion (cf 2.5) to platelet/red cell suspensions (cf 2.1.1) varied with position along

the tube such that the logarithm of the distance from the inlet (z) and the logarithm of the platelet accumulation were directly proportional (Figure 6.1). The slopes of the lines presented in Figure 6.1 are the power-law exponents (m) in the equation, platelet accumulation α z^m. For the treatments studied Table 6.1 gives the power-law exponent as determined by non-linear regression (column D), the average platelet accumulation for the first centimeter of tube length, inlet, (column E) and the average release from deposited platelets over this segment (column F), as well as the average platelet accumulation for the last two centimeters of tube length, outlet, (column G) and the average release from deposited platelets for this segment (column H).

For a control experiment after 600 s of perfusion, Figure 5.1 shows the distribution of platelets on the surface at the inlet of the tube. Kinetic studies (cf Chapter 8) have shown that platelet aggregates formed on the surface in the inlet region of the tube within the first few minutes of perfusion and continued to grow in size as platelet accumulation continued. The bulk of the final platelet accumulation at the inlet, after 600 s of perfusion was due to aggregate formation as shown by the platelet accumulation levels of 237 platelets/1000 μm^2 (Table 6.1, column E, line 1). The level of platelet accumulation required to cover the surface with platelets would be between 65 and 100 platelets/1000 μm^2, (cf Chapter 8). For the control experiments Figure 5.1 shows the distribution of platelets on the surface at the outlet section, accumulation level = 6 platelets/1000 μm^2, where only single platelets were present after 600 s of perfusion.

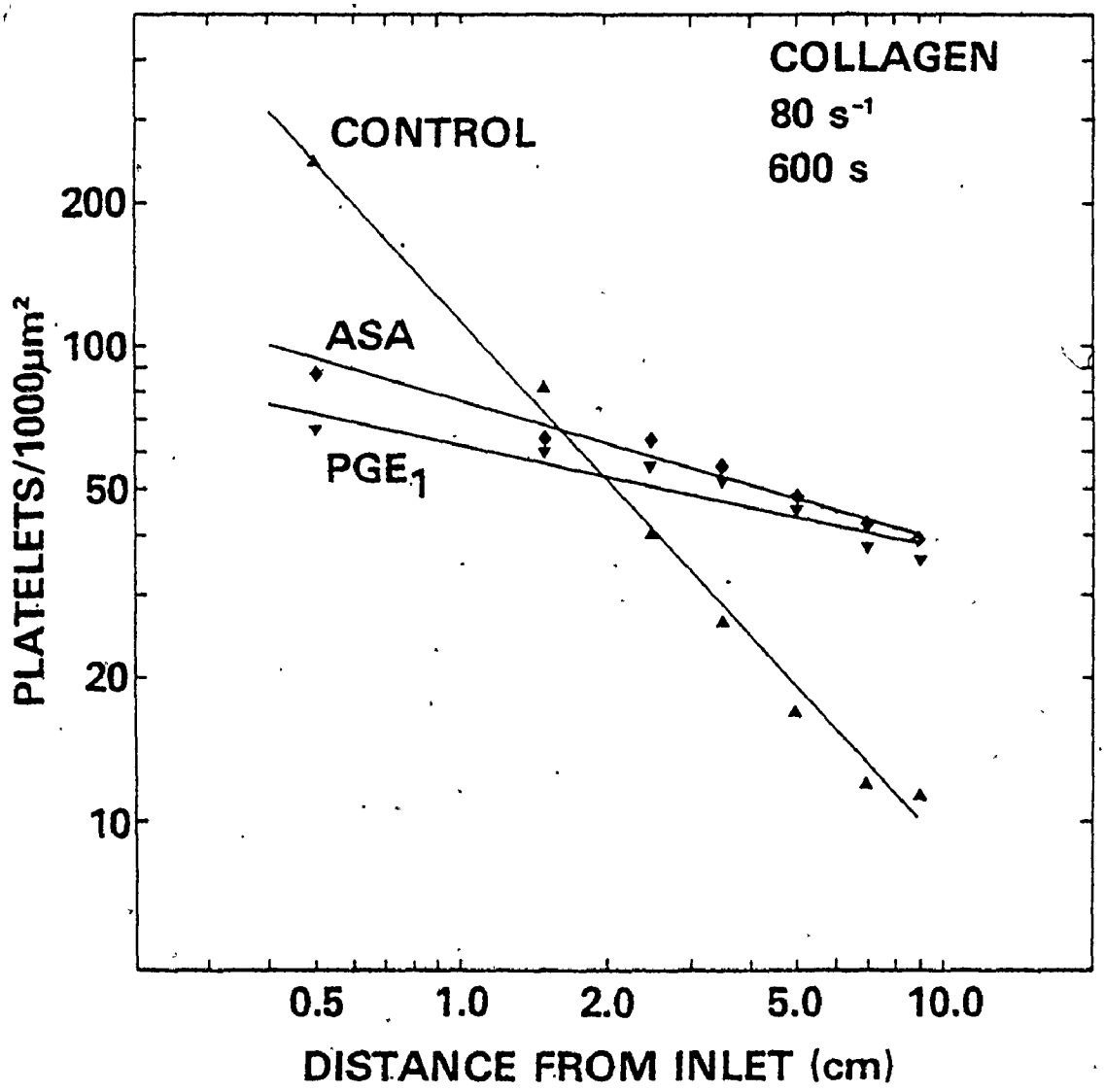


Figure 6.1: Platelet accumulation (PA) on a collagen-coated glass tube after 600 s of perfusion with washed human platelet/red blood cell suspension at a surface shear rate of 80 s^{-1}. Individual data points are means of 51 determinations for the control case, 10 determinations for the PGE_1 case and 16 determinations for the ASA case. The continual decrease in platelet accumulation with distance from the inlet(z) is demonstrated by the straight lines that were determined by regression of the relationship PA = constant $(z)^m$ the slopes of the lines are the power law exponent, m, presented in Table 6.1.

Table 6.1: Human Platelet Accumulation and Release on Collagen-Coated Glass Tubes

Treatment	Conc (μM)	n	m	Inlet		Outlet	
				Platelets/ 1000 μm^2	Release (%)	Platelets/ 1000 μm^2	Release (%)
A	B	C	D	E	F	G	H
1.control	100	58	-1.16(.07)	237(18.4)	48.8(4.4)	11.0(1.8)	83.5(3.8)
Serotonin uptake inhibitor							
2.imipramine	2	10	-1.16(.16)	210(45.5)	70.7(8.3)[b]	11.0(4.8)	91.1(3.3)[c]
Shape change inhibitors							
3.dipyridamole placebo	-	3	-1.18(.38)	248(116.0)	47.5(10.2)	10.3(5.7)	89.4(7.3)
4.dipyridamole	100	8	-.73(.17)[c]	141(34.2)[c]	73.3(10.0)[b]	18.6(4.2)[a]	90.1(4.4)[a]
5.PGE_1	0.1	7	-.23(.08)[c]	65.0(18.1)[c]	83.6(10.2)[c]	35.0(3.8)[c]	86.1(4.3)

Table 6.1 (cont'd)

Treatment	Conc (μM)	n	m	Inlet		Outlet	
				Platelets/ 1000 μm^2	Release (%)	Platelets/ 1000 μm^2	Release (%)
A	B	C	D	E	F	G	H
Thrombin inhibitors							
6.hirudin	4 U/ml	3	-1.10(.25)	228(121.1)	66.1(19.8)	11.9(3.5)	89.2(9.3)
7.heparin	10 U/ml	7	-1.10(.15)	169(25.8)[c]	55.0(21.6)	9.2(4.5)	86.1(5.3)
Inhibitors of arachidonate metabolism							
8.sulfinpyrazone placebo	-	4	-1.12(.10)	222(55.4)	60.4(22.8)	11.9(6.6)	83.2(9.0)
9.sulfinpyrazone	200	6	-.73(.06)[c]	161(20.8)[c]	70.2(16.4)[c]	20.0(1.9)[c]	92.7(8.7)
10.sulfinpyrazone	1000	6	-.38(.18)[c]	97.4(41.3)[c]	85.8(13.3)[c]	32.3(11.3)[c]	89.1(8.7)
11.indomethacin	100	3	-.31(.09)[c]	88.1(13.0)[c]	90.3(6.6)[c]	36.5(5.3)[c]	89.1(4.8)

Table 6.1 (cont'd)

Treatment	Conc (μM)	n	m	Inlet		Outlet	
				Platelets/ 1000 μm^2	Release (%)	Platelets/ 1000 μm^2	Release (%)
A	B	C	D	E	F	G	H
Inhibitors of arachidonate metabolism (cont'd)							
12.ASA	200	16	-.36(.11)[c]	85.1(10.7)[c]	80.2(4.4)[c]	33.0(6.4)[c]	85.7(2.3)
13.mepacrine	50	7	-1.05(.17)	221(30.9)	60.6(12.1)	13.8(5.2)	87.2(3.3)
Combinations of inhibitors							
14.ASA + mepacrine	200+50	4	-.17(.10)[c]	65.1(10.4)[c]	83.6(12.3)[c]	39.7(11.6)[c]	82.4(9.1)
15.ASA + CP/CPK*	200+90U/ml	4	-.30(.03)[c]	69.8(12.7)[c]	82.0(4.7)[c]	32.2(4.2)[c]	83.6(2.1)

mean (± 95% confidence interval)

a) significantly different from the control 2P <.05

Table 6.1 cont'd

b) 2P < .01

c) 2P <.001

* CP concentration 5.0 mM. Inlet values are averages for the first segment of the outlet tube values are averages for the last two centimeters of the tube, m is the least squares power law exponent.

The dipyridamole placebo, sulfinpyrazone placebo, mepacrine, hirudin and heparin (cf 2.7) gave the same results as the controls for all parameters measured (Table 6.1, lines 1, 2, 3, 6, 7, 8, 13) except for the decreased accumulation on the inlet section using heparin treatment (Table 6.1, line 7, column E). Imipramine treatment (Table 6.1, line 2) did not alter the platelet accumulation at the inlet or the outlet as compared with untreated controls. Imipramine treatment did increase percent release values at both the inlet and the outlet sections as compared with untreated controls (Table 6.1, line 2, columns F and H).

Indomethacin, sulfinpyrazone, ASA, PGE_1, dipyridamole, ASA + mepacrine, and ASA + CP/CPK all reduced platelet accumulation at the inlet, increased platelet accumulation at the outlet and reduced the power law exponent as compared with untreated controls. These treatments also gave increased release from platelets accumulated at the inlet (Table 6.1). Only imipramine gave significantly higher release values than controls at the outlet (Table 6.1 line 2 vs line 1, column H). After 600 s of perfusion, Figures 6.2 and 6.3 show the distribution of platelets at the inlet region of a collagen-coated glass tube for PGE_1 and ASA treatments. An evenly distributed layer of platelets that had a rounded spread morphology existed on the collagen-coated surface using PGE_1-treated platelets. ASA-treated platelets were psuedopodic and clustered together on the collagen-coated surface.

The treatment of platelets with combinations of ASA + mepacrine

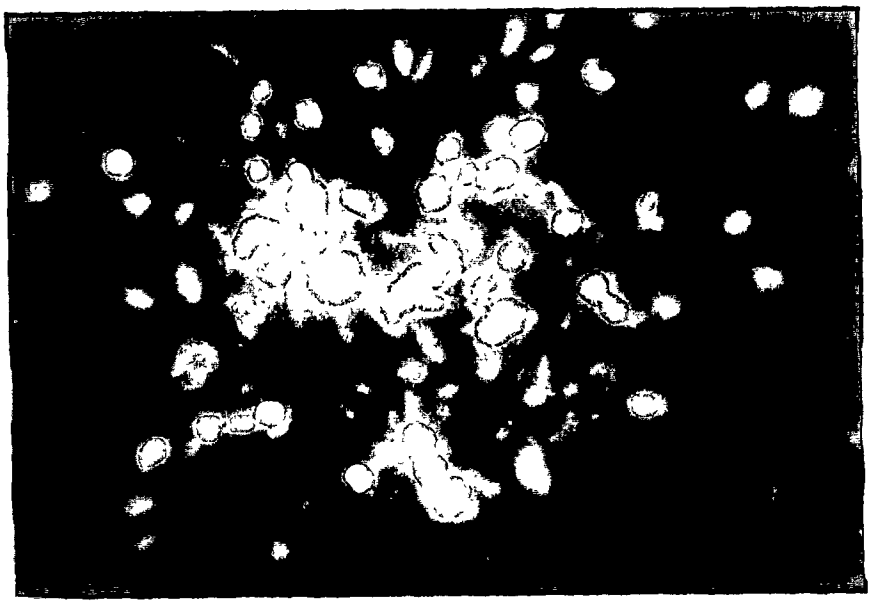


Figure 6.2: Photomicrograph of PGE_1-treated, human platelets adherent to the inlet of a collagen-coated glass tube after 600 s of perfusion at a surface shear rate of 80 s^{-1}. Platelets were fluorescently labeled after the perfusion by including 50 μM mepacrine in the rinsing solution, enabling both cytoplasmic and granule platelet constituents to be seen. Platelets are round, non-psuedopodic individual cells with centralized bright spot which is probably the reminants of dense granules. The long axis of the cross-hair in the center of the photomicrograph is 7.5 μm long.

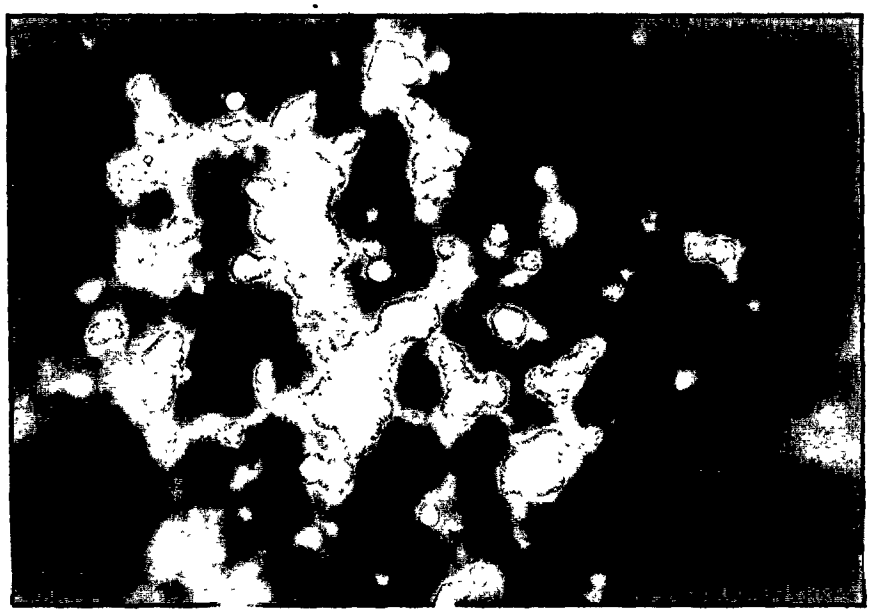


Figure 6.3: Photomicrograph of ASA-treated platelets adherent to the inlet of collagen-coated glass tube after 600 s of perfusion at a surface shear rate of 80 s^{-1}. Platelets were fluorescently labeled after the perfusion by including 50 μM mepacrine in the rinsing solution, enabling both cytoplasmic and granule constituents to be seen. Platelets are psuedopodic spread cells that adhered to the surface close to another forming clusters with platelet-free surface areas between clusters. The long axis of the cross-hair in the center of the photomicrograph is 7.5 μm long.

($2P < 0.01$) and ASA + CP/CPK ($2P < .05$) gave significantly decreased platelet accumulation at the inlet of a collagen-coated tube as compared with ASA treatment of platelets (Table 6.1 column E lines 14 and 15 vs line 12). These combinations did not change any other parameters measured as compared to ASA treatment alone.

6.3 Discussion

6.3.1 Platelet Accumulation

The goal of this work was to study the role of materials that are liberated from platelets during thrombus formation at a surface. Previous chapters in this thesis have suggested that high concentrations of platelet-derived materials could develop near a surface of a blood vessel or a synthetic material. Which of these materials is most important for mural thrombogenesis remains obscure. Studies using aggregometers to probe platelet aggregation and release in suspensions have identified at least three pathways to stimulate platelets, the ADP pathway, the arachidonic acid pathway and a third (or more) as yet undefined pathway (cf 1.2.2). In an aggregation cuvette, collagen-induced aggregation is thought to be initiated by a small fraction of platelets binding to the added collagen fibrils and emitting substances. The emitted substances that stimulate other platelets to aggregate have been identified as ADP and an arachidonic acid metabolite, probably thromboxane A_2(4). Procedures that block arachidonic acid metabolism or remove released ADP, reduce aggregation response to collagen and in combination prevent aggregation induced by collagen(4).

The contention that released ADP is important in thrombin-induced platelet aggregation has recently been challenged(5). The pathway of platelet aggregate formation that is dominant in surface-induced thrombus formation may not be the same as that in platelet aggregation in a cuvette. For example, the ADP pathway does not appear to be important in mural aggregate formation, as neither rapid removal of ADP by creatine phosphate/creative phosphokinase (CP/CPK) conversion nor pretreatment of platelets with adenosine triphosphate, inhibit thrombus formation on a collagen surface, although these procedures inhibit ADP-induced aggregation in an aggregometer (cf Chapter 5).

Figure 6.1 demonstrates the continuous nature of the decrease in platelet accumulation with distance distal to the inlet. The power law exponent (m) is an excellent measure of overall platelet-surface interactions. Those treatments that lowered the inlet platelet accumulations (Table 6.1 lines 4, 5, 9-12, 14, 15 column E) were consistantly associated with increased platelet accumulation at the outlet (Table 6.1 lines 4, 5, 9-12, 14, 15, column G). While the exact reason for this is unclear, it could be that those platelets which failed to adhere at the inlet remained close to the surface adhering downstream (see also 4.3.1).

6.3.1.1 Platelet Adhesion

Cazenave et al. found no reduction in platelet adhesion using ASA-treated platelets on collagen-coated glass(6,7). No reduction in platelet adhesion to subendothelium using ASA, sulfinpyrazone or

dipyridamole treatments have been reported by others(4). PGE_1, PGI_2 and indomethacin have been reported to slightly reduce, platelet adhesion to subendothelium and collagen-coated glass(7).

The outlet levels of platelet accumulation (Table 6.1, column 6) are a measure of platelet adhesion as only singleton platelets were adherent in this region of the tube (Figure 5.1). None of the treatments reduced platelet adhesion at the outlet. In addition, platelet accumulation levels at the inlet of 65 platelets/1000 μm^2 represent an evenly dispersed layer of adherent platelets (cf 8.2). No treatment reduced platelet accumulations at the inlet below this level. Both these results suggest that platelet adhesion was not impaired by any of the treatments studied.

6.3.1.2 Cluster formation

The process of platelet accumulation on a collagen-coated surface using untreated platelets began with adhesion of individual platelets randomly of the surface. After a few minutes, platelets then preferentially adhere to the surface beside the already adherent platelets forming clusters. As the accumulation continued, platelet adhered to already adherent platelets, thus the clusters grew into aggregates with platelet-free areas between aggregates. ASA-treatment prevented aggregate formation (Table 6.1, line 12, column E) but not cluster formation (Figure 6.3). PGE_1 treatment prevented aggregate formation (Table 6.1, line 5, column E) and cluster formation (Figure 6.2). In addition platelets on a collagen-coated tube were psuedopodic

when untreated or treated with ASA but were rounded when treated with PGE_1. The exact relationship between psuedopod formation and cluster formation remains obscure.

6.3.1.3 Mural aggregate formation

The platelet accumulation levels at the inlet (Table 6.1 column E) are a measure of thrombus formation on the collagen-coated glass tubes. Hirudin was used in the present study to rapidly inactivate any thrombin that may have formed on the platelet surface due to residual plasma components on the platelet surface. No difference between controls and hirudin-treated platelets suggest that thrombin formation did not occur in this system (Table 6.1, line 6, vs line 1). Heparin plus antithrombin III also inhibit thrombin rapidly but were not used as platelets contain proteins with antiheparin activity that would make interpretation of these results difficult(8). Heparin addition decreased the platelet accumulation at the inlet slightly (Table 6.1, column E, line 7, vs line 1). Since no antithrombin III was added, the heparin may have antithrombotic properties exclusive of its anticoagulant activity. Heparins are polydisperse materials having a wide range of molecular weights. More monodisperse subfractions of heparin have different anticoagulant activities and may have different activities towards platelets as well(9).

Studies using the annular perfusion chamber of Baumgartner with citrated rabbit blood have demonstrated ASA and sulfinpyrazone greatly reduced thrombi on subendothelium and increased platelet adhesion

slightly(1). However ASA and sulfinpyrazone have no effect on thrombi volume or number of thrombi or platelet adhesion in the same perfusion system when tested using unanticoagulated or heparinized rabbit blood(1) ex vivo. Aspirin similarly has no effect on platelet accumulation on denuded subendothelium in vivo(10). In the present study, ASA, sulfinpyrazone and indomethacin all reduced platelet accumulation at the inlet of the tube, (Table 6.1, column E) which is a measure of platelet thrombus formation. The selective inhibition of large aggregate growth by ASA, sulfinpyrazone and indomethacin is reminiscentof the abilities of these drugs to prevent second phase aggregation in citrated, platelet-rich plasma. These drugs are known to prevent formation of prostaglandin G_2, prostaglandin H_2 and thromboxane A_2 which are metabolites of arachidonic acid and are also potent platelet stimulants (4). The inhibition of platelet accumulation at the inlet of a collagen-coated tube by these drugs suggest that some arachidonic acid metabolite is required for thrombogenesis in a washed platelet system.

Mepacrine inhibits arachidonate liberation from platelets in suspension (cf 1.3.2.6). Mepacrine also inhibits fibrinogen uptake by washed human platelets, a necessary antecedent for platelet aggregation (cf 1.3.2.6). It therefore was surprising that mepacrine had no effect on platelet-surface interactions as compared with controls for all parameters studied here (Table 6.1, line 13 vs. line 1). Suspensions of washed human platelets will not aggregate to ADP without readdition of fibrinogen(4). They will aggregate to thrombin, presumably due to the release of platelet fibrinogen stored in the alpha granules(4).

Mepacrine diminishes platelet aggregation to thrombin but does not abolish it(11). These results suggest that either some other material was released from platelets that supports platelet cohesion or that mepacrine was unable to prevent uptake of fibrinogen released from alpha granules of adherent platelets. The ability of ASA, sulfinpyrazone and indomethacin to prevent thrombus formation indicates some arachidonic acid metabolite was required for platelet aggregate formation on a collagen-coated surface. Once more specific inhibitors of the various stages of arachidonic acid metabolism and methods to measure dense-granule release from adherent platelets become available, more precise location of the dominant materials will be possible.

Prostaglandin E_1, prostaglandin I_2 and dipyridamole are non-specific inhibitors of platelet functions that work through raising intracellular cyclic adenosine monophosphate levels(10). Weiss and Turitto(2) have shown that PGI_2 prevents thrombus formation on subendothelium using citrated human blood. Baumgartner was unable to reduce rabbit platelet thrombi using administration of dipyridamole(1). In the present study, PGE_1 greatly reduced platelet accumulation at the inlet to a level of 65.1 ± 18.1 platelets/1000 μm^2 (mean ±95% confidence interval), (Table 6.1, column E, line 7, vs line 1) which probably represents the level of platelet accumulation possible when platelet cohesion is prevented. The distribution of PGE_1-treated platelets on the collagen-coated surface (Figure 6.2) present at the inlet was the same as that attained on a fibrinogen or fibronectin-coated surfaces which do not support mural aggregate formation (cf 7.2, 8.2).

Dipyridamole reduced platelet accumulations at the inlet of the tube slightly indicating it is a weak inhibitor of platelet aggregate formation.

Combinations of inhibitors such as ASA + mepacrine and ASA + CP/CPK gave significantly reduced platelet accumulations as compared with ASA-treatment alone (Table 6.1, column E, line 14 vs line 12, $2P < 0.01$, line 13 vs line 12, $2P < 0.05$). The final platelet accumulations for the combined drug treatments were the same as that for PGE_1-treatment suggesting that combinations of drugs reduced the small amount of platelet cohesion still present with ASA-treatment alone. Since mepacrine inhibits arachidonate liberation from membrane phospholipids perhaps some arachidonate metabolite other than those in the ASA-sensitive pathway works in cluster formation. Alternately, since CP/CPK was designed to remove ADP, perhaps ADP activation of platelets was required to increase platelet accumulation above the 65 platelets/1000 μm^2 observed in this study.

6.3.2 Release

The release of dense-granule serotonin from deposited platelets was 82-92% for all the treatments at the outlet. For those treatments that blocked thrombus formation at the inlet, (Table 6.1, lines 4, 5, 9-12, 14, 15); a similar release of between 80 to 90% was found. Baumgartner et al(3) reported 92% disappearance of dense granules from platelets adherent to subendothelium. Cazenave et al. has reported 60% release of serotonin from human platelets(7) and Whicher et al. have

reported 49% release from porcine platelets adherent to collagen-coated glass(12). ASA-treatment of platelets has been reported not to prevent release of dense granules from platelets adherent to collagen-coated glass(10) or subendothelium(13). PGE_1 and PGI_2 treatments have been reported not to inhibit release of serotonin from adherent human platelets(7). Clearly platelet-collagen adhesion resulted in maximal release of dense-granule materials even in the presence of agents that prevent platelet aggregation in suspensions and thrombus formation on a collagen surface. Those treatments that failed to inhibit aggregate formation (Table 6.1, lines 1, 2, 3, 6, 7, 8, 13) had reduced release of serotonin at the inlet as compared with the outlet. Platelets adherent to the collagen-coated tube released extensively and those in the mural aggregate probably released slightly less resulting in an average release at the inlet less than at the outlet where only adherent platelets were present. Imipramine treatment resulted in significantly higher release values at the inlet and outlet as compared with controls, although the inlet release value was still less than the outlet release value (Table 6.1 line 5 column E vs column H). Since imipramine inhibits uptake of serotonin by platelets, the release values in the controls and also the treatment cases (except for the imipramine experiments) were net values from initial release and re-uptake. The differences between the release values using imipramine and the other conditions however were slight.

6.3.3 Conclusions

It can be hypothesized, based on the results in this study, that thrombogenesis proceeds through the liberation from adherent platelets of thromboxanes or prostaglandins that move away from the collagen-coated surface inducing incoming platelets to change shape in preparation for cohesion upon contact with adherent platelets. Only those drug treatments that are known to prevent shape change by raising cyclic adenosine monophosphate levels in the platelet or are known to prevent the formation of prostaglandins or thromboxanes were found to inhibit thrombus formation. The observations that PGE_1-treated platelets did not form thrombi, clusters, or psuedopodia on collagen-coated glass while ASA-treated platelets did form clusters and psuedopodia suggests that psuedopodia may be required for platelet cohesion.

A second hypothesized mechanism of thrombogenesis must be considered and is that those drug treatments which inhibited prostaglandin and thromboxane formation or raised cyclic AMP levels within a platelet also prevented the release of alpha-granule substances from adherent platelets. The release of serotonin from the dense granules of adherent platelets was measured and found to be near maximal in all cases studied. Baumgartner et al(3) have reported both alpha-granule and dense-granule release from platelets adherent to subendothelium and Weiss et al.(13) have noted total degranulation of ASA-treated platelets adherent to subendothelium. The measurement of the release of dense granules from adherent platelets does not

implicitly prove that release of alpha granules occurred. The two mechanisms of thrombogenesis must then be tested by measuring the effects of drug treatments on the release of alpha-granule substances from platelets adherent to collagen and the metabolism of arachidonic acid by these adherent platelets. A note of caution should be inserted here since this work was conducted in washed platelet/red blood cell suspensions, in vitro. Plasma components such as thrombin may well be involved in platelet activation, in vivo.

6.4 Summary

Washed human platelet suspensions were used to probe the role of platelet-derived materials in platelet-surface interactions. Imipramine inhibited reuptake of serotonin increasing measured release slightly. Heparin may have antithrombotic properties exclusive of its anticoagulant activity. Thrombin is not important in this washed platelet system. Pharmacological agents that inhibited prostaglandin and thromboxane formation or generally inhibit platelet function prevented thrombus formation but not platelet adhesion or release of dense-granule serotonin from platelets deposited on collagen-coated glass.

CHAPTER 7

PLATELET INTERACTIONS WITH PLASMA PROTEINS COATED ON GLASS TUBES

7.1 Introduction

The previous four chapters have used platelet accumulation in a collagen-coated surface to probe the relationships between platelet-derived materials and platelet-surface interactions. None of the pharmacologic and biologic procedures used in these chapters resulted in a decreased degree of release of dense-granule materials from deposited platelets. However, other surfaces may have different degrees of stimulation of deposited platelets enabling the release to vary independent of flow rate and platelet accumulation rate. In this chapter, albumin, fibrinogen, fibronectin and glass surfaces were studied using the radioisotopic technique used in the previous chapters and a new microscopic technique.

The exposure of synthetic materials to blood results in the rapid adsorption of plasma proteins and adhesion of blood platelets(1,2). Albumin protects against platelet adhesion(3,4,5,6) while gamma-globulin(3,4), fibrinogen(3,4,5), fibronectin(7) and collagen support adhesion. Platelet accumulation on surfaces has been studied by a number of groups(1,2) who have identified contact, spreading, thrombus formation, release of granule contents, embolization, non-adhesive encounters and reattachment of emboli as surface-platelet phenomena. A

knowledge of the type and degree of platelet-surface interaction on various surfaces is required to have an understanding of the mechanisms of thrombosis, as well as to to enable non-thrombotic biomaterials to be rationally developed. To define on-going events such as embolization, non-adhesive encounters and translocation of aggregates requires techniques to monitor platelet-surface dynamics continuously. Accordingly, an epi-fluorescent video microscopy was used to evaluate platelet-surface phenomena on a range of protein surfaces under well defined flow conditions. This dynamic information, coupled with the platelet accumulation and degree of release from adherent platelets obtained radioisotopically enable platelet responses to the protein surfaces studied to be accurately defined and the role of materials released from platelets in each stage of thrombogenesis to be studied.

7.2 Results

7.2.1 Collagen

The pattern of human platelet accumulation on a collagen-coated glass surface (cf 2.3) after 600 s of exposure to cell suspension (cf 2.1.1) as a function of distance distal from the inlet is shown in Figure 7.1. The platelet deposition decreases with distance in an exponential fashion. The exponent obtained for a power law relationship between accumulation and distance from the tube's inlet was -1.14 ± 0.21 (mean $\pm$ S.D., $r^2 = .98$, $n = 39$). (The equation used was accumulation = const. x (distance from tube's inlet)^m; m is the power law exponent.) The release of serotonin from accumulated platelets was $50.3\% \pm 13.2\%$

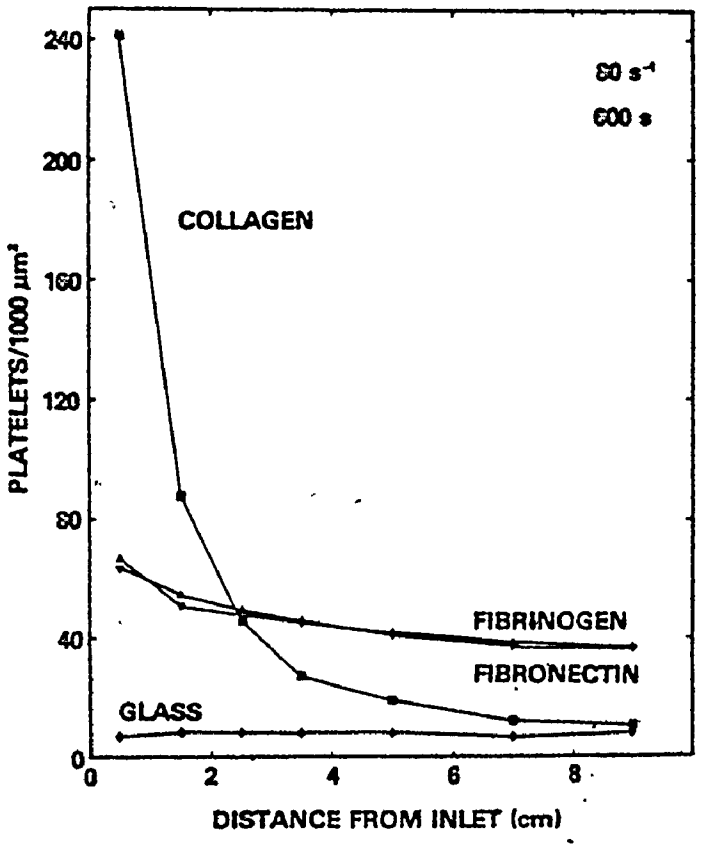


Figure 7.1: Platelet accumulation versus distance from the inlet of 1.3 mm I.D. tubes. Surface shear rate 80 s^{-1}, exposure time 600 s. Standard deviations for all data are less than 12% of the measured value.

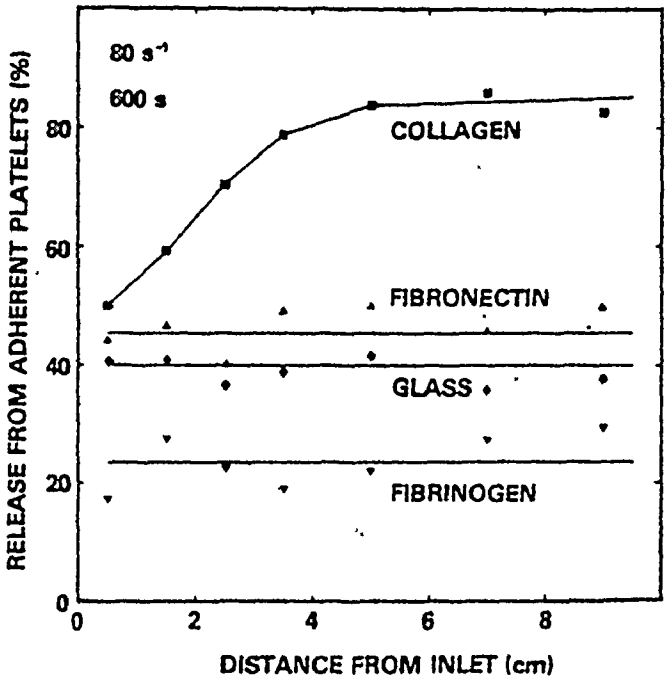


Figure 7.2: Percent release of ^{3}H-serotonin versus distance from the inlet for platelets adherent to 1.3 mm I.D. tubes. Surface shear rate 80 s^{-1}, exposure time 600 s. Standard deviations for all data are less than 12% of the measured value.

(mean ± S.D.) at the inlet and 82.4% ± 9.8% (mean ± S.D.) at the outlet of the tube (Figure 7.2).

Epi-fluorescent microscopy (cf 2.8) was conducted using platelets that were labeled with mepacrine. Since mepacrine was used as a fluorescent label (cf 2.8) the effect of this drug on platelet-surface interactions was evaluated. There was no difference between mepacrine-treated and untreated platelets with respect to platelet accumulation or release from platelets on the collagen surface (Table 7.1). A typical analysis of a video tape recording is shown in Figure 7.3 where the arrival time for platelets adhering 3 cm from the inlet was determined. The solid line in Figure 7.3 connects the origin with the platelet accumulation level after 600 s of perfusion as measured by ^{51}Cr radioactivity. The video system could not be used quantitatively to measure rates of platelet accumulation after 5 minutes on this surface as too many platelets adhered to growing aggregates which were now partially out of the plane of focus.

Platelets arrived individually at all times. The first few platelets adhered with no apparent specific pattern on the collagen surface. Subsequent platelets preferentially adhered beside already deposited platelets forming clusters. These clusters grew into large aggregates, 20-30 μm in diameter at their base (Figure 7.4a). The aggregates, at their bases did not appear to be aligned in any way with the direction of flow. The aggregates were largest at the tube's inlet covering almost the entire surface but some platelet-free areas between aggregates were still present. Clusters of 5 to 10 platelets that

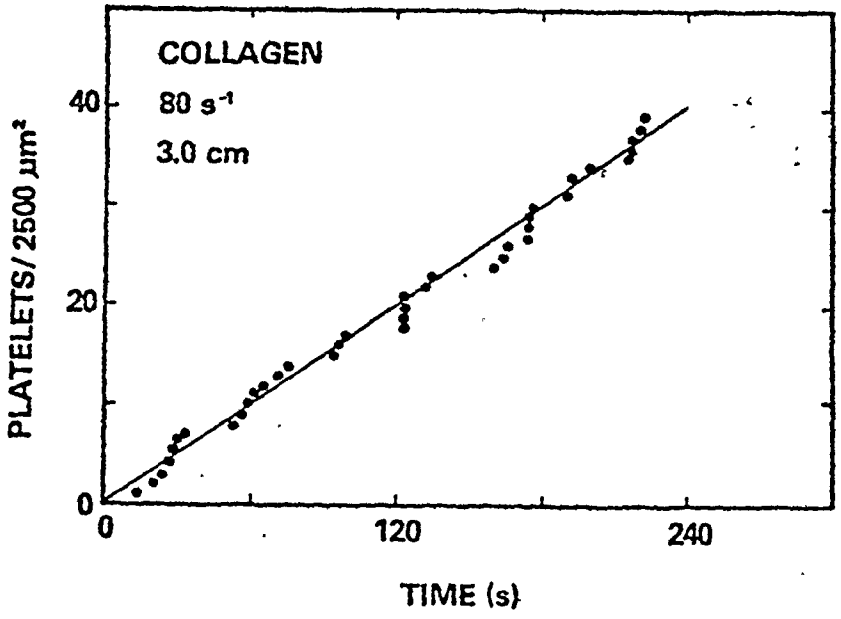


Figure 7.3: Points are deposition of individual platelets as determined by videomicroscopy. Straight line determined via ^{51}Cr labeling. collagen surface, 3.0 cm from tube's inlet, surface shear rate 80 s^{-1}.

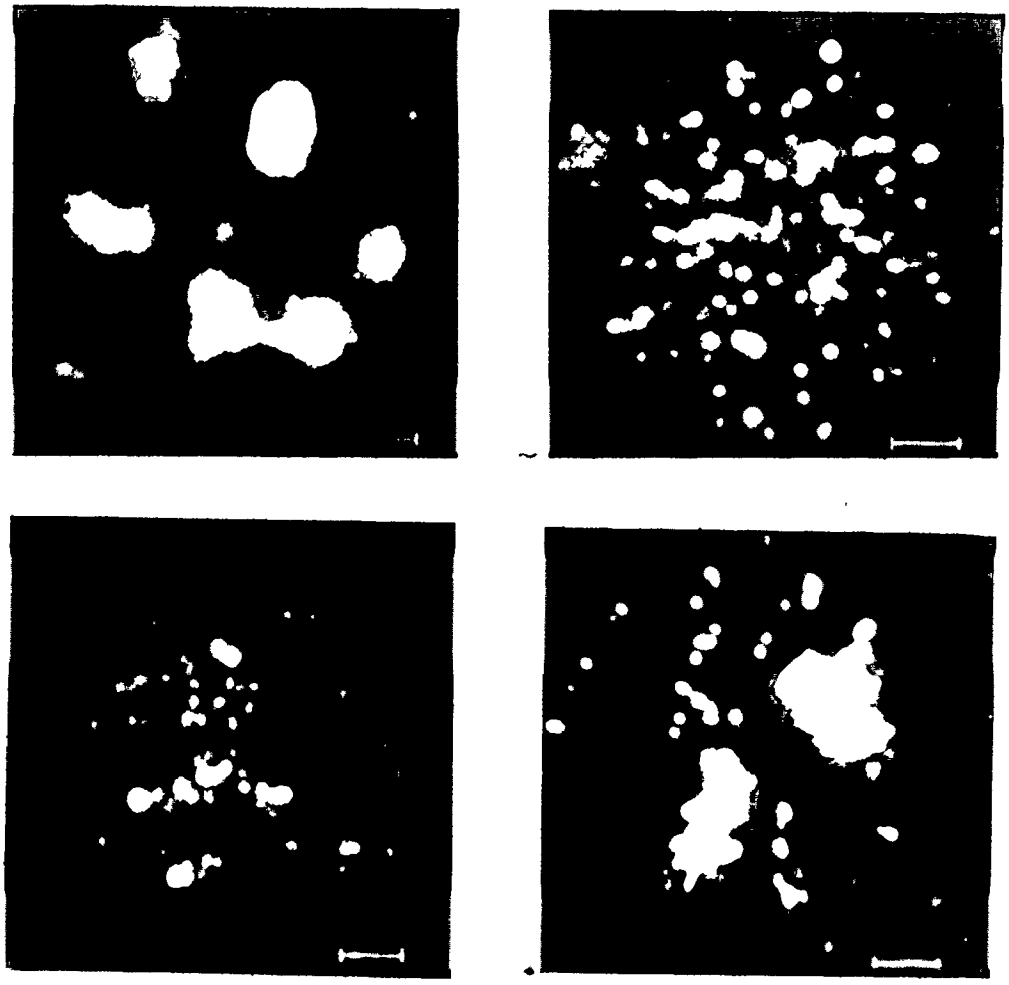


Figure 7.4: Fluorescently labeled platelets at a position 3 cm from the tube's inlet after 600 s of perfusion on (a) collagen, (b) fibrinogen, (c) fibronectin and (d) glass. The long axis of the centrally located cross-hair was 7.5 μm long.

adhered beside one another on the surface were present at the exit of the tube after 600 s of perfusion. No platelets were observed to embolize from the collagen surface once they contacted the surface. The definition of "contact" was taken as any platelet remaining stationary for greater than 33 msec (i.e., the time for one frame on the television). Once clusters began to grow, few platelets contacted the sparcely covered regions between clusters. Cluster growth appeared to occur outward from the first adherent platelet.

7.2.2 Fibrinogen

The platelet accumulation on a fibrinogen-coated glass surface after a 600 s perfusion is shown in Figure 7.1. The power law exponent between accumulation and distance from the inlet was -0.24 ± 0.18 (mean $\pm$ S.D., $n = 16$, $r^2 = .94$). The release of granule serotonin from adherent platelets was $23.7\% \pm 4.7\%$ (mean $\pm$ S.D.) for all positions along the tube (Figure 7.2). Video microscopy revealed an increase in platelet accumulation with time at the 3.0 cm position up to 600 s. The platelets arrived individually with no apparent organized distribution on the surface. As the accumulation increased platelets adhered only to the platelet-free areas. For observations up to 180 s, the process was one in which approximately one half of those cells that had contacted the surface remained. For those cells which embolized, a distribution of residence times upon the surface was found. This distribution reached a maximum at approximately 250 msec with 96% of cells that ultimately embolized did so after less than 500 msec of residence time

upon the surface. After 600 s of perfusion, the surface of the tube was covered with single platelets (Figure 7.4b).

Since fibrinogen is contaminated with fibronectin it could be possible that it is the fibronectin fraction within the fibrinogen that platelets are binding to. Fibronectin-depleted fibrinogen gave the same platelet accumulation and release results as the parent fibrinogen (Table 7.1).

7.2.3 Fibronectin

The platelet accumulation on a fibronectin-coated glass tube after 600 s of perfusion is presented in Figure 7.1. The accumulation was similar to that observed on the fibrinogen-coated glass surface with the inlet levels twice the outlet levels. The power law exponent between platelet accumulation and axial position was -0.23 ± 0.19 (mean $\pm$ S.D., $r^2 = .92$, $n = 14$). The release of granule serotonin from adherent platelets was $45.4\% \pm 4.7\%$ (mean $\pm$ S.D.) for all positions along the tube (Figure 7.2). Mepacrine did not affect platelet accumulation or release from adherent cells as evaluated by radioactive tracers (Table 7.1). Video microscopy revealed similar platelet accumulation sequelae as for the fibrinogen surface. Platelets arrived as single platelets adhering only to platelet-free areas or moving out of the field of view. Those that remained stationary for more than 500 ms adhered and remained. At 600 s an evenly distributed, layer of individual platelets existed near the inlet (Figure 7.4c).

TABLE 7.1: Platelet Accumulation and Release Based on Radioactivity Measurements

Surface	Conditions	n	P1/1000μm^{2+}	m	Release*
Collagen	-	39	245(66)	-1.14(.22)	50.3(13.2)
	mepacrine	7	221(35)	-1.05(.19)	60.6(13.6)
Fibrinogen	-	16	66.5(22.3)	-0.20(.14)	17.5(14.1)
	mepacrine	3	68.8(12.7)	-0.16(.07)	19.2(13.9)
	fibrinogen	3	59.7(10.5)	-0.19(.11)	34.5(20.2)
Fibronectin-depleted Fibrinogen	-	3	57.3(9.5)	-0.14(.05)	17.9(15.4)
Fibronectin	-	14	65.3(17.5)	-0.23(.19)	41.1(12.5)
	mepacrine	3	73.6(16.8)	-0.36(.27)	45.0(26.0)
	fibrinogen	3	55.7(21.3)	-0.30(.21)	51.5(10.2)
Albumin (Human)	-	4	<3.0+	-	-
Albumin (Bovine)	-	6	<3.0	-	-
Glass	-	6	6.6(11.7)	-0.1(.38)	44.0(18.8)

+ Average number of platelets/1000μm^2 for the tube's first centimeter

* Average % release of serotonin from adherent cells for the tube's first centimeter

n Number of determinations, mean (S.D.)

m Power law exponent

7.2.4 Albumin

No detectable platelet accumulation (<3 platelets/1000μm^2) occurred on the human or bovine albumin-coated glass surfaces. The degree of release of serotonin from adherent platelets was thus unmeasurable. Video microscopy revealed that platelets rarely remained stationary on the surface, but rather passed by without slowing down. It is difficult to say if the platelets touched the surface using this technique of en face microscopy.

7.2.5 Glass

Platelets adhered to the glass surface at a position-invarient level of 7.0 $\pm$ 8.0 platelets/1000 μm^2 after a 600 s of perfusion, as determined radioactively. The release of serotonin from adherent platelets was 44.1% $\pm$ 4.2% (mean $\pm$ S.D.) with no axial pattern (Figure 7.2). Video microscopy revealed that only small areas of the glass surface accumulated platelets while the rest of the surface was albumin-like in that platelets came close to the surface but did not slow down. The active sites accumulated platelets that arrived most often as single platelets but occasionally three and four platelet groupings arrived. These groupings were not observed in suspensions before perfusion or on any other surface suggesting they were emboli from upstream. The active sites cyclicly formed clusters that embolized and then new clusters formed (Figure 7.4d).

7.3 Discussion

The interpretation of platelet deposition experiments requires a detailed knowledge of fluid mechanical conditions, blood composition and surface composition. The dynamics of platelet deposition on surfaces has been studied by techniques(11-16) (cf Table 1.1) which at best enable aggregates to be viewed(11,14,15). Others allow even larger surface areas to be studied through indirect measurements(12,13). Other systems used to study platelet-surface interactions exclude red blood cells or employ discrete time points for evaluation of deposited platelets. The mechanism of deposition and the changes that occur between time points can only be speculated upon. The epi-fluorescent technique used in this study presents a new, more powerful way to monitor platelet-surface interactions allowing individual cells and aggregates to be watched continuously. Labeling with ^{51}Cr and ^{3}H-serotonin yields a second measure of accumulation and quantitative release information. These two procedures were used to demonstrate the great variability in platelet-surface interactions and to probe the mechanisms responsible for the variability.

7.3.1 Platelet Accumulation and Embolization

Albumin has been shown to be protective against platelet adhesion(3-6). The results of the experiments presented here support this view. The video microscopic results demonstrate that platelets are not sufficiently attracted to the albumin-coated glass surface to even slow down when near this surface. In contrast, the fibrinogen-coated

and fibronectin-coated glass surfaces have properties that enable platelets to become stationary on these surface. Presumably the nature of attraction changes with time as cells can be pulled off the surface up to 1/2 second (500 msec) after initial contact. After 1/2 second the cells have established permanent bonds that are capable of resisting the forces that would be trying to dislodge the adherent platelets. The collagen-coated surface represents a strongly attractive surface where any platelet that contacts the surface and is stationary for more than 33 msec remains on the surface. The glass surface was a heterogeneous surface with areas where no adhesion occured and areas where aggregates formed and embolized. This was surprising as the albumin in the platelet suspending medium ought to have coated the surface before any platelets arrived rendering the glass surface effectively an albumin-coated surface(2). The active platelet accumulating sites, while their nature is unknown, serve to demonstrate another platelet-surface phenomenon. While these sites had attractive forces sufficient to enable platelets to become stationary and remain attached longer than 500 msec, the growth of platelet aggregates probably increased the dislodging forces allowing removal of the adherent platelets within the embolizing aggregates. This surface shows the danger of using radioactive data alone to characterize a surface as platelet deposition was low but the surface was clearly thrombogenic. Since the fluid mechanical and suspension conditions were the same throughout, the same rate of platelet contact occurred at the inlet for each surface. The decreased accumulation at the inlet on the

fibrinogen-coated and fibronectin-coated glass surfaces in comparison to collagen suggests that only a fraction of the contacting platelets adhere and that these surfaces are saturating with time (cf Chapter 8). This is further supported by the higher outlet accumulations on the fibrinogen-coated and fibronectin-coated glass surfaces relative to the collagen surface.. The saturation of surfaces has been reported by others(2) (cf 8.3).

The decline in platelet accumulation with distance distal to the inlet is consistant with the near-surface region becoming depleted of platelets faster than the delivery rate by diffusion through the suspending medium. These results are better discussed using accumulation versus time information as some surfaces are confounded with platelet saturation phenomenon (cf 8.3).

7.3.2 Release

The release of granule contents from adherent platelets was highest on the collagen-coated surface and varied from 50 to 85% between the tube's inlet and outlet. Since the inlet is also the region of highest accumulation perhaps those platelets within aggregates release less than the platelets adherent to collagen or the released serotonin is trapped in the platelet aggregates. The outlet release probably reflects the release from platelets in contact with collagen. Baumgartner et al.(18) counted residual granules in platelets adherent to subendothelium and collagen and found 92% release. Cazenave et al.(10) have reported 60% release from human platelets on collagen.

Whicher and Brash(3) found 49% release from pig platelets on collagen.

The other surfaces tested in this study gave release from adherent cells which were independent of position along the tube, with the level of release on fibronectin > glass > fibrinogen. These proteins do not elicit shape change aggregation or release when added to our platelet suspension system. The liberation of other materials such as prostaglandins and materials stored in the alpha granules from platelets adherent to these surfaces has not been studied.

7.3.3 Aggregate formation

The collagen and glass surfaces exhibited aggregate formation. With the collagen-coated glass surface, initial clusters grew into large aggregates that remained on the surface. This has been observed by Baumgartner's group also(18). The mechanism of preferential platelet adhesion to aggregates is unknown and arguments of high concentrations of released agents(12,19), altered platelets(12,18,19) and local fluid mechanical disturbances(19) have all been presented as possible mechanisms. Very few platelets contacted the surface once clusters formed resulting in platelet-free areas. The inability of platelets to contact the platelet-free areas after cluster formation along with continued accumulation suggests that platelets only contact the aggregates. The albumin experiments showed that platelets approach the surface with a large axial velocity and smaller normal velocity. This is supported by Goldsmith's work(20) which shows ratios of 10:1 between axial and radial velocities for 2 μm spheres in 40% ghost cell

suspensions. Dr. Goldsmith's work was done at low shear rates ($\approx 10\ s^{-1}$ second). As shear rates increases the axial velocity should increase faster than the radial velocity as the empirically determined diffusion constant (a quantity related to radial velocity fluctuation), increases as the 0.5 power of shear rate(19). The inference here is that platelets approach the surface and skim along it. Anything protruding from the surface would preferentially accumulate platelets and leave the original surface inaccessable to platelets. This is perhaps the reason for the clustering of platelets around the first platelets adherent as even single platelets represent protusions to an incoming platelet.

The linear increase of platelet accumulation with time (Figure 7.3) has been shown by other groups(18,19). The change in the collagen case from platelet-collagen adhesion to platelet-platelet cohesion with increasing surface concentration suggests that both collagen and adherent platelets are sufficiently attractive to enable maximal platelet deposition. The ability of aggregates to form on the collagen surface is interesting since washed human platelets (such as those used in these experiments) will not aggregate in suspension when induced by ADP or low concentrations of collagen without the addition of fibrinogen. Platelets contain alpha-granules that contain fibrinogen and other proteins (cf 1.2.1). These granules are released on stimulation with thrombin enabling washed human platelets to aggregate to thrombin(8). Recent studies have shown that the fibrinogen receptor on the platelet membrane is normally hidden but is exposed during shape change. Fibrinogen rapidly binds to platelets during platelet shape

change allowing aggregation to occur(21). No fibrinogen was added to the suspensions used here. Baumgartner has shown alpha-granule release from platelets adherent to collagen and subendothelial surfaces(18). Also it is generally believed that extensive dense-granule release such as that measured in these experiments is associated with alpha-granule release. It therefore seems likely that fibrinogen from adherent platelets enables aggregate formation to occur on the collagen surface. The release of dense-granule contents from platelets on the glass surface is high (44.1%) and may be the reason for aggregate formation on this surface. But, what about the fibronectin and fibrinogen surfaces? Platelets do not form aggregates on these surfaces. It could be that they do not cause alpha-granule secretion and hence no fibrinogen is available for cohesion. This possibility was explored by adding fibrinogen (0.5 mg/ml) to the suspension prior to exposure to the fibrinogen-coated and fibronectin-coated glass surfaces. No difference was noted between suspensions containing fibrinogen and control suspensions even though the addition of fibrinogen enabled the same platelets to aggregate to ADP in a aggregometer (Table 7.1).

Since platelets must undergo shape change for fibrinogen uptake(21), it may be that the adherent platelets are not sufficiently deformed to allow cohesion to occur. Alternately, the approaching platelets may have to change shape prior to contact. A number of agents are released by platelets (ADP, serotonin, thromboxane A_2) that stimulate platelets to change shape. Collagen induces liberation of all these materials from platelets when added to suspensions and probably

when it is a component of a surface. The release of platelet materials from platelets adherent to fibrinogen-coated and fibronectin-coated glass surfaces is unknown with the exception of the dense-granule materials which have been measured in this study.

7.4 Summary

Platelet accumulation, release from accumulated platelets, thrombus formation, embolization of aggregates and single platelets all occur to different degrees on collagen, fibrinogen, fibronectin, albumin and glass surfaces. There was no correlation between thrombus formation and degree of release of dense-granule materials. Release of materials from other storage sites and progressively formed and liberated materials should be studied. Readdition of fibrinogen does not support aggregate formation on fibrinogen or fibronectin surfaces. Mepacrine had no effect on platelet-surface interactions.

CHAPTER 8

KINETICS OF PLATELET ADHESION AND AGGREGATION ON PROTEIN-COATED SURFACES

8.1 Introduction

The accumulation of platelets on biomaterials and subendothelium with time has been studied by many groups(1-13). While the maximum rate of platelet deposition is controlled by the hemodynamic conditions (2,4,5,9) the extent of and morphological pattern for platelet accumulation varies with exposure time. Some experimental conditions result in an initial rise in platelet accumulation that leads to a constant level of platelet deposition which remain invariant for long periods (1,4,5,7,9,10,13). Other experimental conditions result in an initial rise in platelet accumulation with subsequent embolization of platelets leading to a constant level of platelet deposition some what below the earlier peak in deposition (1,3,4,12,13). Still other experimental conditions result in an initial rise in platelet accumulation with periodic embolization and continued accumulation leading to a cyclic rise and fall in the level of platelet deposition (2,6,8,11). The contributions of hemodynamics, blood composition and surface characteristics responsible for these diverse responses of blood to surfaces are only beginning to be defined (cf 1.3.2).

In Chapter 7, platelet-surface interactions were measured at one duration of perfusion. The work to be described in this chapter

utilizes double labelling of platelets with radiolabels yielding measurements of platelet accumulation on surfaces and percent release of dense-granule bound serotonin from adherent cells. Fluorescent labelling is also used to provide a second measure of platelet accumulation and local information about platelet-surface events such as the pattern of distribution of cells on the surface and the occurrence of embolization of single cells and aggregates. Serial observations with radioisotopes and continuous monitoring with television methods provide a profile with time for this information. We seek here relationships between the amount and rate of platelet accumulation on a surface, the degree of release of dense granule-bound substances, the morphology of accumulated platelets and any local dynamic events observed. These relationships will be used to characterize a number of surface coatings and several drug treatments for platelets with respect to their effect upon the sequence of platelet-surface interactions. The surfaces to be used are glass coated with fibrilar collagen, fibrinogen and fibronectin. The drugs are aspirin, which is known to prevent the formation of thromboxanes and prostaglandins by platelets(14), and imipramine, which is known to prevent uptake of serotonin by platelets(15).

8.2 Results

8.2.1 Collagen

8.2.1.1 Collagen-Control

The accumulation of untreated platelets (cf 2.1.1) on the collagen-coated surface (cf 2.3) is given in Figure 8.1, with time and distance from the inlet as the independent variables. All points along the tube accumulated platelets at constant rates for up to 900 s of perfusion. The rate of accumulation decreased with distance from the inlet, with a rate of 0.32 platelets/1000 $\mu m^2/s$ at the inlet (average for first centimeter of tubing) and a rate of 0.015 platelets/1000 $\mu m^2/s$ at the outlet (average for last two centimeters of tubing), see Table 8.1 for details upon rate computations. Epi- fluorescent videomicroscopic examination (cf 2.8) at the inlet revealed that platelets arrived individually, adhering randomly at first but then preferentially beside already adherent cells, forming clusters in which cells lying on the surface often touched one another. As the accumulation level increased, cell-cell adhesion to clustered platelets resulted in the formation of large aggregates (20-50 μm at the base). For up to 900 s of exposure, no embolization of platelets, clusters or aggregates was observed. There were platelet-free areas between aggregates even after 600 s of perfusion. The outlet section had only individual spread pseudopodic platelets adherent to the collagen-coated glass tube (Figure 8.2). No embolization of platelets at the outlet was observed nor were any aggregates observed.

The percent release of dense granule-bound serotonin from

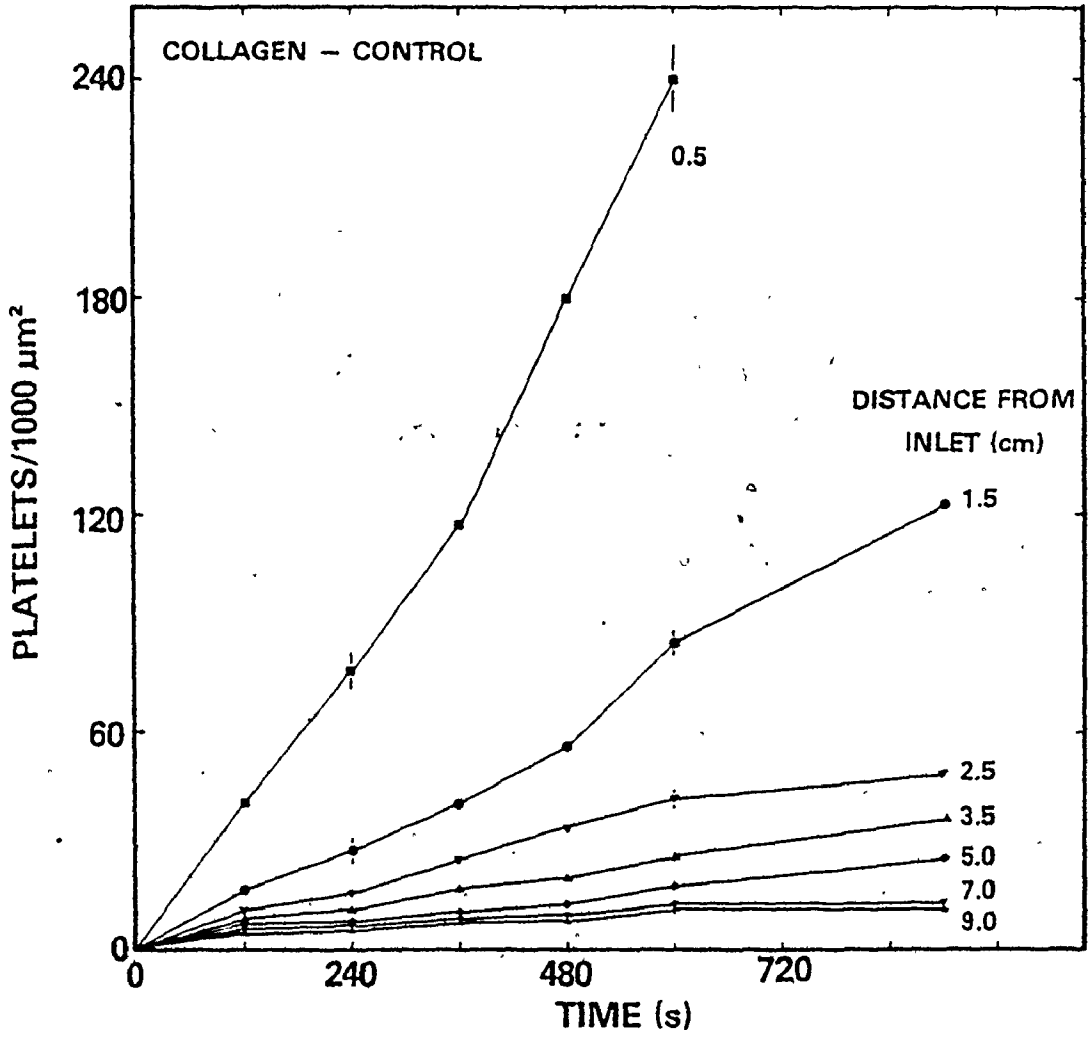


Figure 8.1: Platelet accumulation on a collagen-coated glass tube at a surface shear rate of 80 s^{-1} using untreated, radioisotopically labelled human platelets. Values are means of at least 4 determinations; vertical bars denote standard errors of the means.

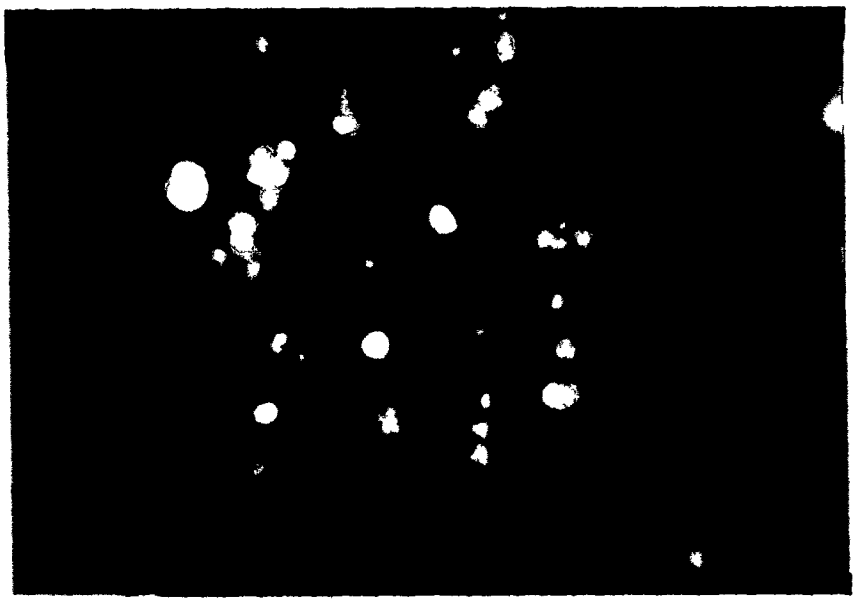
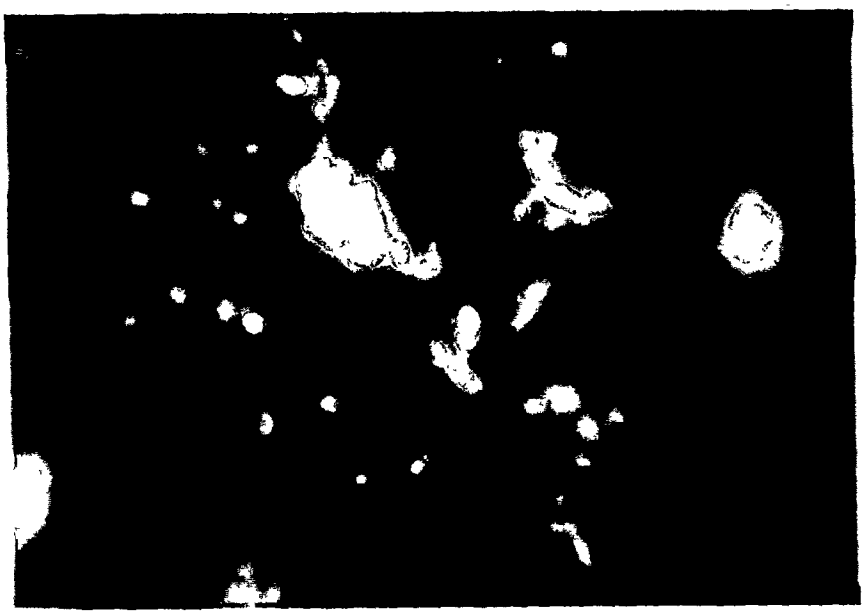
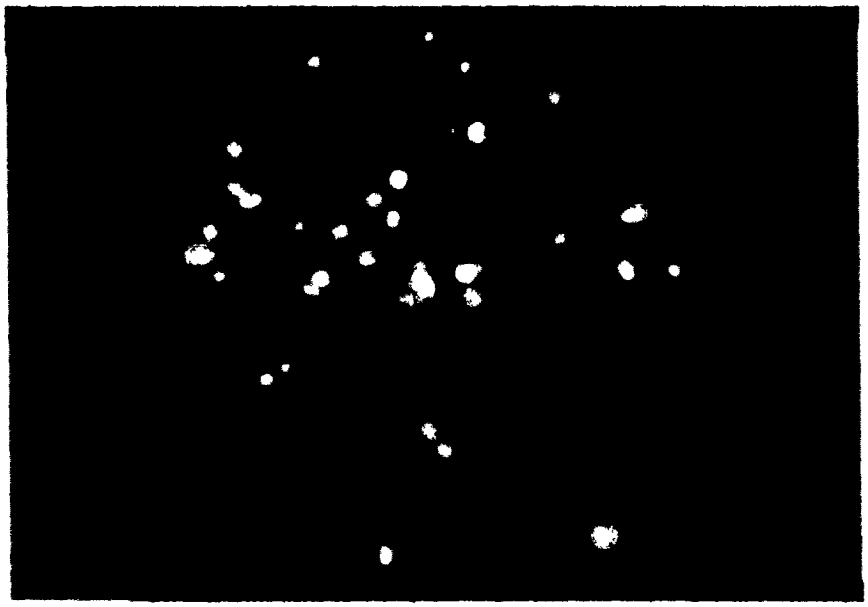


Figure 8.2: Photomicrographs of fluorescent human platelets adherent to the internal surface at the outlet of a glass tube after 600 s of perfusion, shear rate 80 s^{-1}. The long axis of the cross-hair in the center of the photomicrographs is 7.5 μm long. a) Collagen-coated tube with a platelet accumulation of 11 platelets/1000 μm^2 showing individual, pseudopodic, untreated platelets. b) Collagen-coated tube with a platelet accumulation of 33 platelets/1000 μm^2 showing clustered, pseudopodic ASA-treated platelets. c) Fibrinogin-coated tube with a platelet accumulation of 36 platelets/1000 μm^2 showing individual, rounded, untreated platelets.





c)

TABLE 8.1

Initial Platelet Accumulation Rate[a]

Experimental Conditions	Inlet Accumulation Rate n	(Platelets/1000μm^2/s)	Outlet Accumulation Rate n	(Platelets/1000μm^2/s)
collagen-control	23	0.32(0.29-0.35)	95	0.015(0.009-0.020)
collagen-imipramine	6	0.25(0.20-0.31)	20	0.016(0.001-0.032)
collagen-ASA	10	0.32(0.23-0.41)	32	0.057(0.041-0.072)[b]
fibrinogen-control	10	0.23(0.19-0.27)[b]	49	0.054(0.043-0.065)[b]
fibronectin-Control	11	0.20(0.17-0.24)[b]	50	0.049(0.035-0.063)[b]

a Values obtained by regressing accumulation and exposure time data up to 240 seconds for the inlet and up to 600 seconds for the outlet with an equation, accumulation=K_1(time)+K_2, the value reported is K_1(95% confidence limits). n refers total number of data points for each regression.

b Significantly different from collagen-control using 2-tailed t-test on accumulation rate, K_1, $2P<0.05$.

accumulated platelets varied with position along the tube and with duration of perfusion (Figure 8.3). The percent release at the inlet (average for first centimeter of tubing) had lower than the outlet (average over last two centimeters of tubing) section for all exposure times evaluated. Longer perfusion times lowered the measured release at the inlet ($2P < 0.001$) but only slightly lowered the measured release at the outlet (Figure 8.3, Table 8.3).

8.2.1.2 Collagen-Imipramine

Platelet accumulation levels and rates of accumulation were not significantly different between collagen-control and collagen-imipramine experimental conditions at all positions and times studied (Tables 8.1 and 8.2). In the presence of imipramine, which prevents re-uptake of serotonin by platelets, there was increased percent release at the inlet and at the outlet as compared with untreated controls (Figure 8.3, Table 8.3). At the inlet with imipramine present, no significant variation of release with time of exposure was observed (Figure 8.3). The percent release at the inlet after 240 s of perfusion in the collagen-control experiments (62.3%) was higher than the release after 600 s (48.8%). These relationships are shown in Figure 8.3 where at the inlet a continual decrease in percent release with increasing exposure time was found in the collagen-control experiments. No significant decrease in percent release with time was found for imipramine-treated cells. There was a lower release at the inlet than at the outlet for 240 s of perfusion ($2P < 0.05$) and 600 seconds of perfusion ($2P < 0.001$). (This

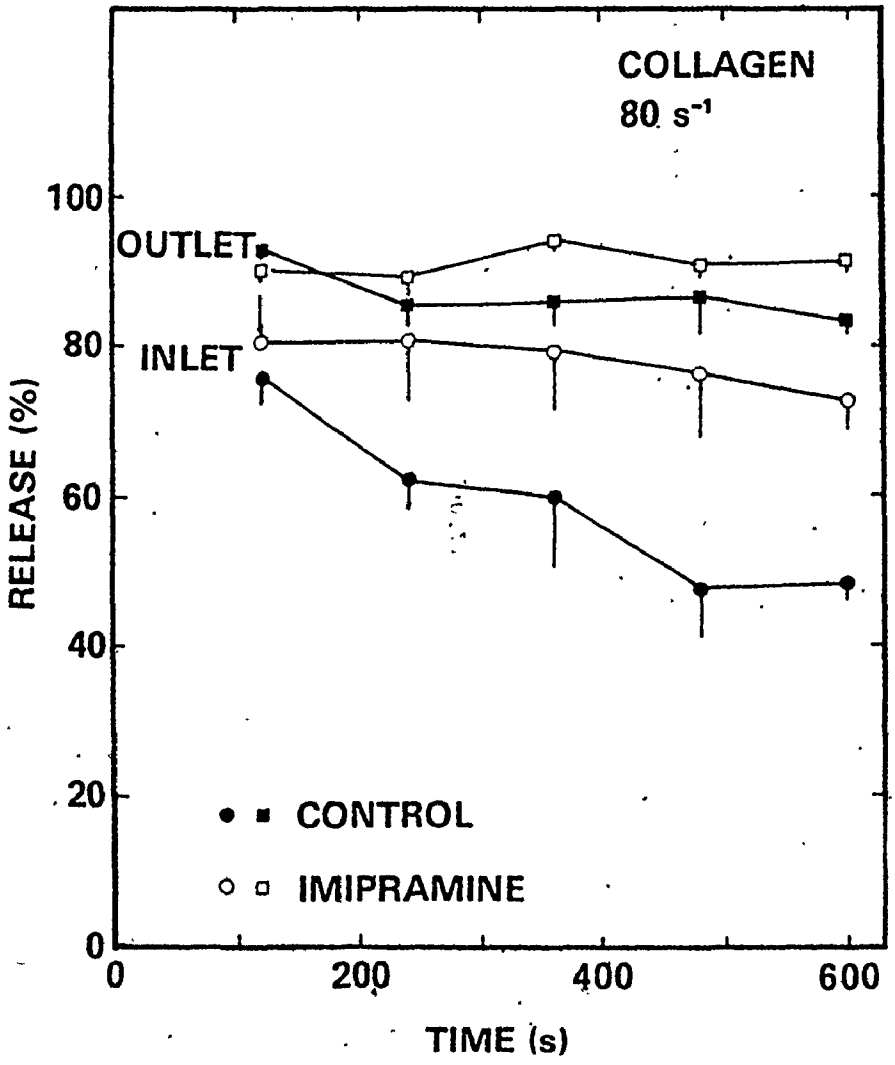


Figure 8.3: The release of ^{3}H-serotonin from platelets deposited on a collagen-coated glass tube. Inlet values are averages of the percent release over the first centimeter and outlet value are averages over the last two centimeters of a 10 cm long tube. Values are means of at least 3 determinations; vertical bars denote standard errors of the means.

TABLE 8.2

Platelet Accumulation on Protein-Coated Glass Tubes[a]

Experimental Conditions	Accumulation After 240 s Perfusion (2) (Platelets/1000μm^2)[a]				Accumulation After 600 s Perfusion (2) (Platelets/1000μm^2)[a]		
	n	m[b]	Inlet	Outlet	n	Inlet	Outlet
collagen-control	5	0.95(0.05)	76.9(4.8)	6.0(0.7)	58	236.6(9.2)	11.0(0.9)
collagen-imipramine	2	0.88(0.21)	77.0(13.3)	7.0(3.3)	10	209.8(20.7)	11.0(2.2)
collagen-ASA	4	0.64(0.07)	61.1(6.2)	10.8(2.0)	16	85.1(5.0)[c]	33.0(3.0)[c]
fibrinogen-control	4	0.44(0.06)	56.3(3.4)	17.0(2.7)	4	70.3(4.9)	36.7(1.6)
fibrinogen-imipramine	-		-	-	6	72.3(14.6)	30.7(2.5)
fibronectin-control	4	0.44(0.10)	48.8(4.5)	15.8(3.4)	26	61.6(4.6)	31.7(2.2)
fibronectin-imipramine	-		-	-	5	77.4(17.9)	28.6(5.0)

[a] Mean (S.E.M) for n determinations. Inlet refers to the average value for the first 1 cm of tubing and outlet for the last 2 cm of tubing.

[b] m refers to the slope in the equation log (Accumulation)=-m log(distance from tube's inlet)+log(const.) and was obtained for n replications of 7 data points taken along each 10 cm tube. m(95% confidence range) is reported.

[c] Mean significantly different from collagen-control, 2-tailed t-test on means, $2P<0.001$.

Table 8.3

Percent Release of ^{3}H-Serotonin from Platelets Adherent to Protein-Coated Glass Tubes

Experimental Conditions	Percent Release After 240 s Perfusion[a]				Percent Release After 600 s Perfusion[a]				Inlet	Outlet
	n	Inlet (%)	Outlet (%)	Inlet vs Outlet	n	Inlet (%)	Outlet (%)	Inlet vs Outlet	240s vs 600s	240s vs 600s
collagen-control	5	62.3(4.3)	85.2(3.0)	2P<0.001	58	48.8(2.2)	82.1(1.9)	2P<0.001	2P<0.01	NS[d]
collagen-imipramine	2	75.6(5.8)	92.0(2.8)	2P<0.05	10	70.8(3.8)[c]	91.1(1.5)[c]	2P<0.001	NS	NS
collagen-ASA	4	80.1(4.5)[b]	90.6(1.5)	NS	16	80.2(2.1)[c]	85.7(1.1)	NS	NS	NS
fibrinogen-control	4	42.2(16.3)	41.6(10.1)	NS	4	22.1(3.7)	34.2(4.6)	NS	NS	NS
fibrinogen-imipramine	-	-	-	-	6	46.3(10.0)[b]	42.0(8.1)	NS	-	-
fibronectin-control	4	52.3(8.6)	67.9(7.2)	NS	26	42.9(4.0)	51.9(4.6)	NS	NS	NS
fibronectin-imipramine	-	-	-	-	5	57.4(4.4)[b]	69.8(6.9)[b]	NS	-	-

[a] Mean (S.E.M) for n determinations, inlet refers to the average for the first 1 cm of tubing and outlet for the last 2cm of tubing.

[b] Mean significantly different from collagen-control, fibrinogen-control or fibronectin-control 2-tailed T-test on means, 2P<0.05.

[c] Mean significnatly different from collagen-control, 2-tailed T-test on means, 2P<0.001

[d] NS means not significantly different using 2-tailed T-test on means, 2P<0.05

last result is common to collagen-control and collagen-imipramine experiments.)

Another approach to demonstrate the statistical significance of imipramine-treatment on measured releases would be to average all the data points (seven per tube) and compare treated and untreated groups. The release however varied with position along the tube making such a comparison difficult to interpret so this was not done.

8.2.1.3 Collagen-ASA

The accumulation of platelets on a collagen-coated glass tube in the presence of aspirin is given in Figure 8.4, with time and distance from the inlet as the independent variables. All positions along the tube accumulated platelets at constant rates until an accumulation level of 70 to 80 platelets/1000 μm^2 was attained whereupon the rate diminished. A continued reduction in accumulation rate with time would result in an invariant surface concentration of platelets. The initial constant rate of platelet accumulation decreased with distance from the inlet; the rate for inlet section was 0.25 platelets/1000 $\mu m^2/s$ and for the outlet section was 0.057 platelets/1000 $\mu m^2/s$, see Table 8.1. Video microscopic examination revealed that platelets arrived individually adhering randomly initially but then preferentially adhering adjacent to (on the surface) already deposited cells forming a discontinuous layer of clustered platelets with platelet-free regions between clusters. After 600 s of exposure platelets were spread on the surface and were pseudopodic. The outlet region also had clusters of

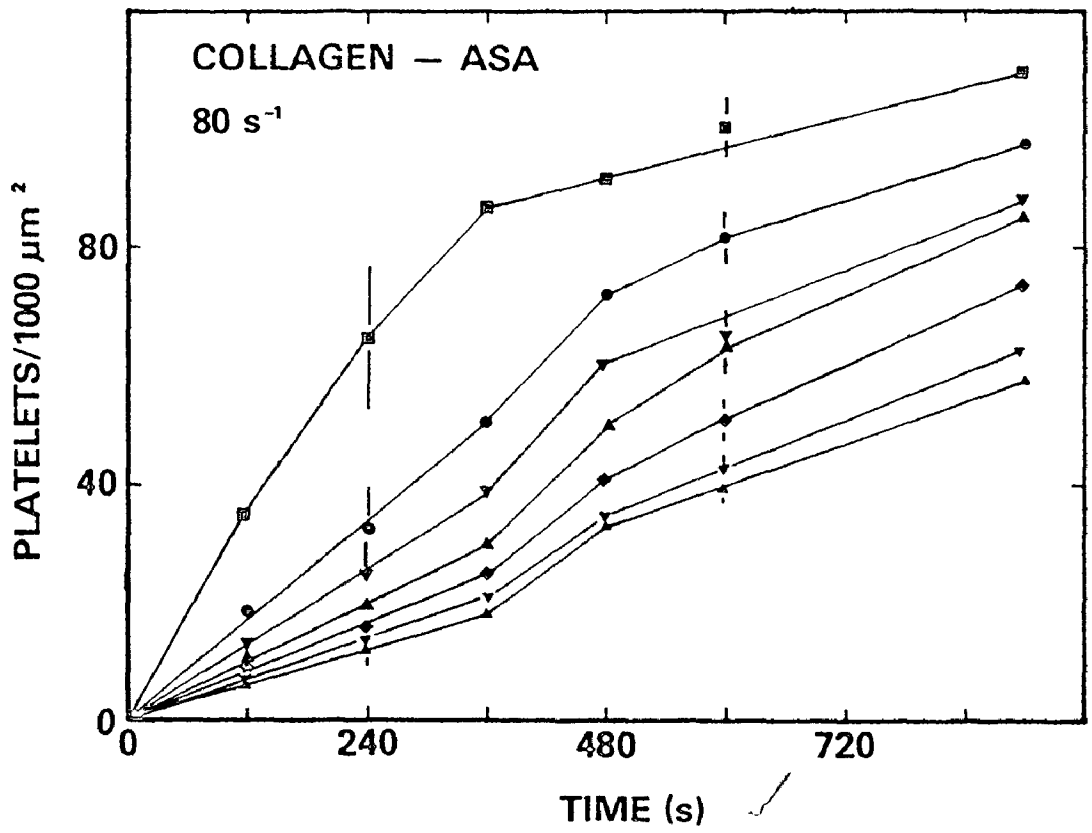


Figure 8.4: Platelet accumulation on a collagen-coated glass tube at a surface shear rate of 80 s^{-1} using ASA-treated, radioisotopically labelled human platelets. Values are means of at least 4 determinations; vertical bars denote standard errors of the means. The symbols used for the data points correspond to the same distances from the inlet as the symbols in Figure 8.1 for 0.5, 1.5, 2.5, 3.5, 5.0, 7.0 and 9.0 cm.

pseudopodic platelets after 600 s of perfusion (Figure 8.1).

The percent release of dense-granule bound serotonin from adherent ASA-treated platelets did not vary with time or position along the tube (Table 8.3). The percent release at the inlet after 600 seconds of perfusion was greater in the collagen-ASA experiments (80.2%) than in the collagen-control experiments (48.8%) $2P < .001$. Since release did not vary with distance from the tube's inlet, the average percent release based on all seven data points for each tube was calculated (Table 8.4) and shows a small decrease in release with increasing time 89.3% to 84.2%, it was non-significant. The 84.2% to 89.3% release range indicates that ASA-treated platelets were maximally stimulated when adherent to a collagen surface.

8.2.2 Fibrinogen

8.2.2.1 Fibrinogen-Control

The accumulation of untreated platelets on a fibrinogen-coated glass tube is given in Figure 8.5, with time and distance from inlet as independent variables. All positions along the tube accumulated platelets at constant rates until a level of 60-70 platelets/1000 μm^2 was attained. The initial rate of accumulation at the inlet was 0.23 platelets/1000 $\mu m^2/s$ and at the outlet was 0.054 platelets/1000 $\mu m^2/s$, see Table 8.1. At the inlet, an invariant accumulation level was reached at approximately 70 platelets/1000 $\mu m^2/s$. Video microscopic examination revealed that platelets arrived individually, initially adhering randomly and then filling in open spaces forming a monolayer on

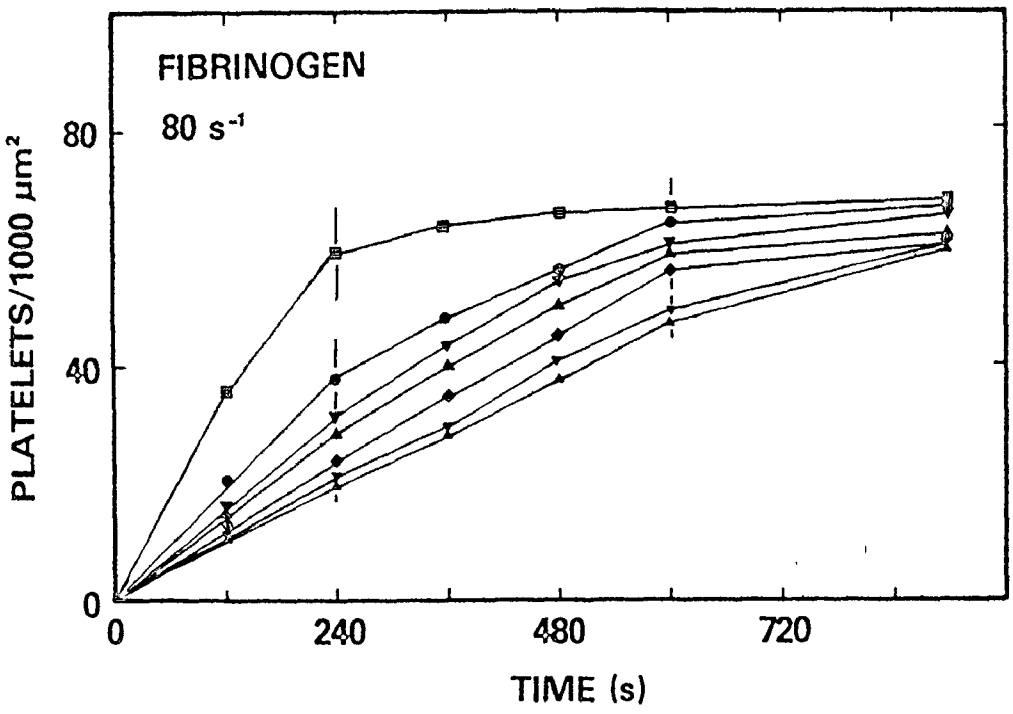


Figure 8.5: Platelet accumulation on a fibrinogen-coated glass tube at a surface shear rate of 80 s^{-1} using untreated, radioisotopically labelled human platelets. Values are means of at least 4 determinations; vertical bars denote standard errors of the means. The symbols used for the data points correspond to the same distances from the inlet as the symbols in Figure 8.1 for 0.5, 1.5, 2.5,, 3.5, 5.0, 7.0 and 9.0 cm.

Table 8.4

Average Percent Release of ^{3}H-Serotonin from Platelets Adherent to Surfaces where Release did not Vary with Position along the Tube.

Experimental Conditions	n	240 s Perfusion[a] (%)	n	600 s Perfusion[a] (%)	240 s vs 600 s Perfusion
collagen-ASA	28	89.3(1.6)	112	84.2(1.0)	NS[b]
fibrinogen-control	28	38.2(1.8)	189	30.8(1.7)	2P < .001
fibrinogen-imipramine	-	-	42	48.8(1.5)[c]	-
fibronectin-control	28	53.4(2.7)	182	48.5(1.7)	NS
fibronectin-imipramine	-	-	35	65.9(2.0)[c]	-

[a] Mean (S.E.M.) for n determinations

[b] NS means not significantly different, 2-tailed t-test on means, $2P<0.05$.

[c] Significnatly different from fibrinogen-control or fibronectin-control, 2-tailed t-test on means, $2P<0.001$

the surface. The platelets were not spread on the surface and were rounded, not pseudopodic (Figure 8.2). For the fibrinogen-coated glass surface, residence-times for individual cells on the surface were evaluated. It was found that cells remaining stationary on the surface for longer than 33 ms (the time for one television frame) either individually embolized within approximately 500 ms or remained on the surface throughout the 600 s observation period, also see reference 11. It is not known if the platelets that embolized reattached downstream.

The percent release from accumulated platelets was constant along the tube after 240 s of perfusion, 42.2% at the inlet and 41.6% at the outlet, but varied slightly after 600 s of perfusion, 22.1% at the inlet and 34.2% at the outlet. No continuous pattern in the change of release with position was found. The percent release decreased with increasing time at the inlet and at the outlet. These trends with time were not statistically significant (Table 8.3). The average percent release computed using all seven data points along the tube showed that there was 38.2% after 240 s and 30.8% release after 600 s of perfusion. These values were significantly different from one another ($2P < .001$, Table 8.4) demonstrating a decrease in percent release with increasing time.

8.2.2.2 Fibrinogen-Imipramine

In the presence of imipramine, platelet accumulation was not significantly different from that in the fibrinogen-control experiments at all positions and times studied (Table 8.2). Imipramine-treatment resulted in higher percent release after 600 s of exposure as compared

to fibrinogen-control experiments (Table 8.3). At the inlet the change was from 22.1% in the control experiments to 46.3% in the imipramine experiments and at the outlet from 34.2% in the control experiments to 42.0% in the imipramine experiments (Table 8.3). The percent release after 600 s with imipramine present (inlet - 46.3%; outlet - 42.0%) was the same as the release measured at an earlier time, 240 s without imipramine (inlet - 42.2%; outlet - 41.6%). Since the per cent release displayed no pattern along the tube, the average release using all seven data points for each tube was computed and showed an increase in percent release with imipramine present to 48.8% at 600 s, as compared with the value at 600 s from the fibrinogen control experiments of 30.8% ($2P < 0.001$, Table 8.4).

8.2.3 Fibronectin

8.2.3.1 Fibronectin-Control

The accumulation of untreated platelets on a fibronectin-coated glass tube is given in Figure 8.6, with time and distance from the inlet as independent variables. The results are qualitatively the same as those of the fibrinogen-control experiments. All positions along the fibronectin-coated tubes accumulated platelets at constant rates up to a level of 60-70 platelets/1000 μm^2 (Figure 8.5). The initial rate of accumulation at the inlet was 0.20 platelets/1000 $\mu m^2/s$ and at the outlet was 0.049 platelets/1000 $\mu m^2/s$, see Table 8.1. Video microscopic examination revealed a sequence of events similar to those in the fibrinogen-control experiments. Platelets arrived individually,

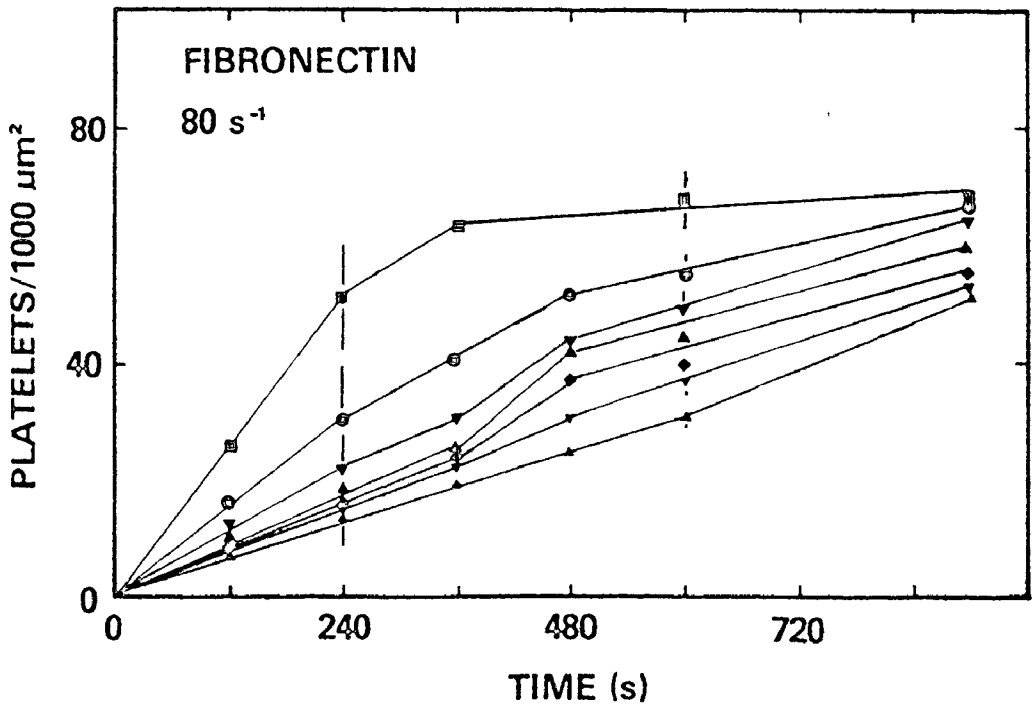


Figure 8.6: Platelet accumulations on a fibronectin-coated glass tube at a surface shear rate of 80 s^{-1} using untreated, radioisotopically labelled human platelets. Values are means of at least 4 determinations; vertical bars denote standard errors of the means. The symbols used for the data points correspond to the same distances from the inlet as the symbols in Figure 8.1 for 0.5, 1.5, 2.5, 3.5, 5.0, 7.0 and 9.0 cm.

contacted the surface either remaining for less than 500 ms and embolizing or remaining throughout the observation period of 600 s. The distribution after 600 s was a monolayer of individual platelets.

The percent release from adherent platelets was relatively constant along the tube with 52.3% at the inlet and 67.9% at the outlet after 240 s of perfusion and 42.9% at the inlet and 51.9% at the outlet after 600 s of perfusion. A not statistically significant trend of decreasing percent release with increasing exposure time was thus present at the inlet and at the outlet. Since release values showed no pattern along the tubes, an average value using all seven data points for each tube was computed. A similar trend of decreasing release with exposure time was found for the average release values. This trend was likewise not statistically significant (Table 8.4). The average release for the fibronectin-control experiments after 240 s of perfusion, 53.4% was significantly higher than the same value for the fibrinogen- control experiments, 38.2%, ($2P < .001$ Table 4). Similarly, after 600 s of exposure the fibronectin surface elicited 48.5% release compared to that of the fibrinogen surface of 30.8% ($2P < 0.001$, Table 8.4).

8.2.3.2 Fibronectin-Imipramine

In the presence of imipramine, platelet accumulation was not significantly different from the fibronectin-control experiments at all positions and times studied (Table 8.2). At the inlet after 600 s of perfusion, the percent release with imipramine present, 51.4%, was higher than the fibronectin-control value, 42.9%, ($2P < 0.05$ Table 8.3)

and at the outlet the release with imipramine present, 69.8%, was higher than the control value, 51.9%, ($2P < 0.05$ Table 8.3). Since release values showed no pattern along the tubes, the average percent release using all seven data points for each tube was computed and showed that after 600 s with imipramine present the release, 65.9%, was higher than the fibronectin-control value, 48.5%, ($2P < 0.001$ Table 8.4).

8.3 DISCUSSION

8.3.1 Platelet Accumulation

8.3.1.1 Platelet Adhesion

Video microscopic examination of platelet accumulation during the first minute of flow demonstrated adhesion of individual platelets to the surfaces for all experimental conditions studied. At the inlet the platelet accumulation rates for the first 240 s of perfusion were not significantly different for collagen-control and collagen-imipramine experiments but were significantly different for collagen-control and collagen-ASA experiments (Table 8.1). This suggests that imipramine does not alter platelet-surface or platelet-platelet binding. At the inlet, the platelet accumulation rate on the fibrinogen-coated and fibronectin-coated tubes for the first 240 s of perfusion was significantly less than the comparable rate for collagen-control experiments. The initial accumulation rates for fibrinogen-control and fibronectin-control experiments were not significantly different. Since no alteration in experimental conditions except for surface coating existed, at the inlet, the same number of platelets per unit time per

unit surface area would collide with all the surfaces. However, the number forming permanent bonds in the collagen-control case was higher than for the collagen-ASA, fibrinogen-control or fibronectin-control cases. Indeed, video microscopy revealed that platelets embolized from fibronectin and fibrinogen-coated glass surfaces after remaining for a fraction of a second (residence times up to 500 ms were previously obtained) but did not embolize in the collagen-coated experiments. The nature of the surface and the state of the platelets then may influence the rate of platelet accumulation.

A result of embolization of single platelets is the possibility of subsequent adherence downstream. From our work, it is not known if such platelets adhered distal to their initial contact site. Butruille et al.(7) have suggested that platelets which undergo non-adhesive encounters become refractory. Baumgartner et al.(1) have suggested that emboli can reattach downstream. We have observed attachment of aggregates to a glass surface under thromboembolic conditions(11). The existence and nature of any refractory phase induced by non-adhesive collisions remains obscure. For the fibrinogen-control and fibronectin-control experiments of this study the constant rate of platelet accumulation up to 240 s of perfusion with a sudden reduction in rate followed by an invariant accumulation (Figure 8.4 and 8.5) is consistent with the experimental findings and a mathematical model presented by Grabowski et al.(5) This model takes into consideration the possibility of non-adhesive encounters and the continued decline in adhesion sites for platelets accumulation on a surface. Calculations using the model

have shown(19) that the opposing mechanisms of decreasing surface availability and increasing concentration of platelets near the surface would maintain the rate of platelet deposition constant until almost complete saturation occurred. The model would predict that when comparing two cases in which the initial platelet accumulation rates at the inlet were different, the case which had the greater rate at the inlet would have a lesser rate at the outlet. Our results, which are consistent with this, show that for the inlet the initial accumulation rates for the collagen-control experiments and collagen-imipramine experiments, which are equal, are greater than the initial accumulation rates for the collagen-ASA, fibrinogen-control and fibronectin-control experiments. For the outlet, the collagen-ASA, fibrinogen-control and fibronectin-control initial accumulation rates are greater than the collagen-control and collagen-imipramine initial accumulation rates, which are equal. The model also predicts for low levels of platelet accumulation that the platelet accumulation would be proportional to the reciprocal of distance from the tube's inlet raised to the 0.33 power and that this represents a maximum in reduction of accumulation with distance from the tube's inlet. For accumulation levels after 240 s of exposure, we have computed, based on our data, comparable power-law exponents for the collagen-control, collagen-imipramine, collagen-ASA, fibrinogen-control and fibronectin-control experiments (Table 8.2) and find absolute values to all be significantly greater than 0.33. This means that the reduction in accumulation levels, with distance from the tube's inlet, found in this work is greater than the maximum predicted

by the model described above; although, a number of consistencies with the model were observed. For the above experimental conditions, the physical principles and assumptions embodied in the model are not consistent with at least one aspect of the platelet accumulation data obtained here.

8.3.1.2 Cluster Formation

In the collagen-control experiments, the process of platelet accumulation at early times was random adhesion changing to cluster formation as platelets preferentially adhered beside already adherent cells. The reason for this anisotropic distribution is unclear but may reside in the adherent platelets causing local roughness elements. Clusters did not form at the outlet of the collagen-control tubes possibly due to the low accumulation levels. They did form at the inlet and outlet collagen-ASA experiments indicating that thromboxanes and prostaglandins are not required for cluster formation since ASA inhibits the formation of these components. On the fibrinogen or fibronectin-coated surfaces cells were more evenly distributed and rounded; clusters did not form at all. Others have also demonstrated on Epon that platelets which are not spread adhere singly and reach a maximum surface coverage of about 20% of total available surface area(1). A correlation between psuedopod formation and cluster formation has been demonstrated and requires more experimentation to see in what way these phenomena may be interrelated.

8.3.1.3 Aggregate Formation

One may hypothesize that platelet aggregate formation on a surface is related to the concentration of substances released from the dense granules of deposited cells. The concentration near the surface due to materials released from platelets has been shown to be proportional to the product of amount of material liberated per platelet and the rate of accumulation of platelets(10). For the same volume flow rates and at the tube's inlet, where upstream release does not influence local fluid concentrations, the ratio of the product of the platelet accumulation rate with the percent release for two sets of experimental condition will also be the ratio of the concentrations of released substances near the tube's surface. At the inlet for the first 240 s of exposure, the platelet accumulation rates were 0.32 and 0.25 platelets/1000 $\mu m^2/s$ for the collagen-control and collagen-ASA experiments respectively. The percent release values at the inlet measured after 240 s of exposure were 62.3% for the collagen-control case and 80.1% for the collagen-ASA case. The collagen surface with ASA-treated platelets would develop almost the same concentration of materials released from dense granules (.25 x 80.1 = 20.0) as the collagen surface with untreated platelets (.32 x 62.3 = 19.9). Yet, for exposures greater than 240 s, the collagen surface with untreated platelets showed aggregate growth while the ASA-treated platelets on the collagen surface did not. The lack of aggregate formation using ASA-treated platelets despite maximal dense-granule release suggests that dense-granule materials which include ADP, ATP, serotonin, calcium

and magnesium, are not influencing mural thrombogenesis. Additional support for the lack of influence of dense granule-bound substances on mural thrombogenesis may be found in our plasma protein-coated surface data. Although the fibronectin-imipramine conditions showed an increase of 35 percent release when compared with the fibrinogen-imipramine conditions, there was no difference between these two cases in: the initial accumulation rates at the inlet and outlet, the accumulation levels at 600 s for both inlet and outlet positions and the morphology of platelets on the surface. Previous studies have shown platelet function inhibitors that specifically block ADP-induced aggregation, do not prevent platelet aggregate formation on a collagen-coated tube(21).

ASA treatment of platelets prevents the fomation of prostaglandins and thromboxanes and inhibited aggregate formation on fibrilar collagen surfaces here suggesting that some arachidonic acid metabolite is responsible for aggregate formation on collagen. The most likely candidate is thromboxane A_2 which is a powerful platelet stimulator(14). Since both the fibrinogen-coated and fibronectin-coated surfaces do not form clusters or aggregates using untreated platelets, it is possible that these surfaces do not permit formation of prostaglandins and thromboxanes. It may also be that aggregate formation on a surface generally occurs through the sequence of steps followed by the platelets in our collagen-control experiments i.e. platelet adhesion, cluster formation with pseudopodic platelets and aggregate growth. Thus, it may be that inhibition of pseudopod formation on fibrinogen-coated and fibronectin-coated surfaces is the limiting step on the pathway to

aggregate formation. Alternately, it may be the release of a material from the alpha granules of platelets that is required for aggregate formation. The release of alpha-granule bound materials need not parallel the release of dense-granule materials, so this possibility remains unresolved for the surfaces and treatments studied here. Previous studies(11) have shown that addition of fibrinogen, a material stored in the alpha granules of platelets, does not envoke aggregate formation on the fibrinogen or fibronectin surfaces. The description of the exact pathways involved in mural thrombogenesis must then await techniques to monitor release from alpha granules and arachidonic acid metabolism of platelets on surfaces.

8.3.2 Release of Dense-Granule Materials

Percent release measurements for platelets localized on surfaces were made with respect to two independent experimental variables which were position along the tube and exposure time to platelet/red cell suspension. All surfaces were evaluated in the presence and absence of imipramine and the collagen-coated glass surface was evaluated in the presence and absence of aspirin.

Only for collagen-control and collagen-imipramine experimental conditions were their the significant differences in the percent release along the tube. In both of these cases, the percent release was less at the inlet section than at the outlet section. Since aggregates were observed with video microscopy at the inlet and individual cells at the outlet under collagen-control conditions and since platelet accumulation

levels were the same for collagen-control and collagen-imipramine conditions, it is possible for these conditions that the platelets within aggregates adherent to a collagen surface release on the average less than platelets adherent directly to a collagen surface. Others have observed that platelets on the lumenal side of mural aggregates tend to contain more dense and alpha granules than cells adherent directly to the surface(23). The difference between inlet and outlet percent release values, for the collagen-control case was greater after 600 s than after 240 s of exposure. There was only a small (not significant) difference between the outlet percent release after 240 s and the outlet percent release after 600 s of exposure. These release data also indicate that the amount of ^{3}H-serotonin remaining at the inlet after 240 s was approximately twice that present at the outlet after 240 s. Since only the inlet release value showed a significant change with time, this change may be related to the granule content of the platelets. In the collagen-imipramine case, no difference in percent release with time was found for the inlet or outlet. This implies that the difference in release values at the inlet between collagen-control values at 240 s and 600 s of exposure was due to reuptake of ^{3}H-serotonin in the collagen-control case. The amount of reuptake of serotonin will also be dependent upon the local concentration of this substance(24) which to some extent will be dependent on local flow conditions(20). Exposure time and flow conditions will thus be important experimental parameters in the use of ^{3}H-serotonin, in the absence of imipramine, as a marker for the release

of dense-granule substances. The presence of imipramine makes ^{3}H-serotonin a better marker for releaseable substances which are stored in the dense granules of platelets such as ADP, ATP and Ca^{++}.

For the plasma protein coatings studied, fibrinogen and fibronectin, in the presence and absence of imipramine, no variation of release along the tube was observed. A significant decrease in percent release with time (between 240 s and 600 s of exposure, see Table 8.4) was also found for fibrinogen-control conditions. For both fibrinogen and fibronectin, the presence of imipramine increased the percent release measured, most likely as a result of preventing reuptake of released serotonin. The collagen-aspirin conditions also showed no variation in percent release with position along the tube. Since no aggregates were found on these surfaces, there was platelet-surface contact only. Using percent release from adherent platelets as a measure of platelet stimulation, these results would rank collagen-ASA experimental conditions > fibronectin-imipramine > fibrinogen-imipramine.

8.4 Summary

Platelet accumulation and release from the dense granules of deposited cells were investigated using radioisotopic and visual fluorescent methods with flow. A flow rate which corresponded to a shear rate of 80 s^{-1} was used. Exposure time to platelet/red blood cell suspension and position along the tube were experimental variables. Platelet deposition on fibrinogen and fibronectin-coated glass tubes

proceeded at a constant rate until an invariant accumulation level was attained whereupon net deposition ceased. At this point single cells without pseudopods were present. A constant release with position along the tube was found on these surfaces. On a collagen-coated tube, platelets accumulated at a constant rate for up to 900 s without saturation. The morphology of deposited cells varied along the collagen-coated tube from aggregates at the inlet, to clusters of surface-adherent cells,to single cells with pseudopods at the outlet. At the inlet, a time-sequence of first single cells with pseudopods, then clusters and then aggregates was followed. There was consistently less release at the inlet than at the outlet of the tube. No correlation existed between release of dense granules and aggregate growth. Aspirin treatment of platelets inhibited aggregate growth but not clustering of platelets, adhesion or release on a collagen surface. For collagen-coated glass, the rate of accumulation at the inlet was less in the presence of ASA than in its absence. This implies that embolization occurred in the presence of ASA. For fibrinogen-coated glass and for fibronectin-coated glass, embolization was visually observed at the inlet. Imipramine does not affect platelet adhesion or thrombus formation but does prevent re-uptake of serotonin by adherent platelets. Using dense granule release as a measure of platelet stimulation, these results would rank collagen-ASA experimental conditions > fibronectin > fibrinogen.

CHAPTER 9

CONCLUSIONS

The work contained in this thesis has been directed at understanding the process of mural thrombogenesis on a more mechanistic level. Using the knowledge of platelet physiology established by biological researchers and using engineering principles, greater insight into platelet-surface interactions and platelet-platelet interactions near a surface has been obtained. Simultaneous measurement of platelet accumulation and dense-granule release and microscopic observation of individual platelets near a surface, allowed a detailed picture of the response of platelets to surfaces to be constructed. Mathematical modelling of the processes involved in transport of platelets to a surface and transport of liberated materials away from the surface resulted in a clearer understanding of the environment near the surface during thrombogenesis. By way of concluding remarks a paradigm of events occuring in thrombogenesis will be constructed based on the experimental and theoretical considerations presented in this thesis and previous work by others.

9.1 A Paradigm of Mural Thrombogenesis

A paradigm of events leading to thrombus formation is presented in Figure 9.1. The degree that each surface studied progresses along this sequence of events is denoted by the bar chart on the right side.

9.1.1 Contact

Platelets are continually colliding with a surface under flowing blood conditions. The rate of collision is controlled by the hemodynamic conditions, principally the shear rate, and the concentration of platelets. The properties that define whether a platelet will bounce off the surface, contact the surface remaining for only a short time or remain on the surface for a long time after a collision have not been studied previously as only a fluorescent microscopic system such as the one employed in this work (cf 2.8) has the capability of such dynamic measurements. Platelets collided with the albumin-coated surface but bounced off indicating no platelet-albumin bond formation was possible. At very much lower shear rates, platelets may be able to bind to albumin-coated surfaces but in the flowing system employed in this work very little platelet accumulation was found (cf. Table 7.1). Indeed, if albumin could remain on the surface, and not be degraded or displaced by other plasma proteins or if a synthetic material could be produced that selectively adsorbed only albumin, this would be an ideal biomaterial. The nonthrombogenic surface of the endothelial cells may be just such a surface which has a coat of albumin. While platelets bounced off the albumin-coated surface, they adhered to the collagen-coated surface upon collision and did not come off. The fibrinogen and fibronectin-coated surfaces exhibited intermediate results where some of the platelets that contacted the surface remained for between 33 to 500 ms, while some remained for greater than 600 s.

Thus, platelets bind to some surfaces and not to others. The nature of the primary interaction between platelets and the surface, that enables platelets to go from being transported along the tube to stationary cells on the surface upon collision is unknown.

9.1.2 Adhesion

Once platelets remain on the surface for a short period of time (500 ms) secondary bonds formed that strongly attached platelets to the surface and platelet adhesion was established. This attachment was overcome on the glass surface by growth of aggregates possibly as aggregates would have increased dislodging forces. The result on the glass surface was embolization of aggregates along the platelet-surface interface removing adherent platelets, suggesting platelet-platelet cohesion is stronger than platelet-glass adhesion. Cyclic adhesion, growth and embolization characterized the glass surface (cf Chapter 7). It is unknown if the secondary bond formation on the fibrinogen or fibronectin-coated surfaces could withstand the removal forces exerted upon aggregates, as these surfaces did not form platelet aggregates (cf Chapter 8). The collagen-coated surface bound platelets strongly and even large aggregates remained adherent. Psuedopod formation does not seem to be important in platelet adhesion as platelets remained on the fibrinogen-coated surface and PGE_1-treated platelets remained on the collagen-coated surface despite their rounded morphology. It is difficult to say if platelet-derived materials are important in adhesion of platelets to the surfaces studied. None of the pharmocological

treatments including those which prevented shape change reduced platelet adhesion to the collagen-coated surface (cf. Chapter 6).

9.1.3 Spreading

Once platelet adhesion is established platelets spread on the surface. A rounded platelet morphology was noted on the fibrinogen-coated surface using untreated platelets and the collagen-coated surface using PGE_1-treated platelets. These conditions resulted in dense-granule release and only platelet adhesion with no platelet aggregates formation. ASA-treated platelets adherent to the collagen-coated surface were psuedopodic and formed clusters but not mural aggregates, suggesting psuedopod formation may be a pre-requisite for cluster formation. The interrelationships between psuedopod formation, spreading release of granules and platelet cohesion require further experimentation to discern their role in thrombogenesis.

9.1.4 Release

9.1.4.1 Dense Granules

A possible feedback loop may exist whereby liberated ADP works in synergy with the surface to increase release. Studies performed on the collagen-coated surface showed inhibition of ADP-induced platelet aggregation in suspensions did not alter was not modulating platelet adhesion, aggregate formation or release (cf Chapter 5). The possibility that ADP released by deposited platelets induced platelet shape change prior to the collision of platelets with the surface and

that this ADP-induced shape change facilitated platelet cohesion could not be ruled out. At the inlet of a collagen-coated tube, CP/CPK in combination with ASA decreased platelet accumulations but not percent release, below the level for ASA-treatment alone (cf Chapter 6). The level of platelet accumulation using CP/CPK with ASA-treated platelets was similar to the level for PGE_1-treated platelets where individual platelets were adherent. These last results suggest ADP may be important in cluster or pseudopod formation. Other dense-granule materials were estimated not to be in sufficient quantity to affect known platelet functions (cf Chapter 3).

No correlation was found between the release of dense granules and mural aggregate formation: the release was 80-90% on the collagen-coated surface for control platelets where aggregates formed and for PGE_1 and ASA-treated platelets where aggreagtes did not form. In addition aggregates formed on the glass surface where the percent release was only 40% yet did not form on the fibrinogen or fibronectin-coated surfaces when the percent release was 49% and 66% respectively. Release from dense granules of deposited platelets does not seem to be sufficient to produce mural aggregate formation nor is total release required for aggregate formation.

9.1.4.2 Alpha Granules

Release of fibrinogen from alpha granules would not raise the level of this material above the normal plasma levels (cf Chapter 3). In the washed cell system used in these experiments, some amount of

fibrinogen may be required for platelet cohesion. Addition of fibrinogen to platelet suspensions did not result in mural aggregate formation on fibrinogen or fibronectin-coated surfaces (cf Table 7.1). The role of fibrinogen and other alpha-granule materials remains speculative and await, specific techniques to block their effects on platelets and ways of measuring their release from adherent platelets.

9.1.5 Arachidonate Metabolism

Platelets probably metabolized arachidonate and liberated TA_2 and PGD_2 upon adhesion to the collagen-coated surface in the same way that platelets liberate these materials in response to collagen addition in aggregometry studies. The exact time sequence of release, spreading and arachidonate metabolism is unknown. TA_2 would diffuse away from the surface stimulating arriving platelets to change shape in preparation for cohesion. The shape change reaction would expose previously cryptic fibrinogen receptors on the platelet membrane. Fibrinogen, normally present in plasma or released from the alpha granules of deposited platelets in the washed platelets used in this study then binds to the platelet and bridges the platelets together upon collision. Aspirin, sulfinpyrazone and indomethacin all inhibited mural-aggregate formation on a collagen-coated surface probably due to the inhibition of TA_2 formation. The ability of PGE_1 to prevent aggregate formation on the collagen-coated surface could be due to inhibition of platelet responses to the TA_2 formed by the adherent platelets or PGE_1 may inhibit the formation of TA_2 or PGE_1 may prevent adherent platelets from forming

psuedopods necessary for platelet cohesion. The PGD_2 intrinsically formed by the platelet performs the same functions as the PGE_1 but it would appear as if TA_2 activation predominates. The resolving of the competing effects of platelet stimulation by TA_2 and platelet inhibition by PGD_2 must await specific inhibitors of the platelet receptors for these materials.

Clearly the paradigm presented in Figure 9.1 is for a system where no plasma is present or activation of plasma components is severely impaired. In whole blood, numerous zymogens exist that are activated during thrombosis. Thrombin especially can stimulate platelets exclusive of arachidonic acid metabolism and ADP release. Thrombin is produced on the surface of the platelets by the [factor Va, Ca^{++}, factor Xa, platelet] complex. Like platelet-derived materials, the thrombin could diffuse into the lumen of the tube or vessel activating incoming platelets much in the same way TA_2 has been speculated to do in the non-plasma system analysed in the present work. The concentration of thrombin would rapidly decrease away from the surface due to diffusion and inactivation by plasma inhibitors. TA_2 may also be inactivated in plasma. A recent report of the presence of platelet factor 4 on vessel subendothelium suggests that, in vivo, platelet-derived materials may not only diffuse away from the wall but may bind to the wall as well. PGI_2 is libertated by the vessel wall during thrombogenesis, in vivo and would diffuse into the blood in the same manner as the platelet-released materials. PGI_2 is a powerful inhibitor of platelet function that works through the same mechanism as

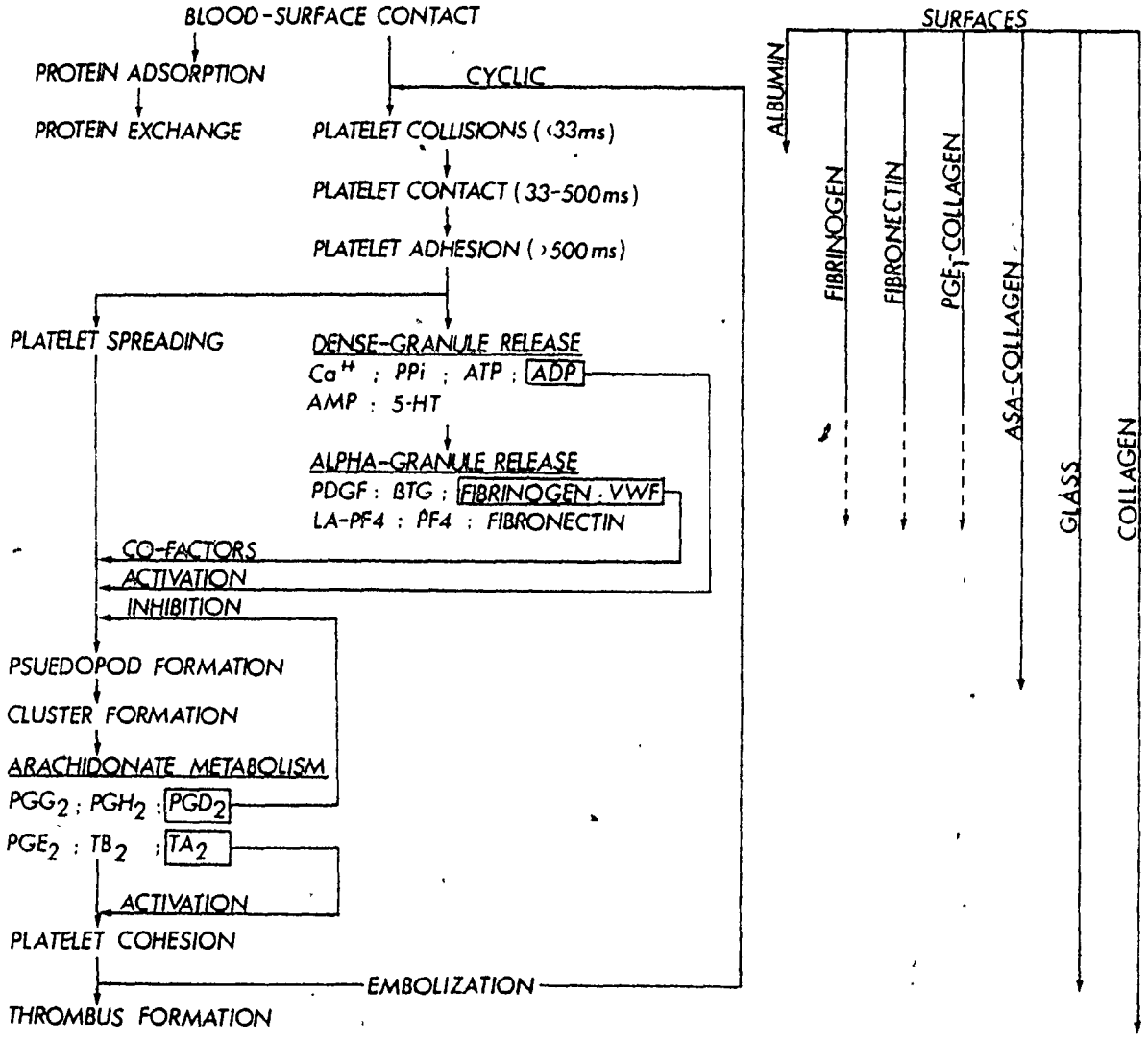


Figure 9.1: Paradigm of mural thrombogenesis.

PGD_2 and PGE_1. The discussion of the effects of PGD_2 and PGE_1 on mural thrombogenesis will apply to PGI_2 as well.

The use of a washed platelet system in this work has enabled exclusion of a number of parameters known to affect thrombogenesis. The simultaneous measurement of platelet deposition and dense-granule release along with continuous observation of platelet-surface interactions enabled a detailed description of the initial stages of mural thrombogenesis. The firm understanding of this simpler blood model establishes a basis to explore the more complicated intact blood case. The mechanisms documented in this work will be operative in whole blood conditions but may be overshadowed by other mechanisms available in whole blood and the vessel wall.

9.2 IFC Model

The model used to estimate interfacial fluid concentrations of platelet-liberated agents demonstrated that increasing shear rate resulted in higher IFCs. If it is a platelet-derived material that is controlling thrombogenesis arterial systems where shear rates are high would be more likely to generate thrombus than venous systems where the shear rates are low. This is the observed clinical pattern of platelet-rich thrombi in arterial circulation and clots in the venous circulation. The model also predicts that concentrations would decrease rapidly away from the site of injury. This is consistent with the observation that thrombosis is a local event. The model also suggests novel ways to approach the hypothesis that TA_2 or some other

platelet-derived material is controlling mural thrombogenesis. For instance decreasing the platelet concentration would decrease the platelet accumulation rate on a surface. This would decrease the flux of platelet-liberated material and therby lower the interfacial fluid concentration provided the flow rate remained the same. This procedure may convert a normally thrombogenic surface like collagen to a surface that saturates much like ASA or PGE_1 treatment does. In the hypothesized instance, production of TA_2 is not inhibited but rather the rate of release of material is not fast enough to develop sufficient concentration to induce aggregate growth.

The model used to calculate the interfacial concentration of platelet-derived materials was designed to give order of magnitude estimates of concentrations. Sophistication of the model by including inactivation or metabolism of platelet-derived materials in the medium, or binding of materials to incoming platelets or binding of materials to platelets on the surface or binding of materials to the surface are all possible. The kinetic parameters and binding affinities are available for numerous pairs of components such as thrombin and platelets, thrombin and ATIII or PGI_2 and platelets etc. No analytical solution of the equations describing inactivation is available for such extensions, however numerical procedures could be employed.

The IFC model allows estimates of the local concentration of numerous materials liberated by platelets. Recently Dangelmaier and Holmsen(1) published an extensive list of the amounts of acid hydrolases in a platelet. These values can easily be used to predict the local

concentrations of these materials in the same manner as presented in Chapters 3 and 4. Further materials such as serotonin and TA_2 have vasoactive properties that may be in the range of concentrations calculated. A survey of the effects of platelet-derived materials on other tissues besides platelets and the necessary concentrations for these effects may allow further insight into thrombogenesis. As more of the suspected materials that exist in platelets are measured and their effects on biological tissues examined predications of important interactions can be made. An understanding of the pathways in thrombogenesis may well depend on the detailed kinetic and spacial analyses begun in this work.

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APPENDIX A

This section deals with methods for estimating local surface concentrations of ADP derived from platelet or red cell sources.

A.1 Local Concentrations of ADP from Adherent Platelets

Platelet aggregation is most often measured as increased transmitted light in a turbidimetric device (aggregometer). The suspension (red cells absent) is mixed by means of a magnetic stir bar allowing platelet-platelet collisions and rapid dispersal of the added aggregating stimulus. The intense mixing motion also rapidly disperses and thereby dilutes any liberated platelet materials such as ADP. In contrast, blood flow in a tube or blood vessel is streamlined so little radial convective mixing occurs. Any material liberated from an adherent platelet would remain close to the tube's surface, diffusing away slowly. Since the releasing platelets are at the tube's surface, the combined effects of many platelets would generate a local region of elevated concentration of released agents adjacent to the surface. In the aggregometer, the combined release of all the platelet contents can create a final suspension concentration of 5 to 10 μM ADP. In the tube flow case, locally elevated concentrations could be reached by the release of the contents of only a small number of surface-adherent platelets due to the absence of mixing and hence less rapid dilution of released material. The early stages of mural thrombosis and hemostasis

correspond to this latter case. Accordingly a procedure to ascertain the local concentration of released agents near a surface was developed.

The experimental procedure and data collection and processing presented were designed to yield values for the flux (amount/unit time/unit of tubular surface area) of released material from platelets adherent to the tube's surface. Of particular interest for biological arguments is the concentration of released agents in the medium adjacent to and near the tube's surface. These quantities may be computed from the surface flux information through the partial differential equation which describes the transport of solutes from the surface. Transport is assumed to occur through the interdependent modes of diffusion perpendicular to and flow parallel to the surface. The equations then represent a model that was used to calculate the interfacial concentration of platelet-derived materials. The model has a number of limitations due to the assumptions required to simplify the mathematics. The model is simplified compared to the complex systems in native blood but retains the essential elements necessary to estimate the concentration near a surface. It must be remembered that it is only a model and cannot be expected to predict things beyond its level of complexity. The model limitations are:

i) There will be a transient period at the beginning of perfusion where the first platelet to adhere is the only cell releasing materials. The flux of materials that are released at the surface will increased from zero to the steady-state level which will be reached when the first platelet is exhausted. After this

time new platelets will be arriving and beginning to release yielding a constant flux of materials at the surface. It has been assumed for this application that the release reaction was completed in a short time, relative to the duration of perfusion, thereby limiting the length of the transient period. There is no information available to estimate the time required for adherent platelets to release. The lag between the onset of perfusion and the steady-state flux of materials then remains undefined. The accumulation of platelets on the surface was found to have a constant rate (see figure 3.1). While this was consistent with the model requirement for a constant flux rate, it was perhaps fortuitous as the mechanism of platelet accumulation changed with time from platelet-collagen adhesion to platelet-platelet cohesion, on the collagen-coated surface. The exact mechanism involved in the net accumulation rate was, after three minutes, not studied. Thus, it is possible that there would be an increase in arrival rate of platelets as aggregates grew and a centerbalancing increase in emobilization rate, yielding the same platelet accumulation rate. The model used to calculate IFCs is consistent with the data acquisition that only considered the release from the net accumulated platelets.

ii) The model used for the IFC calculation does not include release of materials from non-adherent platelets. No difference in ^{3}H radioactivity was found between pre and post perfusion supernatant, samples indicating little if any release occured

from platelets in the suspension. However a small fraction of the platelets could have released their contents and have gone undetected in this assay.

iii) The accumulation and release information were obtained for 1 and 2 cm tube segments. Thus the calculated interfacial concentrations are averages over these surfaces and are not meant to be construed as estimates of concentrations very close to a platelet that is actively releasing. Even higher concentrations of materials released from platelets could exist within a few platelet diameters of an adherent platelet. The IFCs calculated in this work are descriptive of the general environment near the surface where a uniform flux of material is assumed. The heterogeneous nature of the sources of platelet-derived materials was not taken into consideration in this work.

iv) The model used to calculate IFCs is most applicable to surfaces that do not form large aggregates or to the earlier stages of thrombogenesis. The difficulty in extending the model to surfaces with thrombi is related to the roughness created by the aggregates; these may significantly alter the local fluid dynamics. The aggregates preferentially accumulate platelets at the lumenal side, growing in height, creating valleys between aggregates. The quiescent regions in the valleys no longer have the strong convective component necessary to dilute the materials released at the surface. Such a rough surface represents a mathematically more complicated system than was treated here.

v) No provision for removal of the platelet-derived materials (except by dilution) was included in the IFC model. Possible mechanisms to removal materials in whole blood are: adsorption to the surface, to adherent platelets, to arriving platelets, to red blood cells or to proteins, as well as inactivation by spontaneous decay, inhibitor complexes or proteolysis. The mathematical representations of these conditions are more complex and were not approached in this work.

Since both the accumulation and release can be dependent on the distance from the inlet, the analysis was generalized to deal with the case where the flux is dependent upon distance from the tube's inlet. The flux is assumed to begin when the first platelet adheres and to be invariant with time. Conversion of released ADP in the suspension by apyrase was found to be negligible (see A.4). Mathematically, the problem reduces to a partial differential equation (Equation A1) with two boundary conditions, Equations A2 and A3:

$$2 u_m [1 - (r/r_o)^2] \frac{\partial c}{\partial x} = \frac{D}{r} \frac{\partial}{\partial r} \left(r \frac{\partial c}{\partial r}\right) \qquad \text{A1}$$

$$c = {}_o c \quad \text{for } x < 0 \qquad \text{A2}$$

$$N(x) = D \frac{\partial c}{\partial r} \quad \text{at} \quad r = r_o \qquad \text{A3}$$

where x = axial coordinate measured from the tube's entrance, cm

r = radial coordinate, cm

r_o = tube's inner radius, cm

u_m = average velocity for the flow, equal to the volumetric

flow rate divided by cross-sectional area for flow, cm/s

c = concentration of solute or released material, moles/ml of suspension (in Ref. 29 "T")

c_o = concentration of solute at tube's entrance, moles/ml of suspension (in Ref. 29 "T_o")

$N(x)$ = flux of solute at tube's surface, this quantity is determined experimentally and may be variable with x, moles/cm^2/s (in Ref. 29 "q(x)")

D = solute diffusion coefficient in suspension containing red cells, cm^2/s (in Ref. 29 "α")

The solution to the above problem is available in the literature in the form of a mathematically equivalent problem for heat flux from a tube's surface, often referred to as the "Graetz problem" (29). To convert the problem from heat transfer parameters (dependent variable, temperature) to mass transfer parameters (dependent variable concentration) it is necessary to realize that there is a direct analogy between these transport processes (see ref. 4 page 646), where the mass diffusion coefficient, D, is analogous to the thermal diffusivity, α,; the mass flux, N, is analogous to the heat flux, q, and the concentration, C, is equivalent to the temperature, T. By interchanging these parameters the equations A1, A2 and A3 can be shown to be equivalent to the heat transfer equation given in references 4, 28, and 29. The solution is in the form of an infinite series, the coefficients of which have been determined (29). The principle of superposition was used to synthesize a number of solutions for constant flux into a single

solution for variable flux (28). This procedure was used for calculating the concentration of ADP at the tube's surface from experimentally obtained flux data.

The full modal represented by equations A1, A2, and A3 is valid over all axial positions. However, for small distances, that is close to the tube's inlet, the solution requires the summation of a large number of terms of an infinite series. A simplified model considers the velocity profile near the surface to be linear wih the same slope as that of the parabolic velocity profile present in the previous model. The tube can further be modelled by a flat surface thereby removing the curvature considerations. The mathematical formulation becomes

$$4\ Um\ (\frac{s}{r_o})\ \frac{\partial C}{\partial x} = D\ \frac{\partial^2 C}{\partial s^2} \qquad A4$$

where $s = r_o - r$. This equation is presented in reference 4, eq. 11.2-10 and has been converted to mass transfer parameters using the identities previously described ($\alpha = D$, $T = C$, $Z = X$). This simplified model has all the limitations of the first model with the added condition that it is only valid for the first centimeter of tube length at a shear rate of 80 s^{-1} and for 8 cm at a shear rate of 640 s^{-1}. The solution to both these models are in the literature and are reproduced in section A.3 where a detailed explanation of the calculation procedure is given.

Once the accumulation of platelets at the surface and the extent of release are determined the flux, N(x), required in Equation A3, is given by

$$N(x) = \frac{A(x) \times R(x) \times S}{t} \qquad A5$$

where S = amount of released material per platelet, moles/platelet

$A(x)$ = adherent platelets/cm^2 of tube surface

$R(x)$ = fraction of material released

t = duration of run, s

The diffusion coefficient for ADP in a cell-free system has been estimated by Grabowski et al. (16) to be 4.5×10^{-6} cm^2/s. Dworkin and Keller (12) measured the diffusion coefficient for cyclic adenosine monophosphate and found it to be 4.44×10^{-6} cm^2/s. The effect of red blood cell mixing upon the effective diffusion constant of small molecules is believed to be small (18) and was not accounted for here. However, the presence of red cells has been shown to impede diffusion and reduce the diffusion coefficient in suspensions of small molecules such as urea. This has been accounted for here by a method described in reference (11); the effective diffusion coefficient for ADP used in our calculations was 2.0×10^{-6} cm^2/s. Since the platelet is affected by the suspending medium concentration of ADP and the solution to the above partial differential equation yields an average concentration value for the suspension, the values emerging from the solution were adjusted for the red cell portion which contained no ADP. Suspending medium concentrations are reported.

The amount of releasable ADP from porcine platelets has been measured by Thomas et al. (30) and Mills and Thomas (23) and found to be 2.10×10^{-17} moles/platelet. Release data for serotonin were used to

calculate release information for ADP since both are secreted in parallel from the dense granules (14,33). Fifty percent release of ^{3}H-serotonin is assumed to occur simultaneously with 50 percent release of granule ADP.

A.2 Local Concentrations of ADP from Red Blood Cells

Red blood cells contain ADP that can be liberated due to cell damage. Damage can occur due to surface contact at moderate shear rates (3,21). If cells are assumed to be damaged uniformly along the tube's length, the fluid concentration of ADP at the tube's surface arising from red blood cells alone can be determined. The downstream concentration of ADP plus ATP in the supernatant (P_f) minus the upstream concentration in the feed container (P_i) then gives the net amount liberated from red blood cells in the tube provided no platelets are present. The flux of ADP at the tube's surface due to red blood cells is then given by

$$N_{RBC} = \frac{(P_f - P_i) \times \text{flow rate}}{\text{tube surface area}} \times B \qquad A6$$

where B is the ratio of ADP to ADP plus ATP in a red blood cell equal to 1:10 (5).

Once the flux is known the surface concentration of ADP due to red blood cells can be calculated using the solution procedure for an invariant flux (see Appendix for details).

A.3 Details of ADP Surface Concentration Computation

The determination of the surface concentration of ADP requires the solution of partial differential equation (Equation A1) with boundary conditions (Equations A2 and A3). This problem is a linear one, with a variable flux boundary condition. Since the surface flux results from experimental information, a method which could deal with a generalized flux which could either increase or decrease with distance down the tube and which did not need to comply with any specific algebraic form was sought.

The method chosen has been used for a mathematically equivalent heat flux problem and is described in reference (28); it builds upon earlier work dealing with the invariant surface flux case (29). Since Equation A2 is linear, a superposition principle can be applied to obtain results synthesized from a number of judiciously summed constant flux cases. A solution, the surface concentration, is obtained by summing the surface concentration solutions resulting from a number of uniform mass fluxes which are initiated at various positions along the tube (each of these is held at zero for all upstream positions and remains constant for all downstream positions). The sum of these fluxes may be constructed so that the arbitrary flux to be simulated will be approximated, the accuracy of the approximation depending on the smallness of the individual fluxes used. The process is presented pictorially and in greater detail within reference (28). The resultant surface concentration, C_s, then becomes, for any distance from the tube's inlet, x, which is greater than position x_n,

$$C_s = f(\Delta N_0, x) + f(\Delta N_1, x - x_1) + f(\Delta N_2, x - x_2) + \ldots + f(\Delta N_n, x - x_n) \quad \text{A7}$$

Each component flux corresponds to the surface concentration evolving from the constant surface flux ΔN and is dependent upon the longitudinal coordinate $x - x_n$.

Since the ADP flux information was obtained from release measurements taken from discrete lengths of tubing, it was decided to treat the flux information discretely. (Tube segments were: 1 cm long from 0 to 4 cm from the entrance, 2 cm long from 4 to 12 cm and 4 cm long form 14 to 20 cm). Due to the decreasing form of the flux with x (Figure B5), the surface concentration was formulated by subtracting incremental surface concentration values from a base concentration emanating from a constant flux equal to the value of the first segment's average flux over the full length of the tube. The surface concentration along the second segment is then synthesized from the first segment's solution minus a solution for a flux equal to the difference between the fluxes for the first and second segments.

The specific formulations for the invariant flux solution were taken from reference (28). A simplified solution found in (4,29) was utilized for dimensonless distances, λ, less than 10^{-3}. The key relationships are presented below in mass transfer form

$$\theta = 1.22\,\lambda^{1/3} \qquad \text{for } \lambda < 10^{-3} \text{ (ref. 4, eg. 11.2-20, } \chi=0) \quad \text{A8}$$

$$\theta = 4\lambda_{24} + \frac{11}{} + \sum_{n=1}^{7} C_n e^{-\beta_n^2 \lambda} R_n(1) \qquad \text{for } \lambda > 10^{-3} \qquad \text{A9}$$

$$\theta = \frac{D\, C_s}{\Delta N\, r_o} \qquad \lambda = \frac{D\, x}{2 r_o^2\, u_m} \qquad \text{A10}$$

β_n, $R_n(1)$ and C_n values are presented in (28).

A.4 ADP Degradation Kinetics

Since apyrase degrades ADP and is present in the platelet suspension, it could alter the concentration of ADP during platelet adhesion and release. The mathematical problem for simultaneous degradation of ADP in the flow and release from adherent cells at the surface of a tube remains unsolved, and an experimental approach is not practical. To determine if the apyrase could have affected our results two computations were done: (1) an estimate of the percent of ADP entering the flowing suspension which was degraded by apyrase before leaving the 20 cm length of tubing, and (2) in the absence of flow, the radial concentration profile resulting from diffusion of ADP alone was compared with the concentration profile for diffusion plus degradation by ADP.

The estimate of the ADP degradation rate was made by integrating the local volumetric degradation rate (equal to the product of the first order reaction constant with the local concentration of ADP) over the volume of fluid in the tube. This rate was then compared with the integral of the flux at the tube's surface over the tube's length which is the total input rate. Since a detailed description of the

concentration field was available only for the small λ limiting case (5, 29) mentioned above, the comparison was limited to the first 5 cm of tubing, in the 80 s^{-1} case, but could be done for the entire tube length in the 320 s^{-1} case. This, a posteriori, estimate indicated that less than 1% of ADP released at the surface was degraded before leaving, for both shear rates.

The rationale for the second computation is that the non-flow case represents an overestimation of the effect that apyrase could have on ADP concentration. This is so because for a fixed mass flux at a surface the effect of reducing flow is to increase the concentration of diffusing substance, ADP, within the fluid; increased ADP concentration increases the ADP degradation rate. The comparison of concentration profiles was formulated as the ratio (α) of surface concentration minus fluid concentration for diffusion alone, to the same quantity for diffusion plus reaction . This ratio is dependent upon the diffusion coefficient and first order

$$\alpha = \frac{(C_s - C)}{(C_s - C)} \quad \begin{matrix} \text{Diffusion} \\ \text{Diffusion plus reaction} \end{matrix} \qquad \text{A11}$$

reaction constant for ADP degradation and the distance form the tube's surface and is independent of flux at the tube's surface. The above ratio was found to increase linearly with distance from the surface and reached a value of 1.05 at a distance of 50 μm from the surface. This indicates that the concentrations for both cases will be similar and that ADP degradation is not appreciably affecting the concentration of ADP in the near-tube region.

We conclude from the above computations that the presence of apyrase could not have materially influenced the concentration of ADP in the region where platelet diffusion and accumulation were occurring.

APPENDIX B

B.1 Introduction

The results and discussion presented in this chapter are similar to those in Chapter 4 of the thesis. The only differences are that porcine platelets were used in the study presented in Appendix B. The rate of accumulation of porcine platelets was slower than for the human platelets on collagen-coated glass tubes (cf 3.2). The resultant interfacial fluid concentrations are lower than for similar conditions using human platelets. In addition, the contribution of ADP liberated from red blood cells to the interfacial fluid concentration was estimated. For the porcine platelets the concentration of ADP ranged from 0.6 to 1.8 μM which was right at the threshold required to induce platelet aggregation in an aggregometer. It was concluded that insufficient ADP would be present to induce platelet aggregation at the surface but enough ADP is present to work synergistically with collagen to produce mural thrombogenesis.

B.2 Methods

B.2.1 Tube Preparation

Glass tubes (Dow Corning), 1.0 mm internal diameter, were cleaned and coated with acid-soluble bovine tendon collagen (type I, Sigma) according to the technique of Cazenave et al.(10). These tubes were 20 cm long for experiments with platelet suspensions and 120 cm long for

experiments with suspensions containing only red cells.

B.2.2 Flow Apparatus

A flow device was constructed consisting of a glass tube positioned vertically with one end placed in a beaker of suspension which was maintained at 37°C, and the other end connected to a syringe pump (Harvard Apparatus). This apparatus is well defined fluid mechanically and allows for contact of platelets with the test surface without pre-exposure to feeder tubing.

B.2.3 Preparation of Porcine Platelet Suspensions

Pig blood was processed using the method of Mustard et al.(25) for human platelets with the osmolarity of the Tyrode solution adjusted to 340 mosm using a 300 gm/l NaCl solution (contents of Tyrode solution listed in reference 25). Platelets were labelled with ^{51}Cr (New England Nuclear, $2\mu Ci/10^{12}$ platelets) and ^{3}H-serotonin (New England Nuclear, $4\mu Ci/10^{12}$ platelets) in the first wash. The properties of suspensions prepared in this manner have been defined previously (19). The red cells were washed twice in a Sorensen's phosphate buffered saline and twice in platelet-suspending solution. They were further incubated with apyrase, 1 µl/ml, for 15 minutes in the final wash to remove any residual ADP. The final suspension had a hematocrit of 40%, 3×10^{8} platelets/ml, 1 µl/ml apyrase, 3.5 gm/l albumin, 1 gm/l dextrose in Tyrode solution (which contains 2mM $CaCl_2$ and 1 mM $MgCl_2$). No anticoagulant was present.

The apyrase used in these experiments was isolated from potatoes using the method of Molnar and Lorand(24). Apyrase enzymatically degrades adenosine triphosphate (ATP) and ADP to adenosine monophosphate (AMP)(24). The activity of the preparation was determined using the firefly assay for ATP as explained below. At the enzyme concentration used for the platelet suspensions (1 µl/ml) and over the range of substrate concentration, 10^{-6} to 10^{-9} molar ADP, the apyrase exhibited first order kinetics with a rate constant of 1.8×10^{-3} s^{-1} at 37°C. This concentration of apyrase has been shown to be sufficient to prevent the platelet suspensions from spontaneously aggregating but does not affect ADP induced aggregation as measured in a turbidimetric device(19).

B.2.4 Procedure for Flow Experiments with Platelet Suspensions

The 20 cm long tubes were primed with calcium and magnesium-free Tyrode solution before suspension flow to prevent the passage of a blood-air interface which could influence accumulation. The suspension was drawn through the collagen-coated tube by a syringe pump and discarded. Flow rate and exposure time were the experimental variables. After exposure to the cell suspension, the tubes were rinsed with Tyrode solution containing 10 mM EDTA for 5 minutes at a flow rate of 1 ml/min to remove loosely adherent cells.

The tube was cut into segments and each segment counted in a gamma counter to determine the ^{51}Cr activity associated with the tube's surface; the platelet surface concentration (platelets/1000 μm^2) was

then computed (cf 2.5).

B.2.5 Red Blood Cell Flow Experiments

Red blood cells prepared from 3.8% trisodium citrate or acid-citrate-dextrose anticoagulated blood(25) were washed as outlined in preparation of platelet suspensions. The final platelet-free red cell suspension had a hematocrit of 40%, 0.2 µl/ml apyrase, 3.5 gm/l albumin and 1 gm/l dextrose in Tyrode solution. It was allowed to sit for 45 minutes prior to being passed through a 120 cm long, collagen coated tube at 2 ml/min (320 s^{-1}). Since red cell damage is known to increase with flow rate (3,21), the highest flow rate examined in the platelet flow studies was used. The combined concentration of free ATP and ADP in the supernatant of the red blood cell suspension prior to exposure of the suspension to the tube and after exposure to the tube was determined using the method described below. The difference in nucleotide concentration between these two positions was statistically evaluated using a paired-t test.

B.2.6 ATP and ADP Assay

B.2.6.1 Red Blood Cell Studies: Supernatants of the platelet-free red blood cell suspensions were obtained by centrifugation at 9000 g for 2 minutes. The ADP was converted to adenosine triphosphate (ATP) using the Phosphoenolpyruvate/Pyruvate Kinase (PEP/PK) enzymatic system described by Holmsen(17), PK-Boehringer Manheim and PEP-Sigma. The combined concentration of ADP and ATP was determined within 15 minutes

of the end of the flow experiment using the luciferin-luciferase (firefly) assay(17). Although there was apyrase (0.2 µl/ml) in the red cell suspension it did not significantly degrade liberated ADP or ATP in the 15 minutes between the flow experiment and the assay. This was checked by doing two standard curves, one with apyrase (0.2 µl/ml) and one without. No difference was noted after the 15 minute incubation time between the two standard curves. Longer incubation times showed apyrase was degrading ADP.

B.2.6.2 Apyrase Kinetics: The rate of degradation of ADP by apyrase was determined by adding 1 µl/ml (the concentration used in the platelet suspending solution) of apyrase to a solution containing 1.0 µM ADP. The concentration of ADP remaining after 5, 10, 20, 40 and 80 minutes was determined by enzymatic conversion (PEP/PK) of ADP to ATP and measurement of ATP concentration using the firefly assay. The rate constant (1.8×10^{-3} s^{-1}) was determined as the slope of the straight line obtained by linear regression between the natural logarithm of the residual concentration of ADP and the time of incubation. All work was performed in the platelet suspending solution at $37^{\circ}C$.

B.2.7 Aggregation Studies

Platelet suspensions (red cells absent) at a concentration of 5×10^8 platelets/ml were stimulated by ADP (Sigma), acid soluble collagen (type I, Sigma), or ADP plus collagen, in an aggregometer (Payton Instruments). One hundred percent aggregation was set at the level of

transmitted light present with only the suspending medium in the light path. Decreasing concentrations of stimulus were used until no response was recorded. The minimum threshold for response was defined as that concentration of stimulus giving 10% increase in light transmittance (aggregation) or 10% release or last detectable shape change (increased light absorbance). Fibrinogen (Kabi) was added to the suspension at a final concentration of 1.5 μM as washed porcine platelets will not aggregate to ADP or collagen without it.

B.3 Results

B.3.1 Platelet Accumulation on Collagen-Coated Tubes

Three flow rates were chosen for the experiments giving rise to three surface shear rates (80, 160 and 320 s^{-1}). By carefully choosing the duration of exposure, 800, 420 and 300 seconds respectively, it was possible to obtain the same accumulation pattern for each flow rate. Figure B1 shows the extent of platelet deposition versus distance downstream from the inlet for the pooled data, at the three flow rates. It can be seen that the platelet surface concentration falls with distance from the inlet. There is a ratio of 10:1 between inlet and outlet levels of accumulation. A least square regression line had a slope of -0.75 ± 0.11, ($r^2 = .99$) was obtained.

Figure B2 shows the extent of platelet accumulation as a function of time, for a shear rate of 80 s^{-1}, for various positions along the tube. Each accumulation-time curve was a straight line. The slope of these lines (rate of accumulation) decreased with distance from the

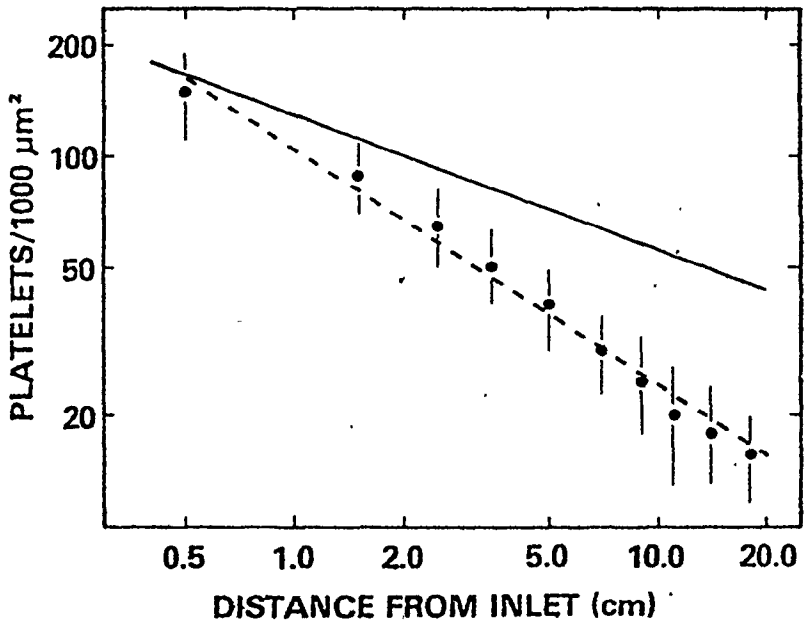

Figure B1: The change in platelet accumulation with distance from the inlet of a 1.0 mm internal diameter, collagen-coated, glass tube. Mean and S.D. of pooled data (n = 22, n = 10, n = 14) for shear rates of 80, 160 and 320 s^{-1} with exposure times of 800, 420 and 300 s, respectively.

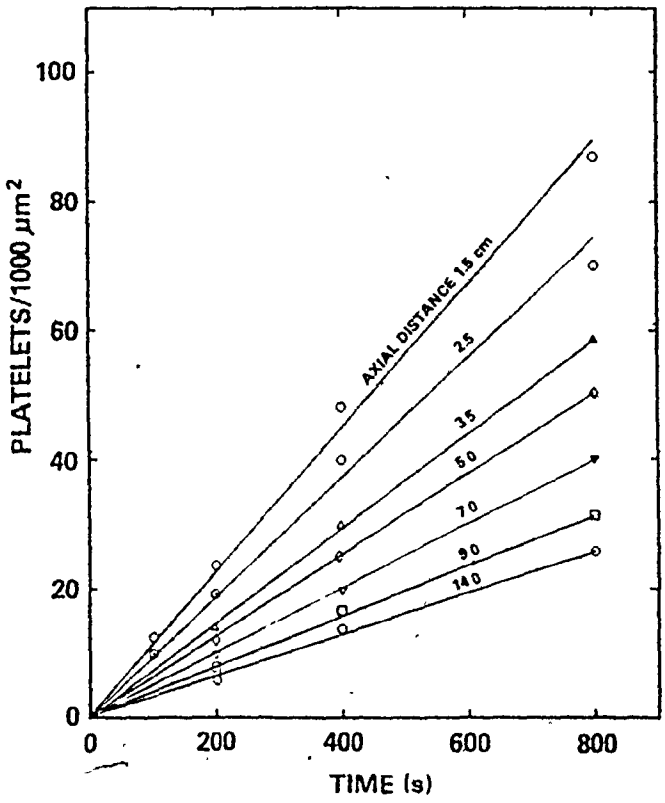

Figure B2: Platelet surface concentration versus exposure time for flow in a 1.0 mm internal diameter, collagen-coated, glass tube at a shear rate of 80 s^{-1}. Measurements at seven axial positions are presented.

inlet.

There exists at the inlet of a tube a segment, called the hydrodynamic entrance region, where the suspension is accelerating and the velocity distribution across the tube is changing. For a Newtonian fluid, this length would be only 0.1 cm for the highest flow rate studied here (4). However, the length of entrance region for cellular suspensions is unknown. The possibility of this entrance region affecting the accumulation pattern was therefore investigated.

A tube was coated with collagen leaving a 5 cm (50 tube diameter) uncoated glass section at the inlet. Glass was found to accumulate few platelets as compared with the collagen surface, see Figure B3. The number of platelets adhering to the glass surface upstream of the collagen region should not affect the platelet concentration near the collagen surface. The result is an entrance region of glass that allows the flow to develop fully while not depleting the suspension platelet concentration near the surface significantly.

The accumulation patterns for the tubes without a glass inlet section and tubes with a glass inlet section are shown in Figure B3. There was no difference in platelet accumulation between the two types of tubes up to a shear rate of 320 s^{-1}. At a shear rate of 640 s^{-1}, the platelet deposition on the first collagen-coated segment of tube (0 to 1 cm) for the tube without a glass inlet section was significantly higher than the corresponding segment (5 to 6 cm) for the tube with a glass inlet section. The accumulation patterns were superimposable at greater distances from the tube's entrance.

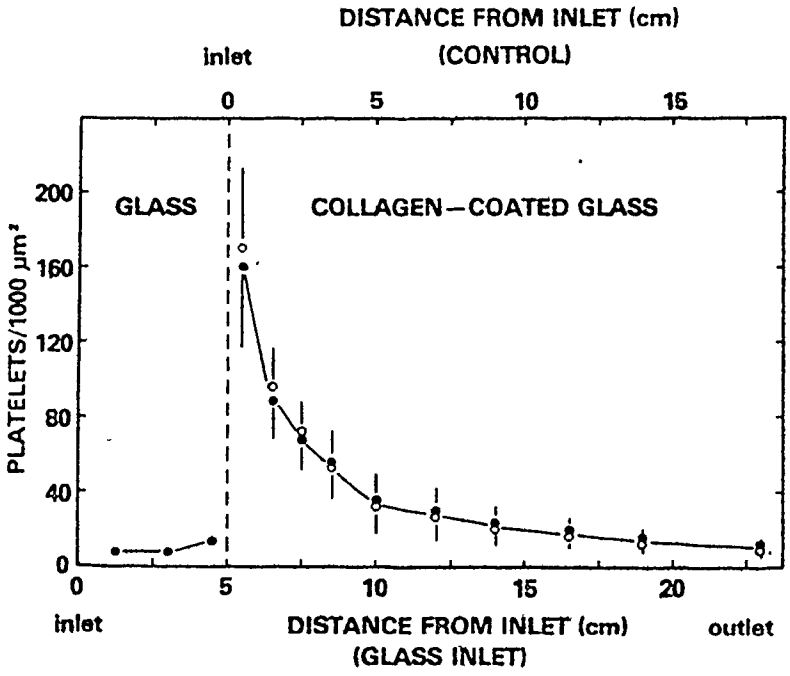

Figure B3: The effect of hydrodynamic entrance region on platelet adhesion. The filled circles refer to the upper coordinate axis, where collagen was coated along the entire length of the tube. The open circles refer to the lower coordinate axis where collagen was coated leaving a 5.0 cm glass inlet section. Mean $\pm$ S.D. of grouped 80 and 320 s^{-1} shear rate experiments (n = 6).

B.3.2 Release of Granule Contents from Adherent Platelets

Percent release of ^{3}H-serotonin from platelets adherent to collagen-coated glass was moderate at the entrance (50-60%) and reached its maximal value (85-90%) at some downstream position (Figure B4). There was no relationship found between percent release and shear rate, paired t-test between data at 80 s^{-1} and 320 s^{-1} done separately at each position along the tube.

B.3.3 Rate of Release of Granule Materials

As outlined in the Appendix A, it is necessary to know the rate of liberation of material being released at the tube's surface, to calculate its concentration near the surface. Figure B5 shows the calculated ADP flux from platelets using Eq. A4 (Appendix A) for the three flow rates studied. In each case, the flux is highest at the inlet and decreases with distance from the tube's entrance. This is due to the overall effects of high accumulation and moderate release at the inlet and low accumulation and maximal release at the outlet.

B.3.4 Surface ADP Concentration (Longitudinal Dependence)

The ADP concentration results are presented in Figure B6. The surface concentration along the tube was relatively invariant for each flow rate. The range in magnitude was from 0.6 μM at 80 s^{-1} to 1.8 μM at 320 s^{-1}.

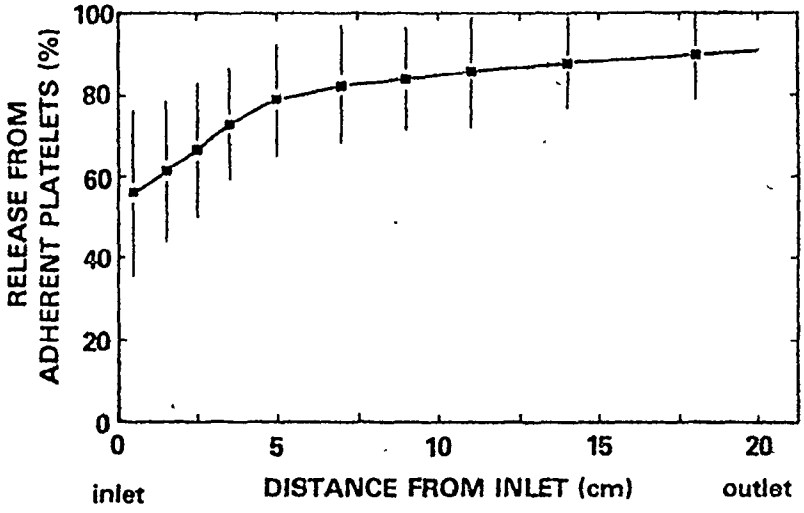

Figure B4: The change in release of dense granule contents from adherent platelets with distance from the inlet of a 1.0 mm internal diameter, collagen-coated, glass tube. Mean $\pm$ S.D. of pooled data (n = 46) for shear rates of 80, 160 and 320 s^{-1}.

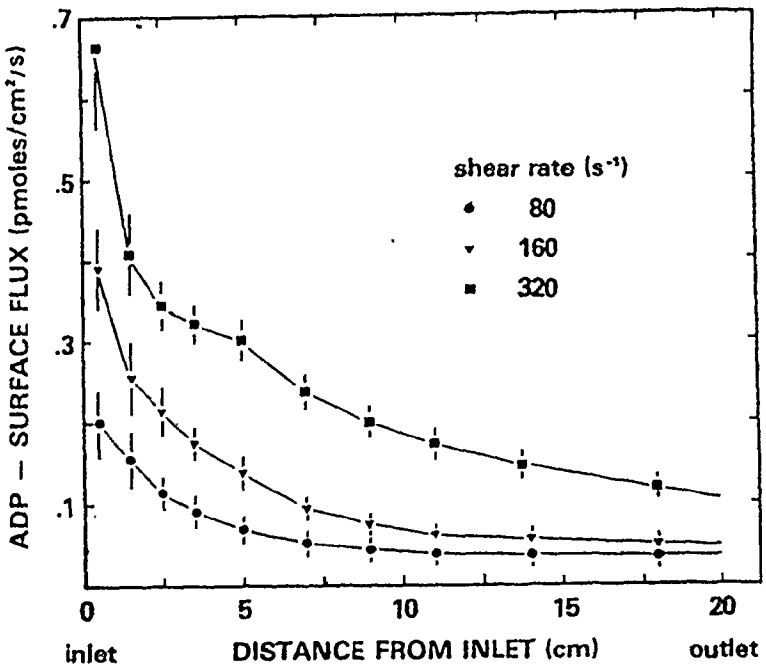

Figure B5: The calculated flux of ADP at the tube's surface from adherent platelets as a function of distance from the inlet of a 1.0 mm internal diameter, collagen-coated, glass tube. Mean $\pm$ S.E. at shear rates of 80 s^{-1}, n = 22; 160 s^{-1}, n = 10; 320 s^{-1}, n = 14.

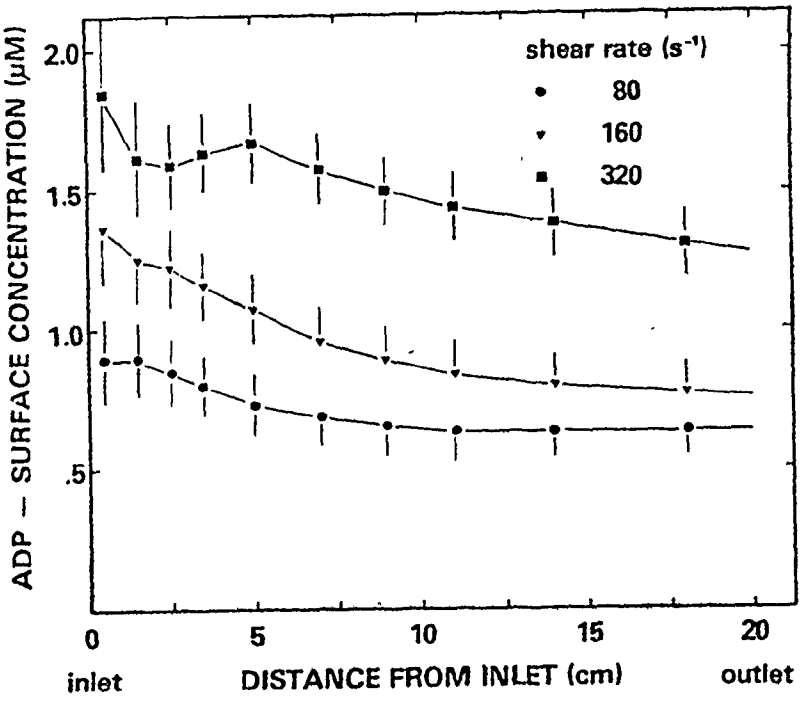

Figure B6: The concentration of ADP at the surface of a 1.0 mm diameter, collagen-coated, glass tube. Mean $\pm$ S.E. at shear rates of 80 s^{-1}, n = 22; 160 s^{-1}, n = 10; 320 s^{-1}, n = 14.

B.3.5 ADP from Red Blood Cells

The combined amount of ADP and ATP librated from red blood cells was determined by measuring the supernatant concentration of the red blood cell suspensions before and after exposure to the tube at a shear rate of 320 s^{-1}. The inlet supernatant concentration of ATP plus ADP was 0.089 $\pm$ 0.016 μM (mean $\pm$ S.D., n=19). The outlet supernatant concentration was 0.082 $\pm$ 0.015 (mean $\pm$ S.D., n=19). The paired difference (outlet minus inlet) was 0.000 $\pm$ 0.011 μM (mean $\pm$ 95% confidence interval, n=19) which was not significantly different from zero. The upper 95% confidence limit (0.011 μM) can be used as a representative upper bound to determine if there could be sufficient material to raise the surface concentration of ADP above that already present due to ADP from platelets. The procedure for this is outlined in the Theory section. The resultant flux of ADP from the tube's surface (EquationA6) would be 0.009 pmoles/cm^2/s. This results in calculated surface concentrations of 0.03 μM and 0.06 μM at the 1 cm and 20 cm positions (measured from the inlet) respectively for a shear rate of 320 s^{-1}. The corresponding surface concentrations due to platelet released ADP are 1.78 μM and 1.25 μM. It may then be surmised that ADP from red cells does not make a significant contribution to the local concentration of ADP adjacent to the tube's surface in the platelet experiments reported here.

B.3.6 Aggregations

The minimum concentration of stimulus for shape change, aggregation and release of ^{3}H-serotonin was evaluated in an aggregometer. The results are presented in Table B1 where the level of ADP required for shape change is 0.5 μM, and for aggregation in the presence of sub-threshold collagen is 0.5 μM. These levels are in agreement with a recently published study of porcine platelet suspensions (1).

B.4 Discussion

B.4.1 Platelet Accumulation

The accumulation versus distance from the inlet curve (Figure B1) shows a more rapid reduction in deposition with distance from the inlet than predicted by classical transport theory (2, 13). The accumulation level was found to be proportional to the distance from the inlet raised to the exponent -0.75 in this study as compared to -0.33 predicted by classical transport theory. Dr. Baumgartner's group reported an exponent of -0.17 (2) and Freidman and Leonard (13) obtained a -0.41 dependence. Some of the possible reasons for the greater dependence of platelet accumulation on distance from the inlet obtained in this study were investigated.

One possibility could be an effect of the removal of priming fluid by the incoming suspension. There will exist a lag time between the beginning of suspension flow and the commencement of platelet arrival at the surface. This lag period will be longer at the tube's

TABLE B1: MINIMUM CONCENTRATIONS OF STIMULI TO CAUSE PLATELETS TO RESPOND IN AN AGGREGOMETER+

STIMULUS	RESPONSE			
	Shape Change	10% Aggregation	80% Aggregation	10% Release
ADP (μM)	0.5	1.0	5.0	*
Collagen (μg/ml)	2.5	12.5	25.0	12.5
Collagen (μg/ml) + ADP (μM)	-	2.5 + 0.5	-	-

+ platelet concentration 5 x 10^8/ml; fibrinogen 1.5 μM.

* no release occurs with ADP for washed porcine platelets.

outlet and shorter at the inlet, according to a theory developed by Butruille et al. (9). However, a similar lag time would occur due to washout of the cell suspension adjacent to the surface with the rinsing fluid at the end of the exposure. The combination of these two processes results in a constant duration of exposure along the tube. This is supported by the data shown in Figure B2 where the platelet accumulation is linearly increasing with time for all axial positions. Dr. Baumgartner's group (2) similarly found no delay in platelet deposition in their annular flow device possibly due to the balancing of blood entrance and removal phenomena. It, therefore, is unlikely that washout phenomena are producing the larger than expected reduction in accumulation with distance from the inlet.

A second possibility could be the preferential deposition at the inlet of platelets that are more highly labelled with ^{51}Cr than those adhering downstream. This possibility was explored by visually counting stained platelets in a 2500 μm^2 area of tube's surface using a light microscope at 1250 X magnification. This accumulation level was compared with the one based on ^{51}Cr activity. The ratio of accumulation based on ^{51}Cr to accumulation based on microscopy was 0.95 ± 0.10 (mean + S.D.) at the inlet and 0.95 ± 0.14 (mean $\pm$ S.D.) at the outlet. This shows that the platelet accumulation was accurately described by ^{51}Cr activity and that the 10 to 1 ratio of platelet accumulation between inlet and outlet was not due to heterogeneous ^{51}Cr labelling of platelets.

A third possibility to explain the experimentally obtained

deposition pattern could be preferential accumulation at the inlet due to hydrodynamic entrance effects. The results obtained, using an inlet region to allow the suspension flow to become fully developed (Figure B3), showed that only at shear rates higher that those used in this study did platelets accumulate at the inlet excessively. The linearity of the curve in Figure B1, further indicates that a rapid decline in platelet accumulation is ongoing along the entire length of the tube and does not occur just at the inlet.

The linear increase in accumulation with time (Figure B2) is consistent with classical mass transport theory (2, 13). The linear increase in platelet deposition with time and the observation that these curves, extrapolated to zero pass through the origin, suggests that the 10 to 1 ratio of inlet to outlet accumulation levels would be present at short times and perhaps at the onset of adhesion. The exact reason for this decline in platelet accumulation then remains obscure. Other possible mechanisms are: preferential aggregate formation at the tube's entrance, embolization, preferential adhesion of a sub-population of platelets and a platelet-derived inhibitor of accumulation which has an increased downstream concentration.

The effect of shear rate on the rate of platelet accumulation was determined by plotting the logarithms of these parameters. In order to compare our results with those of others who have worked with shorter tube lengths, plots of the logarithm of accumulation rate for the first 1 cm segment of tubing against the logarithm of shear rate were prepared (plots not shown). The resultant straight line had a slope of 0.77.

This is in agreement with Butruille et al (8) and Baumgartner et al. (2) who obtained values of 0.75 and 0.61, respectively. Classical transport theory predicts that the flux of a substance to a surface, or in this case the rate of platelet accumulation, is proportional to the 0.33 power of the shear rate (2, 13). Inherent in clasical transport theory for flow in tubes is a constant diffusion coefficient. Platelet diffusion is enhanced in the presence of red cells (15) and by increasing the shear rate (2, 8). The difference between the 0.77 power dependence of shear rate upon the rate of platelet accumulation obtained here and the 0.33 classical value may be a result of the presence of red blood cells and their effect on platelet diffusivity.

B.4.2 Release

The release data (Figure B4) shows that the degree of release from adherent platelets at the inlet of a tube is lower than that at the outlet. This suggests that the possibility some material, released by the platelets, is convected downstream inducing platelets adhering there to release more extensively. The degree of release being dependent on the local concentration of released material. It does not appear that the change in release with distance from the inlet (Figure B4) can be directly related to the surface concentration of ADP, or any other dense granule material as these quantities change little with distance from the inlet (Figure B6). It may then be that other factors are working in parallel with released substances to produce the observed release pattern. Another possibility could be the time dependent sensitization

of platelets by the released ADP. The platelets that had longer periods of incubation may have had time to become fully sensitive to the collagen stimulus while those incubated for a short time may not have been fully sensitized. In this work, release was found to be independent of shear rate. The degree of release could then be dependent on the time from entry into the tube and thus the distance from the inlet since cells which adhere downstream are exposed to ADP longer than cells which adhere upstream. This scheme is consistent with the observed platelet release pattern along the tube. Platelets aggregate and release in response to collagen more extensively when small concentrations of ADP are present than in the absence of ADP (Table B1). The time required for sensitization by ADP incubation has not been studied in detail.

A third possibility may be that released agents from other granules or progressively secreted agents such as thromboxane A_2 could be modulating release. The concentration of such a material may vary with distance from the inlet in some manner other than that shown for dense-granule materials.

B.4.3 Surface Concentration

Although the conditions for aggregometer studies are different from those of our tube flow studies, concentration-response data from this device are the only information available for comparison with concentrationresponse information from this study. The calculated values of ADP surface concentration, 0.6 μm to 1.8 μM, are in the range

where ADP and collagen act synergistically to aggregate platelets (Table B1). Since both of these stimuli are available in our system, we may surmise that platelet shape change could have occured adjacent to the surface in preparation for adhesion, aggregation and release at the surface and that ADP and collagen acting together produced the accumulation and release demonstrated. Grabowski et al. (16) have shown that a concentration of 1.5 μM ADP, obtained by infusion of ADP through a semi-permeable membrane, will cause thrombus growth on a cuprophane surface, provided a layer of platelets was already on the surface. The ADP released from platelets or red blood cells was not taken into consideration by these workers. Studies using glass bead columns have identified the platelet as the principle source of ADP in retention experiments (22). Other workers maintain that ADP from red blood cells is important in thrombogenesis (7) while others feel they serve a physical purpose (2). The contribution of red blood cell ADP to the surface concentration for the studies presented here, was shown to be at least an order of magnitude less than that for platelet derived ADP.

A number of synergistic pairs of platelet stimulatory agents have been reported (20, 27). Although the concentration of any individual released material may not be greatly above the threshold required for platelet response, the combined effects of two or more agents at subthreshold concentrations may be very significant. More detailed experiments dealing with combined effects of the various granule materials should be undertaken especially in regard to their synergistic nature in pairs and higher number of combinations, as this represents as

reasonable a picture of the physiological process as the higher concentrations of single stimuli normally used in aggregometer studies.

The increase of surface concentration with increasing shear rate is an interesting phenomenon. The final surface concentration of ADP depends on the rate of liberation of the material and the ability of the flowing blood to carry the released material away. Increasing the shear rate increases the rate of accumulation and, hence, the flux of ADP. The experimentally determined dependence of the rate of adhesion on shear rate is a power law with exponent 0.77. The ability of the fluid to remove ADP at the same surface concentration would also increase with increasing shear rate although the exact relationship is unknown. Since the calculated surface concentration rose on increasing the shear rate, the increased ability to remove ADP by increased shear rate was insufficient to cope with an increased rate of liberation of material. The greater enhancement of platelet deposition due to increased shear rates over removal of ADP due to increased shear rate necessitated an increase in surface concentration to maintain steady state. This result suggests that arteries should be more dependent on release-induced aggregation than veins where the shear rates are lower.

Weiss et al (32) have reported a platelet adhesion defect in blood from patients with von Willebrand disease, which was only demonstrable at high shear rates. These workers suggested that the high shear rates result in maximal arrival of platelets at the surface making the rate of adherence of the platelets to the surface the rate limiting step (32). Since platelets contain von Willebrand factor (26), it may

be hypothesized, based on the work presented here, that the release of this protein could be producing the difference between normal and von Willebrand factor defficient platelets by enhancing the adhesion rate of normal cells at high shear rates. The experiments and calculations in this report predict that higher surface concentrations of released materials would be present at higher shear rates. The ability of von Willebrand platelets to adhere to collagen as well as normal cells at low shear rates (2, 32) suggests that von Willebrand factor is not required for normal platelet adhesion at low shear rates.

B.5 Summary

These results are pertinent to the process of thrombosis in blood vessels and on biomaterials, and to hemostasis. Calculations based on experimental data show that ADP released from platelets produces sufficiently high concentrations at a potential site of injury to induce platelet shape change but not platelet aggregation. However, trace amounts of other released agents or vessel wall constituents such as collagen may act synergistically to aggregate platelets. The concentration of ADP does not change quickly along the surface. Higher shear rates result in enhanced arrival of platelets at the surface and higher concentrations of released materials. The amount of ADP released from red blood cells was found to be negligible relative to that released from platelets. The proceding results suggest that the contribution of platelet released agents to thrombosis is quite possibly through synergistic action of combinations of agents acting close to the site of thrombus formation.

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