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Experimental and mathematical analyses of mural thrombogenesis under flow

Folie, Bernard Jean, Ph.D.

Rice University, 1989
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EXPERIMENTAL AND MATHEMATICAL ANALYSES
OF MURAL THROMBOGENESIS UNDER FLOW

by

BERNARD J. FOLIE

A THESIS SUBMITTED
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DOCTOR OF PHILOSOPHY

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ABSTRACT

An epifluorescence video microscopy system, coupled with digital image processing techniques, and a computerized microphotometric measurement system were used to visualize and analyze the effects of a novel antiplatelet agent (GT-12), a carbamoylpeperidine congener, and a novel anti-von Willebrand Factor (ATA) agent, a triphenyl-methyl compound, on surface platelet aggregation and on the kinetics of thrombus growth induced by collagen under controlled flow. In addition, the relative reactivity of the various human collagen subtypes found in the vasculature (types I, III, IV, V and VI) towards human blood platelets was also investigated as a function of wall shear rate. Finally, the system was used to assess the roles played by plasma von Willebrand Factor (vWF), endogenous vWF released from platelets' α-granules, and the unusually large vWF multimers secreted by endothelial cells in mural thrombogenesis under different flow conditions. A parallel plate flow chamber, 200 μm-thick, served as the model blood vessel in those studies.

Both macroscopic and microscopic measurements revealed that increasing concentrations of GT-12 correspondingly decreased the reaction rate between platelets at the surface, thereby reducing thrombus rate of growth at the surface. In the presence of GT-12 average thrombus size and number of platelets per thrombus were both strikingly lowered. The drug's effectiveness relative to controls in impeding
platelet/platelet interactions was found to increase with decreasing incubation time and increasing perfusion time. Similarly, ATA was found to reduce significantly both thrombus rate of growth and size, when compared to controls, in a dose-dependent manner. At low concentrations (< 115 \mu M), platelet adhesion to collagen was not as readily affected by the drug as platelet to platelet adhesion.

Both macroscopic and microscopic measurements indicated that, in native-fibrillar form, type I collagen is the most thrombogenic of the collagens at high wall shear rate (1500 sec\(^{-1}\)), while type III is the most reactive at low (100 sec\(^{-1}\)) and intermediate (800 sec\(^{-1}\)) wall shear rates. Whereas platelet surface deposition continually increased on type I and VI collagen fibrils with increasing wall shear rates, it reached a maximum value at 800 sec\(^{-1}\) on types III, IV and V fibrils. Type V fibrillar collagen was found to be the least thrombogenic among the collagens of the vasculature at all wall shear rates considered in this study. There were significant platelet surface deposition defects on collagen with heparinized whole blood of a patient with severe von Willebrand's disease. Those defects were especially important at high wall shear rates (\(\geq 1500 \text{ sec}^{-1}\)). Whereas platelet-released vWF slightly augmented platelet surface deposition on collagen at low wall shear rate (100 sec\(^{-1}\)), no conclusive evidence on the effect of the unusually large vWF multimers has been found in this study.

The concentration profiles of adenosine diphosphate (ADP), thromboxane A\(_2\) (TxA\(_2\)), thrombin, and von Willebrand factor (vWF) released extracellularly from the platelet granules or produced metabolically on the platelet membrane during thrombus growth, were estimated using finite element simulation of blood flow over model thrombi of various shapes and dimensions. It was found that thrombin concentrations were large
enough to cause irreversible platelet aggregation, even in presence of heparin. While ADP concentrations were large enough to cause irreversible platelet aggregation at low shear rates and for small aggregate sizes, TxA2 concentrations were only sufficient to induce platelet shape change over the entire range of wall shear rates and thrombi dimensions studied. The local concentration of vWF multimers released from the platelet α-granules may be sufficient to modulate platelet aggregation at low and intermediate wall shear rates (< 1000 sec⁻¹). It was also found that standing vortices developed on both sides of the thrombus even at low wall shear rates. Their sizes increased with thrombus size and wall shear rate, and were largely dependent upon thrombus geometry. The average shear stress and normal stress as well as the torque, acting to detach the thrombus, increased with increasing wall shear rate. Both stresses were found to be nearly independent of thrombus size and only weakly dependent upon thrombus geometry. Although both stresses had similar values at low wall shear rates, the average shear stress became the predominant embolizing stress at high wall shear rates.

Platelet wall fluxes were predicted by finite element simulation of platelet transport in flowing whole blood, coupled with platelet reaction at the wall, and compared with experimentally-measured platelet wall fluxes obtained under identical flow conditions. It was found that the lateral convective term is only of minor importance compared to the diffusive term in modeling platelet transport. For a bulk hematocrit of 30% and a wall shear rate of 500 sec⁻¹, the average effective platelet diffusivity in best agreement with the experimental data was estimated to be two orders of magnitude larger than the Brownian diffusivity. The enhanced platelet transport to the surface probably occurred through an "impeded" red blood cell rotation mechanism.
To Françoise
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CHAPTER 1
INTRODUCTION

1.1 General Characteristics of Platelets

Platelets originate from the cytoplasm of bone marrow megakaryocytes, and their formation is regulated by a humoral mediator, thrombopoetin. Each megakaryocyte cell can generate between 1,000 to 1,500 platelets.

In normal blood, platelets circulate as flat discs with a diameter of 1 to 3 μm, a mean volume of 10 μm³, and in concentrations ranging from 180,000 to 400,000 per μl. Their lifespan is about 9 to 10 days, during which time they undergo changes in both biochemical composition and function. Indeed, as platelets age, they undergo progressive reduction in proteins, phospholipids, cholesterol, ATP, etc. and become less capable to aggregate in response to stimuli. Platelets are eventually removed from the circulation by the reticuloendothelial system in the spleen and liver. Studies using platelets labelled with $^{51}$Cr and $^{111}$In have indicated that platelet removal is predominantly an age-related process (Weiss, 1982).

1.2 Platelet Morphology
Platelets are covered with an amorphous coat, 10-20 nm thick, absent in RBCs and leukocytes. The platelet's plasma membrane is composed of a lipid bilayer, with sphingomyelin located primarily on the outer layer of the membrane, phosphatidylcholine (PC) located symmetrically across the bilayer, and the acidic phospholipids phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) located predominantly in the inner half of the bilayer. Interpolated between the phospholipids are cholesterol molecules which play an important role in the fluidity of the membrane in conjunction with the saturation of the fatty acids of the phospholipids. The more cholesterol the greater the membrane viscosity and the more saturated the fatty acids the greater the stability. Platelet surfaces contain many invaginations that form a network of tortuous channels through the platelet cytoplasm, called the surface connecting (or canicular) system (SCS). It essentially serves as a conduit for the uptake of substances found in plasma and for the extrusion of granule-bound secretory substances during the release reaction. Interdigitating with the SCS is a system of narrow tubules known as the dense tubular system (DTS), which serves to take up and sequester Ca\(^{2+}\) in presence of ATP in a very effective manner. At least five different glycoproteins (Gp) have been identified on the platelet membrane. Gplb (missing in Bernard-Soulier syndrome) is involved mostly in platelet adhesion to the subendothelium; Gplb and GpIIia (absent in thrombasthenia) are involved in platelet-platelet adhesion; GpV binds thrombin very specifically; finally, there is GplV whose function is not known. In addition, the platelet membrane contains receptors for a number of other substances known to react with platelets, such as ADP, serotonin, fibrinogen, factor VIII/von Willebrand Factor (vWF), and the coagulation
factors Va and Xa.

The platelet cytosol contains two major fiber systems, the microtubules and submembranous filaments of actin and myosin immediately adjacent to the unit membrane, which act to support the discoid shape of unaltered platelets and provide a contractile mechanism involved in shape change, pseudopod formation, and secretion.

Two major types of granules, known as the α-granules and the dense granules, can be found in platelets. The dense granules contain serotonin absorbed from plasma, 65% of the total platelet content of adenines nucleotides (ADP and ATP), and 60 to 70% of the platelets' Ca²⁺ and pyrophosphates. Studies have shown that, when platelets are stimulated by thrombin or collagen, the nucleotides released are derived entirely from the granules. Therefore, a defect in platelet aggregation can be expected in patients with congenital storage pool deficiency, lacking most granules-bound proaggregatory substances, such as ADP. The α-granules, on the other hand, contain two platelet-specific proteins that bind heparin, platelet factor 4 (PF4) and β-thromboglobulin (βTG), a cationic growth factor, the platelet-derived growth factor (PDGF), a number of coagulation proteins including fibrinogen, fibronectin, factor V, and factor VIII/vWF, a glycoprotein, thrombospondin, and other cationic proteins, including a protein which increases vascular permeability, a chemotactic factor, and a bactericidal factor. Other subcellular compartments, such as lysosomes, mitochondria, clumps of glycogen granules, peroxisomes, and vesicles, can be found in the platelet cytosol as well. The various morphological components of blood platelets are shown diagrammatically in Figure 1.1 (Weiss, 1982; Firkin, 1984).
Figure 1.1: Platelet morphology: longitudinal (top) and coronal (bottom) sections. (From Firkin, 1984).
1.3 Platelet Functions

When blood platelets flow near the subendothelium of an injured blood vessel, an artificial polymeric surface used in a prosthetic blood-contacting device, or an atherosclerotic lesion, they undergo a variety of changes which collectively may be called platelet activation. Four general types of interrelated platelet responses have been observed: shape change, involving the transformation of a platelet from a flat disc to a "spiny" sphere, perhaps by reversion of the SCS, adhesion, a process by which platelets adhere to "foreign" surfaces, aggregation, a process by which platelets adhere to each other, and secretion, a process by which granules-bound substances are released extracellularly.

Although platelets do not adhere to intact endothelium, they do adhere on several components of the subendothelium (Baumgartner et al., 1980a), including collagen fibers and microfibrils associated with elastin, and on the basement membrane. Several studies have shown that collagen is probably the most reactive material in the vessel wall. The precise mechanism by which platelets adhere to the subendothelium in general and to collagen in particular is not well understood. In one theory, it was suggested that rigidly-spaced polar groups, particularly the ε-amino groups of lysine, are required for collagen to support platelet adhesion. Other mechanisms have been proposed, but no convincing evidence has been found to favor any of them. It should be pointed out, however, that all surfaces exposed to blood are coated within a few seconds with certain plasma proteins, which may influence their attractiveness towards platelets. For instance, both gamma globulin and fibrinogen absorbed onto glass surfaces greatly
enhance platelet surface deposition, whereas precoating with albumin has the opposite effect (Weiss, 1982).

A number of substances are known to induce platelet aggregation including ADP, epinephrine, serotonin, TxA$_2$, arachidonic acid, Ca$^{2+}$ ionophores, thrombin, trypsin, collagen, ristocitin, etc. Epinephrine and ADP induce secretion by an aggregation-mediated response; this secretion can be blocked by preventing aggregation, for instance, by not stirring the platelet-rich plasma suspension or by using inhibitors to the prostaglandin pathways, such as aspirin. Stronger agonists, such as collagen and thrombin, may cause secretion without inducing aggregation. In general, the initial step probably involves the binding of the proaggregatory substance to its receptor(s) on the platelet plasma membrane. The formation of this agonist-receptor complex initiates a signal which somehow renders the platelets more attractive to each other, little being known on this last step.

In general, secretion is induced in platelets by one of the following processes: (a) aggregating agents, such as ADP and epinephrine, (b) platelet to surface interaction, as with collagen, and (c) another mechanism as with thrombin or the divalent cation ionophore A23187. It is likely that platelet secretion shares some of the characteristics of secretory processes in other cells. In endocrine cells, for example, secretion, also called exocytosis, is associated with movement of the secretory granules to the cell surface, fusion of granules and cell membranes, and extrusion of the granule contents through openings in the fused membranes (Weiss, 1982; Firkin, 1984; Report of the National Heart, Lung, and Blood Institute Working Group, 1985a).
1.4 Regulatory Mechanisms

The various metabolic processes that regulate platelet responses, including those involving Ca$^{2+}$, cyclic AMP, platelet prostaglandins, and endothelial cell PGI$_2$, are briefly reviewed in this section.

The role of Ca$^{2+}$ in mediating platelet reaction is complex. Indeed, whereas the initial change in shape occurs in the absence of Ca$^{2+}$, platelet aggregation induced by most physiological agents has an absolute requirement for Ca$^{2+}$. An increase in cytoplasmic Ca$^{2+}$, released from its intracellular storage sites in the platelet membrane, induces the release reaction. The precise mechanism by which Ca$^{2+}$ induces platelet activation probably involves activation of actomyosin-mediated contractile mechanisms in platelets. Actomyosin constitutes about 15 to 20 % of the total protein content in platelets, and many cellular activities, including pseudopod formation, aggregation, secretion, and clot retraction, have been attributed to it. It is associated with the formation of actin-like microfilaments in stimulated platelets.

Platelet cAMP may be increased by agents which either increase the activity of adenyl cyclase, an enzyme that catalyses the conversion of ATP to cAMP, or decrease the activity of phosphodiesterase, an enzyme that hydrolizes cAMP to ATP. It is thought that cAMP inhibits both platelet secretion and aggregation by regulating the storage and release of Ca$^{2+}$ from its storage sites in the platelet plasma membrane into the cytosol. In fact, it has been postulated that the effects of cAMP depend on a protein kinase which phosphorylates myosin kinase, decreasing this protein's ability to bind calmodulin. Increases in free Ca$^{2+}$ in the cytosol lead to the formation of calmodulin-Ca$^{2+}$
complexes, which may activate the inactive myosin kinase. This enzyme may then interact with actin causing contraction and the release reaction.

Prostaglandins are produced metabolically in stimulated platelets. In the first step, arachidonic acid (AA) is hydrolyzed from membrane phospholipids by the phospholipases. The proposed two major phospholipid sources of AA are PE, which liberates AA by the action of the calmodulin-modulated enzyme, phospholipase A$_2$, and PI, which liberates AA in two steps, phospholipase C releasing diglyceride which is then attacked by diglyceride lipase and monoglyceride lipase to produce free AA. The AA liberated is then converted by a cyclo-oxygenase enzyme to the endoperoxides intermediates, PGG$_2$ and PGH$_2$, which are potent platelet aggregating agents, or through the lipoxygenase pathway, to 12-hydroxyeicosatetraenoic acid, HETE, a chemotactic factor which may result in the attraction of leukocytes at the site of platelet aggregation. PGH$_2$ can be converted to other prostaglandins, PGE$_2$ and PGF$_2\alpha$, which do not directly aggregate platelets, but may potentiate aggregation by other substances, PGD$_2$, which is an inhibitor of platelet aggregation, HHT, another chemotactic factor, and malonaldehyde.

The most important transformation of PGH$_2$ is to the nonprostaglandin compound thromboxane A$_2$ (TxA$_2$), the most powerful platelet-activating agent derived from AA and a potent vasoconstrictor, capable of reducing vessel diameter and flow. TxA$_2$ is very unstable and is rapidly converted in 10 to 30 seconds to TxB$_2$. TxA$_2$ inhibits the cAMP increases stimulated by substances such as PGE$_1$ and PGI$_2$, by inhibiting their effect on adenyl cyclase. Since Ca$^{2+}$ also inhibits PGI$_2$-stimulated cAMP increases in platelets,
\( \text{TxA}_2 \) could also act by releasing \( \text{Ca}^{2+} \) from its bound state in the platelet membrane, thus inhibiting adenyl cyclase. The AA pathways are shown in Figure 1.2.

Prostacyclin or \( \text{PGI}_2 \) is known as a very potent platelet aggregation inhibitor, due to its ability to stimulate platelet cAMP through activation of adenyl cyclase, and as a vasodilator. It is synthesized by ECs from AA or \( \text{PGH}_2 \). This compound is very unstable and is rapidly converted to the end product, 6-keto-\( \text{PGF}_{1\alpha} \). The ECs may be stimulated to produce \( \text{PGI}_2 \) by exposure to thrombin, AA, the ionophore A23187, trypsin, or mechanical stress. \( \text{PGI}_2 \) produced in the blood vessels may play an important role in regulating platelet behavior in vivo and in preventing platelet deposition on vascular walls. \( \text{PGI}_2 \) production can be inhibited by lipid peroxides, such as may be found in advanced atherosclerotic lesions, vitamin E deficiency, aging, etc. (Weiss, 1982; Firkin, 1984; Report of the National Heart, Lung, and Blood Institute Working Group, 1985a).

1.5 Roles of Platelets in the Coagulation Pathways

Both the intrinsic and extrinsic coagulation pathways lead to the activation of factor \( X \) and the subsequent conversion of prothrombin to thrombin on the platelet surface. The extrinsic pathway is independent of platelets and is unique in that it requires tissue factor thromboplastin and factor VII for the activation of factor \( X \), neither of those are required in the intrinsic pathway. Platelets, on the other hand, are required in the intrinsic pathway, which consists in a cascade of clotting factors being
Figure 1.2: The arachidonic acid pathways in platelets. (From Firkin, 1984).
activated, commencing with the activation of factor XII (or contact factor) and ending with the activation of factors X and V. The platelet activity required in this pathway is referred to as platelet factor 3 (PF3) and has been attributed to phospholipids or lipoproteins present in platelet membranes and granules. Indeed, platelet membrane phospholipids are required in the reaction by which factor IXa, factor VIII, and Ca\(^{2+}\) activate factor X, and also in the reaction by which factor Xa, factor Va, and Ca\(^{2+}\) activate prothrombin to form thrombin. Although the classic clotting pathways are important in the diagnosis of hemostatic defects, they appear to be less relevant in the hemostatic and thrombotic processes in man, which are now considered by many to occur as solid phase surface reactions involving predominantly the platelet (Firkin, 1984).

1.6 Physiology of Hemostasis

Blood platelets have been recognized as the major contributors in the normal hemostatic mechanism for decades (Bounameaux, 1959; Spaet and Zucker, 1964; Sixma and Wester, 1977). This mechanism is better understood if divided arbitrarily in several steps. The first step involves platelet adhesion to the exposed collagen fibers or to other microfibrils lying adjacent to the vessel, and requires plasma vWF and GpIb on the platelet membrane. The second step is called primary reversible aggregation and describes platelet aggregation before the onset of the release reaction. This process is triggered by the collagen fibers of the blood vessel wall, by release of ADP either from the damaged tissues or from lysed RBCs, by small amounts of thrombin generated by the extrinsic coagulation pathway, or by other metabolites such as epinephrine and
serotonin. The third phase is irreversible aggregation which results after the release reaction has occurred, and includes the secretion of the prostaglandins metabolites and of granule-bound substances. The last step involves the formation of fibrin from thrombin which consolidates the platelet plug.

There are a number of safety mechanisms that prevent widespread thrombosis. First, there is stasis which prevents fresh platelets from arriving at the site of trauma and results in the local consumption of all the clotting factors so that further thrombin and fibrin can not be produced. Other mechanisms include elevation of cAMP levels, degradation of ADP on the surface of ECs by the ADPases, release of the aggregation inhibitor, 2,3-diphosphoglycerate, from lysed RBCs, inactivation of thrombin by binding to ECs and fibrin strands, or by the plasma factors, antithrombin III (AT III) and α2-macroglobulin, release of PG12 by damaged ECs, or also thrombin-mediated or thrombomodulin-mediated activation of protein C, which inactivates the important clotting factors V and VIII. Finally, plasmin can also be produced from its precursor plasminogen, bound to the fibrin strands in the thrombus, by the action of tissue plasminogen activator released from ECs, causing the dissolution of the thrombus. A summary of the interactions between the thrombotic agents and their regulators is shown in Figure 1.3 (Firkin,1984; Report of the National Heart, Lung, and Blood Institute Working Group, 1985a).

1.7 Thrombosis

Platelets are involved in thrombosis caused by inappropriate activation or
Figure 1.3: Relationships between the thrombotic agents and their regulators during thrombus formation on injured endothelium. (From Report of the National Heart, Lung, and Blood Institute Working Group, 1985a).
defective modulation of the normal hemostatic mechanism (Hirsh and Brain; 1983). There are three major contributors to the formation of a thrombus: alteration in blood flow, damage to the vessel wall, and enhanced coagulability of the blood. Venous thrombi are usually different in appearance than arterial thrombi, because of the different rheological conditions under which they form. Because of the relatively slow wall shear rates in the veins, the blood cells (RBCs, platelets, and leukocytes) are distributed randomly throughout the fibrin mesh of the thrombus, which resembles a traditional blood clot. In the arteries, on the other hand, the high shear rates favor the diffusion of platelets to the vessel wall through mechanisms involving RBCs, and, consequently, arterial thrombi are composed essentially of platelets with only a few RBCs and leukocytes trapped in the fibrin mesh on the top of the thrombus. In addition, whereas venous thrombi usually begin in valve pockets, where rheological conditions favor formation of relatively stagnant pools of blood, arterial thrombi form predominantly at arterial bifurcations, where local shear stresses are elevated and endothelial injury is more likely to occur (Weiss, 1982).

1.8 Roles of Platelets in Atherosclerosis

Atherosclerosis is a process characterized by localized lesions in the aorta and large distributing arteries consisting of raised thickenings or plaques containing fibrous and fatty materials. Platelets contribute to this process by either forming mural thrombi which are covered by ECs and incorporated in the vessel wall, or by secreting a growth factor, the PDGF, which stimulates the proliferation of smooth muscle cells
(Weiss, 1982; Sussman, 1985).

1.9 Thesis Overview

Because of the crucial role played by blood platelets in such life-threatening diseases as thrombosis and atherosclerosis, it is important to develop the technology that would allow one to visualize and quantify the processes of platelet adhesion and aggregation on various substrates under physiological flow conditions. In order to study the cellular and hemodynamic mechanisms involved in mural thrombogenesis "in vitro", one would desire to perfuse whole blood inside a model blood vessel under well-defined rheological conditions, and, at the same time, to analyze the microscopic phenomena associated with individual thrombus formation as well as the macroscopic dependence of platelet surface deposition on axial position along the reactive surface. The microscopic analysis provides precious information on the intercellular and cell-surface interactions, while the macroscopic analysis give further insights into the coupling of bulk platelet mass transport and surface reaction kinetics. Such a technology would allow one to test antithrombotic agents, to investigate the thrombogenicity of various purified components of the blood vessel walls or of polymeric materials with potential use in artificial organs, or yet to identify key proteins involved in the adhesion and/or aggregation processes.

With those objectives in mind, a parallel plate flow chamber, a video microscopy equipment, and a microphotometric measurement system were developed and/or used in this study to analyze quantitatively and qualitatively the process of mural
thrombogenesis on a surface in contact with flowing whole blood. Chapter 2 gives a
detailed description of the apparatus, the experimental techniques, and the data analysis
methods. Chapters 3 and 4 are two application chapters with mostly experimental
results obtained with the above equipment. In Chapter 3, the effects of two novel
antithrombotic agents, an antiplatelet agent and an anti-vWF compound, on mural
thrombogenesis on bovine collagen-coated glass are reported. In Chapter 4, the
thrombogenicity of the various subtypes of human collagen found in the vasculature was
tested under different flow conditions, spanning the physiological range. In addition,
results from preliminary experiments performed to investigate the role played by vWF
in mural thrombogenesis under flow are also reported in that chapter. Because
hemodynamic considerations are often crucial to the thrombosis process in vivo,
accurate mathematical models are needed if one is to understand the physical forces
affecting, for example, the transport of cells and macromolecules involved in thrombus
formation, the local concentration of those substances at the site of thrombosis, or the
stability of the thrombus forming at the surface. In Chapter 5, a microscopic
mathematical model was developed to investigate the importance of several key
platelet-activating agents, released extracellularly from a growing thrombus, in the
process of thrombus growth, and to study the effects of viscous shear flow, from a fluid
mechanical point of view, on thrombus growth and stability. A macroscopic mathematical
model of platelet transport in flowing whole blood, aimed at determining the mechanisms
involved in the augmentation of platelet transport from the fluid bulk to the reactive
surface during the thrombosis process, is presented in Chapter 6. Finally, several
suggestions for the extension of this work are presented in Chapter 7.
CHAPTER 2

MATERIALS AND METHODS

2.1 Blood Sample Preparation

Blood was collected from the antecubital vein of consenting donors into 10 units/ml of heparin (anticoagulant; liquaemin sodium, Elkins-Sinn Inc., Cherry Hill, NJ) and 10 μmolar mepacrine (fluorescent probe; quinacrine dihydrochloride; Sigma Chemical Co., St. Louis, MO). The fluorescent probe, mepacrine, has an absorbance peak at 440 nm and an emission peak at 505 nm, and is actively taken up by the platelets' dense granules (Adams et al., 1983; Feuerstein and Kush, 1985; Hubbell and McIntire, 1986b,c). Since the dye did not contaminate significantly the red blood cells (RBCs) or the leukocytes, it was used to directly label platelets in whole blood, eliminating the need of washing platelets, as required by most other techniques (Houdijk et al., 1985; Baumgartner et al., 1980a,b). Mepacrine is known to affect platelet aggregation by interfering with the mobilization of intracellular Ca$^{2+}$ and/or by penetrating the platelet membrane and interacting with negatively charged phospholipids, phospholipase A$_2$ and phospholipase C (Lasslo, 1984). At low concentrations, however, it was reported not to affect platelet function (Dise et al., 1982). The donors were carefully chosen as being non-smokers and aspirin-free for at least two weeks prior to the day of the experiment. Blood was then immediately poured into polypropylene test tubes (Sarstedt
Inc., Princeton, NJ) and kept at room temperature. Thirty minutes before perfusion the blood sample was immersed in a water bath (Precision Scientific Group, Chicago, IL) maintained at 37°C. Blood was always used within 3 hours after venipuncture.

2.2 Perfusion Chamber

Whole blood was aspirated by a syringe pump (Model 935, Harvard Apparatus, South Natick, MA) at a controlled wall shear rate from a test tube into a parallel plate flow chamber, in order to establish a well-defined fluid-dynamic and rheologic environment, suitable for precise analysis of mural thrombogenesis. The test tube, the tubing, and the flow chamber were maintained at 37°C by a thermostatic air bath (Model 279, Laboratory Products, Boston, MA). The flow chamber, shown in detail in Figure 2.1, consisted of a polycarbonate distributor, a plastic gasket, a glass cover slip and a metallic base plate. Before being assembled as part of the flow chamber, the glass cover slip (No.1, 24 x 50 mm; Corning Glass Works, Corning, NY) was coated with the material of interest. This coated-glass cover slip, together with the gasket and the base plate, formed the bottom part of the flow chamber. The base plate was made of stainless steel, 3.2 mm thick, and contained a precision milled lower deck, 0.38 mm deep. The plastic gasket was made of 0.01 inch non-reinforced Silastic brand medical-grade silicone rubber (Dow Corning, Midland, MI). The cover slip and gasket fitted into the deck of the base plate, as shown in Figure 2.1. The distributor, in turn, was made of 12.7 mm-thick polycarbonate sheet and formed the upper part of the flow chamber. Six nuts and bolts were used to tighten the distributor to the base plate. The assembled flow
Figure 2.1: Parallel plate flow chamber used for whole blood perfusion studies in a well-defined and controlled hemodynamic environment.
chamber, with a final slit width of 205 μm, served as a model of an injured blood vessel of the size of a small artery or vein. Assuming that the fluid entered the flow chamber as a Newtonian fluid in a fully-developed laminar flow regime with a typical parabolic profile, the wall shear rate was calculated from (Bird et al, 1960):

\[ \dot{\gamma}_{\text{wall}} = 1.5 \times \frac{Q}{B^2 W} \]

where W is the width of the flow chamber (1.27 cm); B is half the thickness of the flow chamber (102.5 μm); and Q is the volumetric flow rate.

2.3 Microscopic Measurements

2.3.1 Video Microscopy Equipment

The flow chamber was mounted on an inverted-stage microscope (DIAPHOT-TMD; Nikon, Garden City, NY) equipped with an epi-fluorescent illumination attachment (TMD-EF; Nikon), a high-power 100 magnification oil immersion objective (CF Achromat, numerical aperture [NA] = 1.25 to 0.9; Nikon), and a silicon-intensified target (SiT) camera (Model C-1012; Hamamatsu, Waltham, MA) suitable for very low light levels and equipped with gain and offset adjustments for control of the video signal and a 5 x projection lens. For those experiments, it was necessary to operate in a low light level environment in order to use low fluorophore concentrations, which has the advantage of not altering platelet function (Dise et al., 1982) and of reducing the rate of photobleaching (Hubbell, 1986). The microscopy was performed in EPI or
incident-light mode due to the opacity of whole blood. In this system, the UV-light, powered by a high pressure mercury lamp (HBO 100W/2; OSRAM GmbH Berlin-Munchen, Germany), is first filtered by a 400 nm interference filter (IF) before passing through a standard Nikon BV filter cassette. This cassette is made of an IF (400-450 nm), a dichroic mirror which reflects light with wavelength smaller than 455 nm and passes light with wavelength greater than 455 nm, and an eyepiece-side absorption filter (480 nm). In epi-illumination, the high-frequency excitation light is reflected by the mirror into the objective and then absorbed by the fluorescent objects. The low-frequency emitted light is collected back by the objective, passes through the mirror and is focused through the eye pieces or on the tube of the SIT camera. The experiments were recorded real-time on a 1/2 " color video cassette recorder (Model BR-3100U, JVC Audio/Video, Houston, TX) or a 3/4 " video editing system (Sony, Park Ridge, NY). At the same time, the video signal coming out of the camera control unit (Vidicon Camera C-1000, Hamamatsu, Waltham, MA) was displayed on two black and white monitors (Model VM 4509; Sanyo, Compton, CA), one color monitor (Model VM-8PRW; Videotek, Pottstown, PA), and a waveform monitor (Model TSM-5A; Videotek, Pottstown, PA), an instrument necessary for fine tuning the optimum gain and offset levels of the video signal. A time-base corrector (Model 690; Harris, Sunnyvale, CA) was used to electronically correct the improper timing relationships of the synchronization signals coming out of the 1/2 " VCR and created by mechanical and electronic errors in the VCR (Inoué, 1986). This improved analog signal was then digitized by a digital image processor (Model 327; Perceptive Systems, Houston, TX) for further image processing and analysis. Data files from the digital image processor were
transferred to a VAX-11/750 computer system for fast data processing and curve fitting. Slides and prints of the processed digitized images were obtained using a photographic module (Model 635; Dunn Instruments, San Francisco, CA). A color bar generator (Model 210; 3M, St. Paul, MN) was also used before the experiments as a reference for adjusting picture brightness, contrast, and color intensity. This experimental set-up is shown diagrammatically in Figure 2.2 and graphically in Figure 2.3.

2.3.2 Digital Image Processing

Digital image processing was performed with a LSI-11/23 (Digital Equipment Corporation, Maynard, MA), a 16-bit high performance, high speed micro-computer, integrated with additional ITI (Image Technology Inc., Woburn, MA) IP-512 image processing boards and two menu driven software packages, PSIXEC (Perceptive Systems Inc., Houston, TX) and TURBO. The hardware configuration of the system is as following: one LSI-11/23 CPU with MMU and FPU chips, 256 Kb solid-state MOS memory, one multifunctional board, a 4-channel serial interface, three ITI frame buffers, one ITI RGB video digitizer, one ITI arithmetic logic unit, one 0.5 Mb floppy disk, and one 80 Mb Winchester hard disk. The frame buffers served to temporarily display digitized images in a video format, while the arithmetic-logic unit allowed numerical manipulations of images at video rates.

The first step in digital image processing is the conversion of the optical image into a form that can be stored in computer memory. This conversion is performed by the optical digitizer, which produces coded numbers that are a measure of light intensity,
Figure 2.2: Block diagram showing the relationship between the inverted-stage microscope, the computerized microphotometric measurement system, and the video and digital image processing equipment.
Figure 2.3: Experimental set-up used for both microscopic and macroscopic measurements of platelet surface deposition during dynamic study. This figure illustrates graphically the relationship between the microscope, the perfusion system, the image analysis equipment and the microphotometric measurement system.
from 0 for black to 255 for white. The image is divided into small rectangular regions (512 by 480) called picture elements, or pixels for short. At each pixel location, the image brightness is sampled and quantized. This step generates an integer at each pixel representing the brightness or darkness of the image at that point. After this has been done for all pixels, the image is represented by a rectangular array (512 x 480) of integers, also called grey levels. Once the image has been digitized, it can be manipulated by standard digital computing techniques. All such manipulations can be placed in one of two general categories: image analysis or image enhancement. Image analysis refers to computer routines that produce descriptive information about the image, such as counting the number of cells or providing quantitative information on cell dimensions. This type of numerical analysis is also referred to as feature extraction. Routines for image enhancement, on the other hand, manipulate the image in such a way that it is more useful to a human observer. Those include image subtraction and addition, color enhancement, digital filtering, etc. (Inoué, 1986; Castleman, 1979).

2.3.3 Data Analysis

Epifluorescence video microscopy, coupled with digital image processing techniques, were utilized to study qualitatively and quantitatively local blood platelet adhesion and individual thrombus formation under physiological flow conditions on a flat surface coated with a material of interest. This technique involved the real-time, local visualization and analysis of mural thrombogenesis and was used to analyze the kinetics of thrombus growth, to model the three-dimensional structure of individual thrombi, to estimate the number of platelets per thrombus, and to obtain different morphological
information on each thrombus including basal area, length, width, average height, and average volume. Quantitative information concerning the thrombi' shapes and the direction in which they grow with respect to the flow field were also estimated with the digital image processor in this study. The circularity factor (CIRC) was computed as a measure of the circularity of an object according to:

\[ \text{CIRC} = \left( \frac{\text{PER}^2}{4 \times \pi \times \text{EAR}} \right) - 1.0 \]

where PER is the distance around the object boundary and EAR is the area inside the object perimeter. CIRC is zero for perfect circles and takes on larger values for more complex shapes. Next, the aspect ratio (ASPR) was estimated for each object. It is defined as the ratio of the width to the length of a rectangle having the same area and perimeter as the object of interest. It is 1.0 for square objects and decreases for more slender objects. Last, the angle of rotation, defined as the angle of the major axis of the objects (in degrees) as determined from invariant moments (Castleman, 1979), was computed to determine how the aggregates are aligned in the flow field. In most cases, this microscopic analysis of mural thrombogenesis was performed at the location of maximum macroscopic platelet accumulation along the collagen-coated glass, as determined by the microphotometric measurement system described in the next section.

The analytical procedure consisted of several steps. First, images from the video tapes were digitized at given time intervals. Second, a number of specific picture enhancement operations were performed on each digitized image in order to eliminate the noise and increase object contrast. Those include, in order, background subtraction, grey
scale linear stretching, and a convolution with a 12 x 12 smoothing mask or kernel (Box Filter) which suppresses the contribution of high spatial frequencies in the image. Third, image segmentation was performed to identify the boundaries in the image that separate one image feature from another. Next, boundary editing routines were used to enhance object boundaries. Last, different feature extraction routines were applied to the processed image to identify the platelets or thrombi by tracing their perimeters and performing numerical computations on each object.

In order to estimate the number of platelets per thrombus, one had to assume that the integral of the grey level intensity over the area of the thrombus was proportional to the number of platelets constituting that thrombus:

\[ P_T = K \iint_{XY} i(x,y) \, dx \, dy \quad (2.1) \]

where \( K \) is a proportionality constant; \( P_T \) is the total number of platelets per thrombus; and \( i(x,y) \) is the grey level intensity, a function of pixel location in the image. \( K \) was estimated by visually counting the number of cells adhered on the surface in the entire field of view after 15 seconds of flow, at which time platelet/platelet adhesion was minimal. The assumption of linearity between fluorescence intensity and number of platelets is valid for small to medium size thrombi, as those analyzed in this study, for which intensity loss due to out-of-focus and reabsorption is negligible (Hubbell, 1986).

Similarly, the three-dimensional structures of individual thrombi were estimated by assuming that, at each pixel in the two-dimensional image, the height, \( h(x,y) \), is
proportional to the grey level intensity, \( i(x,y) \), at that location:

\[
h(x,y) = k \cdot i(x,y) \tag{2.2}
\]

where \( k \) is a proportionality constant. In addition, the total thrombus volume \( V_T \) is given by:

\[
V_T = P_T V_P = \int \int_{XY} h(x,y) \, dx \, dy \tag{2.3}
\]

where \( V_P \) is the platelet volume (about 10 \( \mu \text{m}^3 \)). Substituting equation (2.2) into equation (2.3), and solving for \( k \) gives:

\[
k = \frac{P_T V_P}{\int \int_{XY} i(x,y) \, dx \, dy} \tag{2.4}
\]

where \( P_T \) was estimated from equation (2.1) and the double integral by the image processor. The background intensity, measured between thrombi, was subtracted from the measured intensity, prior to evaluation of the double integral (Hubbell, 1986).

The three-dimensional image was constructed by isometric projection.

2.4 Macroscopic Measurements
2.4.1 Microphotometric Measurement System

Automatic scanning and direct recording of the locally averaged fluorescent intensity level in the entire field of view were performed by a motorized microscope stage and a computerized microphotometric measurement system consisting of a photodiode (Model PIN-10DP/SB, United Detector Technology, Hawthorne, CA), an amplifier with variable transimpedance gain (Model 101C, United Detector Technology), an input/output terminal panel, a 12-bit analog to digital converter board (ACSE-12; Strawberry Tree Computers, Inc., Sunnyvale, CA) and a micro-computer (Apple Macintosh SE). The amplifier also acted as a current to voltage converter. A 40 x FLUOR objective (CF Fluor, NA = 0.85; Nikon) and a 1 x projection lens were used in those measurements. The high performance data acquisition board was designed for measurement of any analog signal in the range of 2 μVolts to 10 Volts, with a maximum sampling rate of 10,000 samples per second. A menu driven software (Analog Connection Workbench; Strawberry Tree Computers Inc.) was used to select voltage range, sampling rate, and create data files. A dial indicator was used to measure the position of the flow chamber. This microphotometric measurement system and its relationship to the rest of the equipment is shown diagrammatically in Figure 2.2 and graphically in Figure 2.3.

2.4.2 Data Analysis

Macroscopic analysis of the end-point platelet accumulation along the coated surface was performed by a motorized microscope stage and a computerized microphotometric measurement system. This technique allows one to determine the axial dependence of platelet deposition along the reactive surface.
In order to convert locally-averaged fluorescent intensity to locally-averaged platelet density at any given axial position along the coated surface, the analysis required the knowledge of the total number of platelets accumulated on the coated surface during the perfusion period. For that purpose, the glass cover slip was carefully removed from the flow chamber at the end of the perfusion period. The excess blood was flushed off the cover slip with 3.0 ml of isotonic saline; the cover slip was then dipped for 30 seconds in a stromatolysing agent (ZAP-OGLOBIN II; Coulter Electronics Inc., Hialeah, FL) solution consisting of 20.0 ml of ISOTON II and 10 drops of the agent. This step was necessary to lyse all remaining RBCs sticking on the surface. The cover slip was then crushed in a 50.0 ml conical test tube containing 1.0 ml of 1% triton X-100 cell lysing buffer. The sample was then sonicated for 5 seconds, centrifuged on 1300 rpm for 10 minutes to remove the glass fragments and stored at 4°C before being assayed.

Two biochemical assays were performed on the experimental and/or the control samples with known concentrations of platelets and RBCs. All experimental and control samples were assayed for the platelet cytoplasmic enzyme lactate dehydrogenase (LDH) by a standard spectrophotometric technique (Wroblewski and LaDue, 1955). Because RBCs contain a large amount of LDH activity, the possible contamination of the control samples by a few RBCs may invalidate the results. Therefore, the samples were also assayed for the conjugated protein hemoglobin by an other spectrophotometric technique (Eilers, 1967). Both assays are detailed in Appendix A.

In order to convert light level to number of cells, one had to assume again that the local average platelet surface density, PD(z), was proportional to the local average fluorescence intensity, AI(z), as measured by the photodiode, at any axial position z
along the reactive surface:

\[ PD(z) = c A(z) \quad (2.5) \]

where \( c \) is a proportionality constant. The total number of cells accumulated on the surface during the perfusion period, \( P_T \), is given by:

\[ P_T = w \int_{Z}^{P_D(z)} dz \quad (2.6) \]

where \( w \) is the width of the surface (12.7 mm). Substituting equation (2.5) into (2.6), and solving for \( c \) gives:

\[ c = \frac{P_T}{w \int_{Z}^{A(z)} dz} \quad (2.7) \]

where \( P_T \) is obtained from the biochemical assays and the integral estimated from the microphotometric measurements. In those calculations, the background intensity, measured upstream of the reactive coating and consisting of platelet adhesion on bare glass, was subtracted from the measured average intensity, before estimation of the constant \( c \) (Hubbell, 1986). The computer programs used to calculate the constant \( c \) as well as to curve fit the digital data are shown in Appendix A. The global average platelet density on the slide was also computed according to:

\[ APD = \frac{P_T}{l \times w} \quad (2.8) \]
where I is the length of the reactive surface (26.3 mm).

Because the LDH assay is not sensitive enough to measure very low cell concentrations, an alternative method was used in certain cases to estimate the constant c. The digital image processor was used to visually count the number of cells adhering on the reactive surface in a chosen field of view, while the average fluorescence intensity in the same field of view was recorded as before by the microphotometric measurement system. Of course, corrections were made for the fact that the actual areas scanned by the photodiode and the video camera were different. First, the fluorescence intensity per platelet, b(plt), was estimated according to:

$$b(plt) = \left( \frac{\Delta AI}{\Delta \text{platelets}} \right) \times \left( \frac{\text{area DIP}}{\text{area photodiode}} \right)$$  \hspace{1cm} (2.9)

where $\Delta AI$ is the difference in average fluorescence intensity between two fields of view and $\Delta \text{platelets}$ is the difference in platelet number between the same two fields of view.

Next, assuming that $PD(z)$ in equation (2.5) is given in platelets/1000 $\mu$m$^2$, the constant c was computed from:

$$c = \left( \frac{1}{b(\text{plt})} \right) \times \left( \frac{1000}{\text{area photodiode}} \right)$$  \hspace{1cm} (2.10)

Because those two techniques did not always give comparable results in terms of PD(z) or APD, the second one was used mainly for comparison purposes, rather than for estimating absolute values of platelet density.
CHAPTER 3
EFFECTS OF ANTITHROMBOTIC AGENTS IN MURAL
THROMBOGENESIS ON COLLAGEN-COATED GLASS

3.1 Background and Objectives

The search for agents that would prevent thrombus growth and formation, either by altering normal platelet function, by inducing dissolution of thrombi, or by preventing their stabilization with fibrin has been an important area of research in the field of medicinal chemistry. Antiplatelet agents are drugs which interfere with the chains of intracellular events leading to platelet activation, thereby being capable of preventing aggregation as well as thrombus formation on vascular subendothelium (Report of the National Heart, Lung, and Blood Institute Working Group, 1985; Gordon, 1981). Because the release of intracellular Ca$^{2+}$ from storage sites in the dense tubular system (DTS) into platelet cytosol plays a fundamental role in mediating platelet activation and platelet aggregation, drugs that block release of intracellular Ca$^{2+}$ have received much attention from researchers (Lasslo et al., 1983). Indeed, calcium is
maintained at low concentrations in the cytosol of resting platelets. Stimuli at the platelet plasma membrane, such as chemical agents (thrombin, ADP, etc.) or shear forces, are transmitted through the open canalicular system (OCS) to the DTS, resulting in (a) the release of calcium sequestered there, (b) increased Ca$^{2+}$ concentration in the cytosol, and (c) platelet activation and subsequent aggregation. On the other hand, the platelet cytosol's Ca$^{2+}$ content can be reduced by cyclic AMP (cAMP)-stimulated transfer of calcium to the DTS and the vesicle storage sites, which then restores the platelet to its inactivated state (Lasslo, 1984).

The initial step in platelet activation, antecedent to thrombus formation, is the activation of the membrane-bound enzyme phospholipase A$_2$ (PLA$_2$) and the cytosolic enzyme phospholipase C (PLC) by mobilized Ca$^{2+}$. PLA$_2$ is known to split arachidonic acid (AA) from the membrane phospholipids. While Kinlough-Rathbone and Mustard (1987) feel that phosphatidylinositol (PI), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) are primarily involved, Chap et al. (1987) emphasize that PI, PE and phosphatidylserine (PS) have the highest AA content and that PC contains considerably less. The liberated AA is then converted by cyclo-oxygenase to endoperoxides prostaglandin G$_2$ (PGG$_2$) and prostaglandin H$_2$ (PGH$_2$), which are subsequently transformed by thromboxane synthetase to thromboxane A$_2$ (TXA$_2$), one of the most potent inducers of platelet activation and release reaction. Although this pathway is thought to produce most of the free AA in platelets, PLC also leads to the liberation of AA, by hydrolyzing phosphatidylinositol-4,5-biphosphate (PIP$_2$) to 1,2-diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP$_3$). AA is then freed
from DAG upon interaction first with the enzyme diglyceride lipase (DGL) and then with monoglyceride lipase (MGL). It should be noted that, in a recent reference work, Kinlough-Rathbone and Mustard (1987) emphasized that elevation of cytosolic calcium is not inevitably a prerequisite for the activation of PLC and that the enzyme can be energized by other means. At the same time, DAG may also be converted to phosphatidic acid (PA), a possible Ca\(^{2+}\)-ionophore, by the enzyme diacylglycerolkinase (DAGk). IP\(_3\), which emerges from the aforementioned hydrolysis as a water-soluble product, is also able to release calcium from the platelet's DTS (Chap et al., 1987) and increase the cytosol's Ca\(^{2+}\) content which, in turn, energizes PLA\(_2\). Some investigators (Gerrard et al., 1978; Gerrard et al., 1979) have mentioned that the first AA molecules liberated come from this second pathway and that PI-derived PA is necessary to release membrane-bound Ca\(^{2+}\) in large enough quantities to activate PLA\(_2\) into generating massive quantities of AA from PC. In this context, it should also be pointed out that activation of adenylate cyclase (AC) and/or inhibition of cAMP phosphodiesterase (cAMPPDE) may yield high levels of cAMP, this process probably functioning as an endogenous balancing process for limiting free Ca\(^{2+}\) (Lasslo, 1984). The pathways for Ca\(^{2+}\) release and platelet activation are illustrated in Figure 3.1.

Among the chemical agents that have been known to affect platelet functions, propranolol-type compounds, tricyclic entities such as chlorpromazine and mepacrine, and molecules of local anesthetics act by interfering with Ca\(^{2+}\)-related platelet functions. The common denominators in these compounds are the nonaromatic amine functions, either alicyclic or aliphatic, secondary or tertiary amines, as shown in Figure 3.2. More recently, Lasslo and associates (Lasslo et al., 1983) have been
Figure 3.1: Pathways showing the chemical reactions and associated enzymes involved in the release of intracellular Ca^{2+}, leading to platelet activation. The sites of action of GT-12 and aspirin are also shown in this figure. The notation is similar to that used in the text.
Figure 3.2: Examples of antiplatelet agents known for interfering with normal Ca$^{2+}$ functions and/or reducing response sensitivity of anionic phospholipids in platelet's plasma membrane. Their common structural denominators, the aliphatic or alicyclic amines, are marked with shaded circles in the figure. (a) Lidocaine. (b) TMB-8. (c) Mepacrine. (d) Chlorpromazine. (e) Trifluoperazine. (f) Sulocitidil. (g) Propranolol. (h) Imipramine. (i) Methysergide. (j) Reserpine. (from Lasslo and Quintana, 1984).
developing a series of novel carbamoylpiperidine congeners which act by a similar type of mechanism. In structuring those compounds, they were looking for molecular designs that would exert maximal platelet aggregation-inhibitory potency and minimize undesirable effects, thereby favoring their potential use as pharmaceutical products. In fact, the tertiary amines in the heterocyclic rings, identified as platelet aggregation-inhibitory specific functions in those compounds, are deemed capable of interacting with and reducing the response-sensitivity of anionic phospholipids (PI and PS) in the platelet membrane’s lipid bilayer (Quintana et al., 1982) and, thereby, stabilize membrane complexes of the DTS and of other storage sites sequestering calcium in the platelets (Lasslo, 1984). Since this impedes or blocks mobilization of additional Ca$^{2+}$ into the platelet’s cytosol by conventional stimuli, the threshold for triggering or sustaining platelet aggregation should be elevated, and only stimuli of considerably greater intensity could actuate the process (Lasslo and Quintana, 1984; Lasslo, 1984; Quintana et al., 1981b; Quintana et al., 1982). The main reason why tertiary and secondary aliphatic and alicyclic amines are potent platelet-aggregation inhibitory functions is that they are subject to broad changes in protonation, depending on the pH of their immediate environment and on the pK$_a$ of their compound. This characteristic enables them to assume the appropriate hydrophobic character for the penetration of the platelet membrane’s lipid bilayer without subsequently interfering with their transformation into corresponding cations for interaction with the platelet membrane’s anionic phospholipids. Their quaternary analogs, on the other hand, are unable to penetrate the membrane’s lipid bilayer because they retain the same charge regardless of the environment they are in (Lasslo, 1984).
Acting by a mechanism distinct from the compounds mentioned above, a triphenyl-methyl compound, aurin tricarboxylic acid (ATA), has been recently recognized as a potential platelet aggregation inhibitor by Phillips and associates (1988). They showed that ATA inhibits shear-induced as well as ristocetin-induced, von Willebrand Factor (vWF)-mediated platelet aggregation, yet preserving platelet metabolic functions. ATA binds to the largest vWF multimers found in normal plasma and to the unusually large vWF multimers secreted by endothelial cells (ECs). By doing so, ATA interferes with the interaction between glycoproteins Ib located on the platelet membrane and the circulating vWF multimers, thereby preventing platelet to platelet binding.

Many other medicinal agents affecting directly or indirectly blood platelets have been developed over the years. Among the most well-known are streptokinase, urokinase, and the tissue-culture-derived plasminogen activator, which are fibrinolytic drugs; heparin, a thrombin inhibitor and anticoagulant; aspirin, known to irreversibly acetylates cyclooxygenase in platelets, blocking synthesis of TxA2; etc. Good reviews on antiplatelet drugs can be found in Packam and Mustard (1983), Weiss (1982), Lasslo and Quintana (1984), and De Gaetano et al. (1987).

In this study, epifluorescence video microscopy coupled with digital image processing techniques and a microphotometric measurement system were used to analyze macroscopically and microscopically the effect of one of the most potent carbamoylpiperidine congeners, α, α'-bis [3-(N,N-diethylcarbamoyl)piperidino]-p-xylene dihydrobromide (GT-12) (Lasslo et al., 1987), on platelet accumulation on a collagen-coated surface. The results were
compared to those obtained with a well-known reference compound, chlorpromazine hydrochloride. A few preliminary microscopic experiments were also performed to investigate the inhibitory effect of ATA in the thrombosis process. Previous work with GT-12 has shown the compound’s capacity to inhibit platelet aggregation in response to various aggregation-inducing agents, including ADP and thrombin, without blocking platelet responses associated with shape change and internal transformation (Quintana et al., 1981b; Lasslo and White, 1984; Lasslo et al., 1984). All those studies were performed with platelet-rich plasma (PRP) in an aggregometer. ATA, on the other hand, was shown (Phillips et al., 1988) not to inhibit ADP- or arachidonic acid-induced platelet aggregation in an aggregometer with PRP. The compound did inhibit, however, ristocetin-induced, vWF-mediated platelet clumping in both fresh and formaldehyde-fixed platelet suspensions in the same apparatus. In addition, experiments were done under elevated shear stresses in a cone and plate viscometer with that compound (Phillips et al., 1988). In that system, ATA inhibited shear-induced, vWF-mediated platelet aggregation in PRP in concentrations above 200 μM and in buffer suspensions of washed platelets at a concentration of 0.1 μM. Note that their system only measures bulk platelet aggregation in a shear field and that no platelet to surface adhesion is necessary prior to aggregation, as is the case "in vivo". Consequently, the objective in the studies presented was to investigate the effect of GT-12, as well as ATA, on collagen-induced surface platelet aggregation in a more physiological environment, namely under dynamic flow conditions and in the presence of physiological concentrations of red blood cells (RBC). The presence of RBCs is important in testing pharmacological compounds for several reasons. First, the RBCs act physically to
augment platelet transport from the bulk of the fluid to the reactive surface (Turitto and Weiss, 1980). Second, they may act hormonally as a source of proaggregating ADP and as a buffering sink for substances released by platelets and other cells. Finally, it has been reported that the capacity of RBCs and plasma proteins for the compound under test must be exceeded before platelets are affected (Adams, 1985).

3.2 Experimental Technique

3.2.1 Drug Sample Preparation

Drug samples were always prepared fresh daily. Chlorpromazine hydrochloride (35.53 mg) (F.W. C₁₇H₂₀N₂Cl₂S, 355.52, Sigma Chemical Co., St. Louis, MO) was weighed into a 1.0-ml volumetric flask filled with distilled water and covered with parafilm to make a 100 millimolar (mM) solution; 1 μl of this solution for each ml of blood yielded a 100 μmolar (μM) final concentration. Similarly, 31.63 mg of GT-12 (F.W. C₂₈H₄₈Br₂N₄O₂, 632.57) was weighed into a 1.0-ml volumetric flask filled with 95% ethyl alcohol and covered with parafilm to make a 50 mM solution; 2 μl of this solution for each ml of blood yielded a 100 μM final concentration. The flask was then placed in a water bath at 37°C for 15 to 30 minutes to facilitate dissolution of the drug and was then allowed to cool at room temperature. Concentrations of 95% ethyl alcohol up to 2.0 μl per ml of test medium were reported not to affect normal platelet function in aggregometric studies (Quintana et al., 1980). For higher doses of the drug, distilled water was used as dissolving medium. In the case of ATA, 6.33 mg and 0.633 mg of the compound (F.W. C₂₂H₁₄O₉, 422.35) were individually dissolved in 3 ml of
platelet-poor plasma (PPP) in a water bath at 37°C and then allowed to cool at room
temperature; each sample was mixed with 10 ml of whole blood for 10 minutes at 37°C
prior to perfusion. The final drug concentration in those samples was 1.15 mM and 115
µM, respectively.

3.2.2 Collagen Preparation

One gram of type I acid-insoluble collagen (Sigma Chemical Co, St. Louis, MO)
from bovine achille tendon was mixed with 12 ml of glacial acetic acid and 198 ml of
distilled water for 10 minutes at room temperature in a stirrer. This solution was
placed in a homogenizer (The Virtis Co., Gardiner, NY) for 2 hours and after addition of
200 ml of distilled water, it was again homogenized for one more hour. The final collagen
solution was centrifuged (Model J-21B, Beckman, Palo Alto, CA) for 10 minutes at
1,000 rpm. The supernatant was collected and stored at 4°C. The final collagen solution
was assayed for its peptide bonds by a standard spectrophotometric technique (Peterson,
1977) (Protein Assay Kit No. P 5656, Sigma Chemical Co., St Louis, MO) and the final
collagen concentration was calculated to be about 850 µg/ml in a 0.522 M acetic acid
solution at a final pH of 2.8.

3.2.3 Flow Studies and Collagen Coating

Heparinized whole blood with fluorescent-labeled platelets was perfused for 2
minutes in a parallel plate flow chamber suitable for development of a laminar parabolic
flow profile, as described in detail in Chapter 2. Twelve different donors were used for
those experiments. Because mass-transfer considerations are important to the kinetics
of thrombus growth on surfaces, a high but still physiologically relevant wall shear rate of 1000/sec (5.337 ml/min) was used in all experiments. At this wall shear rate, it has been shown previously (Hubbell and McIntire, 1986c) that the rate of platelet transport to the surface is not limiting thrombus growth when compared to the surface rate of reaction. Before being assembled as part of the flow chamber, a glass cover slip was coated with 200 µl of type I acid-insoluble collagen suspended in acetic acid. The fibrillar collagen solution was allowed to rest for 45 to 60 minutes on the glass cover slip before the supernatant was rinsed off with 10 ml of sterile isotonic saline. The amount of collagen in the supernatant was calculated to be on the average 14 µg/ml by the same spectrophotometric method as previously mentioned (Peterson, 1977). This leads to an average collagen density on the glass surface of 3.50 µg/cm². Finally the flow chamber was assembled and filled with sterile isotonic saline.

3.3 Macroscopic Measurements - Results

The results of the macroscopic measurements are presented in Figures 3.3 to 3.6 and Table 3.1. Figures 3.3 to 3.6 show the platelet surface density, in number of platelets per 1000 µm², versus the axial position along the collagen-coated surface. As can be seen from the control curves in Figures 3.3 and 3.6, after short term perfusion (1 or 2 minutes) of heparin anticoagulated whole blood, most platelet aggregation occurs at the entrance of the reactive surface. Figure 3.3 illustrates the difference in the inhibitory effects of chlorpromazine and GT-12 on platelet aggregation, both drugs tested at a same concentration of 100 µM. At that concentration, chlorpromazine has
Figure 3.3: Platelet accumulation on collagen-coated glass in contact with control or drug-treated whole blood, as indicated in the figure. The blood was heparin anticoagulated and allowed to contact the surface for 2 minutes at a wall shear rate of 1000/sec. The abscissa represents the axial position along the reactive surface.
Figure 3.4: Effect of GT-12 concentration on platelet accumulation along a collagen-coated glass. The antiplatelet agent was tested at four different concentrations, as indicated in the figure. The flow conditions were the same as for Figure 3.3.
Figure 3.5: Effect of incubation time of GT-12 and chlorpromazine on platelet accumulation on a collagen-coated surface. Both drugs were incubated for 5 or 30 minutes in a water bath at 37°C before perfusion in the flow chamber during 2 minutes at 1000/sec. In those experiments, GT-12 was used in 50 μM concentration and chlorpromazine in 200 μM concentration.
Figure 3.6: Effect of perfusion time of GT-12 on platelet accumulation on a collagen-coated surface. Heparinized whole blood was perfused untreated or treated with 50 μM GT-12 for 1 minute and/or 2 minutes, as indicated in the figure. The flow conditions are identical to those in Figure 3.3.
Table 3.1: Global Average Platelet Density as a Function of Drug Concentration.

<table>
<thead>
<tr>
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<th>CONCENTRATION</th>
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</thead>
<tbody>
<tr>
<td>DRUG</td>
<td>20 μM</td>
</tr>
<tr>
<td>GT-12</td>
<td>17.4±8.0*</td>
</tr>
<tr>
<td></td>
<td>24.3±1.4†</td>
</tr>
<tr>
<td>CHLORPROMAZINE</td>
<td>-</td>
</tr>
</tbody>
</table>

The average platelet density is expressed as percentage of the control value, after perfusion of chlorpromazine- or GT-12-treated blood at 1000/sec on collagen. The perfusion time and incubation time are, respectively, 2 min and 30 min, unless indicated. Results are presented as mean±SD. (*): 5 min incubation, 2 min perfusion; (†): 5 min incubation, 1 min perfusion.
essentially no effect on platelet/platelet interactions on the collagen-coated surface, while the effect of GT-12 on platelet aggregation and thrombus formation is quite dramatic, as shown by a significant decrease in platelet density at the inlet of the coated surface. The effects of varying GT-12 concentrations on platelet surface aggregation are shown in Figure 3.4. As the drug concentration is increased from 20 to 50 µM, there is a significant decrease in platelet density, especially near the inlet of the reactive surface. However, as the drug concentration is further increased to 100 and 200 µM, surface platelet density increases again. The effect of incubation time on the aggregation-inhibitory potency of chlorpromazine and GT-12 is shown in Figure 3.5. Both drugs are more effective in inhibiting platelet aggregation when left only for a short period of time (5 minutes) in contact with blood before perfusion. Finally, Figure 3.6 shows the effect of perfusion time on the aggregation-inhibitory potency of GT-12 at 50 µM when incubated for 5 minutes. As shown by the two bottom curves in that figure, only a small increase in platelet density occurs between 1 and 2 minutes of blood flow, indicating that the net rate of platelet deposition on the reactive surface is very low.

Table 3.1 shows the global average platelet density (APD) for different drug concentrations, incubation periods, and perfusion periods, expressed as percentage of the control value and calculated from the total number of platelets accumulated on the surface during the perfusion period (see Chapter 2) and the total active area of the collagen-coated surface. The APD for chlorpromazine-treated blood decreases with increasing concentration and decreasing incubation time. On the other hand, the APD for GT-12 decreases with increasing concentration up to 50 µM but paradoxically increases again at higher concentrations, as was observed in Figure 3.4. The data for GT-12 at 50
μM also indicate that the compound's effectiveness in preventing platelet accumulation on a collagen-coated surface increases with decreasing incubation and increasing perfusion time, which is consistent with the data presented in Figures 3.5 and 3.6.

3.4 Microscopic Measurements - Results

In order to analyze in detail the effect of an antiplatelet agent on platelet adhesion and aggregation on collagen, it is very informative to look at the morphology and the rate of growth of individual thrombi. The results of this microscopic study are presented quantitatively in Figures 3.7 and 3.8, and Table 3.2, and qualitatively in terms of two-dimensional contour images and three-dimensional models of thrombi, both pseudo-color enhanced, in Figures 3.9 to 3.11.

The typical morphologic information, length, width, average height, and basal area, are shown as a function of time in Figures 3.7 and 3.8 when the control or the 50 μM GT-12-treated whole blood is exposed to collagen-coated glass for 120 seconds at a wall shear rate of 1000/sec. Also shown in Figure 3.8 is the total number of platelets constituting a given thrombus as a function of time. As can be seen from the shape of the control curves, the rate of growth of the thrombus, in terms of length, width, basal area, and number of platelets, is, in general, steadily increasing with time, as reported earlier (Hubbell and McIntire, 1986b; Adams et al., 1983). The average height of the thrombus, however, does not follow this pattern and levels off after about 75 seconds of flow. This can also be verified by looking at the three-dimensional structure of the thrombi in Figure 3.9, where there is no apparent difference in average thrombus
Figure 3.7: Length, width, and average height of a single growing thrombus as a function of time for the control (——) and for 50 μM GT-12-treated (— —) blood. The flow conditions are identical to those shown in Figure 3.3.
Figure 3.8: Basal area and platelet number per thrombus as a function of time for the control (—) and for 50 μM GT-12-treated (——) blood. The flow conditions are identical to those shown in Figure 3.3.
Figure 3.9: Kinetics of thrombus growth on collagen-coated glass in three dimensions for the control blood. Images were digitized at four different perfusion times as indicated on the figure and the three-dimensional structures of the thrombi generated digitally from local intensity measurements and pseudo-color enhanced. The length scale applies to both the length and the height.
Figure 3.10: Kinetics of thrombus growth on collagen-coated glass in three dimensions for the 50 μM Gt-12-treated blood. The same comments as those in Figure 3.9 apply.
Figure 3.11: Kinetics of thrombus growth on collagen-coated glass in two dimensions respectively for the control blood (1), the 200 μM GT-12-treated blood (2), and the 50 μM GT-12-treated blood (3). These time contour diagrams show the growing basal area of a single thrombus as a function of time. Different times are represented by different colors.
<table>
<thead>
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<th>DRUG</th>
<th>50 µM</th>
<th>100 µM</th>
<th>200 µM</th>
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<tr>
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</tr>
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<td></td>
<td>16.2±5.2</td>
<td>24.6±3.8</td>
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</tr>
<tr>
<td>CHLORPROMAZINE</td>
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<td>20.9±5.1</td>
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<tr>
<td>(a)</td>
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</tr>
<tr>
<td>(b)</td>
<td>–</td>
<td>94.2±10.1</td>
<td>65.6±23.1</td>
</tr>
<tr>
<td>(c)</td>
<td>–</td>
<td>93.6±14.2</td>
<td>25.3±0.8</td>
</tr>
<tr>
<td>(d)</td>
<td>–</td>
<td>101.4±8.3</td>
<td>37.6±4.4</td>
</tr>
</tbody>
</table>

Basal area (a), average thrombus height (b), total number of platelets per thrombus (c), and minimum number of aggregated platelets per thrombus (d) are expressed as percentages of the control values. Blood was perfused for 2 min at 1000/sec on collagen after a 30 min incubation period with the drug.

Results are presented as mean±SD.

* GT-12 was dissolved in distilled water.
height after 90 or 120 seconds of flow. The thrombus grows mostly from its basal area during that period of time. The rate of growth of the basal area is shown in 30-second intervals in the contour diagram (1) of Figure 3.11 for the control case. After 90 seconds of flow the thrombus grows mostly from its downstream edge, supporting the fact, noted by others, that platelets may become activated as they flow over the growing thrombus (Hubbell and McIntire, 1986a). It is likely, however, that the thrombus has to reach a certain size so that sufficient proaggregating substances are released in the medium and so that the flowing platelets have the time to become activated before this process occurs.

Very large differences in thrombus morphology and rate of growth, as it develops on collagen, can be observed in Figures 3.7 and 3.8 when heparinized whole blood is treated with 50 µM GT-12. The shape of the curves in Figure 3.8 indicates that the thrombus rate of growth, in terms of number of platelets and basal area, practically vanishes after 45 to 60 seconds of flow. This is also shown in terms of the rate of change of length, width, and average thrombus height in Figure 3.7. The steady state maximum average height is about 4 µm. The dramatic effect of the compound on thrombus morphology and rate of growth can also be observed qualitatively by comparing the three-dimensional structures of thrombi at four different perfusion times in Figures 3.9 and 3.10, respectively, for the control case and the 50 µM GT-12-treated case. Also, the contour diagrams (2) and (3) in Figure 3.11, when compared to diagram (1) for the control case, illustrate the effect of GT-12 at 200 µM and 50 µM, respectively, on the rate of growth of the thrombus basal area.

The data in Table 3.2 show the final inhibitory effects of both drugs on thrombus
morphology in terms of basal area, average height, and total number of platelets, expressed as percentages of the control values, after two minutes of perfusion. Because the total number of platelets per thrombus includes both platelets involved in adhesion and aggregation, an effort to estimate the number of platelets involved in aggregation solely was undertaken to compare the results of this study with previous data obtained in aggregometric studies with PRP (Quintana et al., 1981b; Lasslo and White, 1984). For that purpose, the surface coverage of a single platelet, adhered to a collagen-coated surface, was estimated to be approximately 4.35 µm² from measurements made from electron microphotographs (White, 1984) and with the digital image processor. Using that number and the basal area of the thrombus, the maximum number of platelets adhered to the surface was computed and the minimum number of aggregated platelets, expressed as percentage of the control value, inferred as shown also in Table 3.2.

As previously mentioned and as illustrated in Table 3.2, chlorpromazine at 100 µM has little effect on thrombus morphology or reaction rate between platelets. On the contrary, at that concentration, the compound might potentiate slightly the process of platelet aggregation as reported elsewhere (Lasslo and Quintana, 1984). Chlorpromazine at 200 µM and GT-12 at concentrations ranging from 50 to 200 µM, on the other hand, greatly reduce both thrombus size and the aggregation process after 2 minutes of flow. The data also indicate that, increasing the concentration of GT-12 from 50 to 200 µM has little effect on thrombus morphology.

Because Owens et al. (1989) recently showed that ethanol might affect platelet aggregation on collagen in the same flow system as the one used here, this matter was briefly investigated. For that purpose, additional microscopic experiments were
performed with GT-12 dissolved in distilled water. The results are shown in Table 3.2 when GT-12 is used in 50 μM and 100 μM concentrations. In terms of total number of platelets per thrombus, the percentages inhibition at 50 μM are very similar with or without alcohol present. At 100 μM, the percentage inhibition is slightly larger in presence of ethanol, probably due to the fact that twice as much volume was added. In terms of thrombus basal area, the drug dissolved in water was more effective in inhibiting normal platelet functions than when dissolved in ethanol. Those data indicate that GT-12 remains effective in impeding platelet adhesion and aggregation on collagen, even in absence of ethanol, the dissolving medium.

Quantitative results on individual thrombus size are shown in Figures 3.12 and 3.13 as a function of time in the cases of 1.15 mM and 115 μM ATA-treated whole blood. The controls for the measurements presented in those two figures, corresponding to the untreated blood case, are the same as those shown previously in Figures 3.7 and 3.8. Figure 3.12 clearly indicates that both thrombus rate of growth and average size, in terms of basal area and platelet number, significantly decrease with increasing drug concentration. Indeed, after 2 minutes of flow, 1.15 mM ATA reduces thrombus basal area by 90.3% and number of platelets per thrombus by 97.3%, while, in 115 μM concentration, the compound reduces thrombus basal area by 46.5% and the number of platelets per thrombus by 80.0%, when compared to controls. Figure 3.13 shows that, although thrombus length decreases significantly when increasing the drug concentration from 115 μM to 1.15 mM, thrombus width and average height decrease considerably less. Again, the steady-state average thrombus height is reached after 45 seconds of flow and varies between 2.5 and 3.0 μm. After 2 minutes of flow, 1.15 mM ATA reduces
thrombus length by 66.2\%, thrombus width by 74.6\%, and thrombus average height by 71.2\%, whereas 115 \( \mu \)M ATA reduces thrombus length by 3.0\%, thrombus width by 61.6\%, and thrombus average height by 61.9\%, when compared to controls. The three-dimensional reconstructions of thrombi formed in the field of view and shown in Figure 3.14 illustrate the fact that increasing concentrations of ATA reduce both individual thrombus size as well as platelet surface coverage, after 2 minutes of flow on a collagen-coated surface. Again the corresponding three-dimensional image for the untreated blood is shown in Figure 3.9 after 120 seconds of flow.

3.5 Discussion and Conclusions

The results obtained in those experiments and reported elsewhere (Folie and McIntire, 1988) showed that the carbamoyl-piperidine congener GT-12 profoundly interferes with the normal platelet function during blood/materials interaction in response to a strong stimulus such as a collagen-coated surface in vitro. Indeed, both the macroscopic and microscopic measurements revealed that the synthetic compound inhibits the chain reaction of platelet activation-secretion-aggregation and the subsequent thrombus formation and stabilization. In fact, the current findings corroborate previously reported observations. Specifically, GT-12 appears to act by interacting with and reducing the response sensitivity of anionic phospholipids (Lasslo, 1984; Quintana et al., 1981a), stabilizing membrane storage sites and impeding mobilization of \( \text{Ca}^{2+} \) into platelet cytosol (Lasslo and Quintana, 1984). Through reinforcement of platelet membrane surfaces (Lasslo and White, 1984), it
**Figure 3.12:** Basal area and platelet number per thrombus as a function of time for the 1.15 mM ATA-treated (——) and the 115 μM ATA-treated (— —) whole blood. The flow conditions are identical to those shown in Figure 3.3.
Figure 3.13: Length, width, and average height for a single growing thrombus as a function of time for the 1.15 mM ATA-treated (—) and the 115 μM ATA-treated (—−) whole blood. The flow conditions are identical to those shown in Figure 3.3.
Figure 3.14: Three-dimensional reconstructions of thrombi formed on a collagen-coated glass after 2 minutes of perfusion at 1000/sec. The blood was treated for 10 minutes with 1.15 mM ATA (1), and 115 μM ATA (2), respectively, prior to perfusion.
inhibits activation of PIC and thereby precludes formation of IP₃. Consequently, as a result of reduced Ca²⁺ flux, it blocks stimulation of PI₄,₂0 along with the pathway this generates.

When discussing the results of the macroscopic measurements, it is worthwhile pointing out, as mentioned previously (Hubbell and McIntire, 1989), that the shape of the curves in Figures 3.3 to 3.6 represents the balance between platelet transport to the surface and platelet adhesion on, and aggregation at, the surface. Therefore, both mass transfer to the surface and rate of reaction at the surface must be considered when analyzing those results in this type of dynamic system. As illustrated by the control curves in Figures 3.3 and 3.6, most platelet aggregation occurs at the entrance of the coated surface, indicating that the process of mural thrombogenesis is limited downstream by platelet diffusion from the bulk fluid to the reactive surface. Indeed, after most platelets contained in the fluid layer adjacent to the reactive surface have reacted, this layer is depleted of platelets and only slowly replenished with platelets due to the transport-limited process. The curve exhibited by chlorpromazine at a concentration of 100 μM in Figure 3.3 is similar to the control curve; it is monotonically decreasing, showing that most platelet surface aggregation occurs at the inlet and indicating again that platelet transport to the surface further downstream is the rate-limiting step in the thrombosis process. It is clear that chlorpromazine, at that concentration, has virtually no effect on surface platelet aggregation. On the other hand, the nonmonotonic shape of the curve exhibited by GT-12, at a same concentration of 100 μM (see Figure 3.3), indicates that the compound weakens platelet-platelet bonds and that the larger thrombi at the inlet, become more fragile, are dislodged from the surface.
by the fluid drag forces. In addition, by altering normal platelet function, the compound also decreases the rate of surface reaction between platelets, making the process somewhat less diffusion-limited downstream. At the lowest concentration (20 μM), the inhibitory effect of the compound is the least, as shown in Figure 3.4; platelet accumulation is decreased at the inlet but also spread out over a larger surface area; the process remains primarily diffusion-limited downstream. At 50 μM some embolization of the larger thrombi occurs at the inlet as indicated by the nonmonotonic shape of the curve. The process is still diffusion-limited, although the reaction rate between platelets is much lower. At 100 μM, as mentioned above, the larger thrombi embolize from the reactive surface (but in a more restrained area than at 50 μM), indicating that fewer large thrombi develop on the surface and that the compound reduces platelet to platelet interaction more effectively. As shown in Figure 3.4, the surface platelet density at that concentration is also becoming independent of axial position, meaning that the thrombogenesis process, under the effect of the antiplatelet agent, is slowly changing from a diffusion-limited to a reaction-limited process. The axial dependence is nearly completely removed when the compound is used at 200 μM. Platelet reaction rate at the surface, mostly the rate of aggregation, becomes the rate-limiting step, preventing the formation of large thrombi and their subsequent embolization from the surface, as seen by the monotonic shape of the curve in Figure 3.4.

The average platelet density for GT-12 decreases with increasing concentration up to 50 μM, but increases again at higher concentrations (see Table 3.1). This apparent anomaly can be explained by two facts: first, at 50 and 100 μM, the larger thrombi
embolize from the surface, decreasing significantly the number of platelets accumulated on the surface; second, as the compound concentration is increased and platelet to platelet interactions are increasingly inhibited, it is likely, as shown by others (Baumgartner et al., 1980a), that platelet to collagen interaction (adhesion) is enhanced, leading to an increasing number of platelets accumulated on the reactive surface in the form of a monolayer. The results from the macroscopic measurements clearly show that as GT-12 concentration is increased, the reaction rate between platelets at the surface (aggregation) also decreases, and that the platelet aggregates become so unstable that embolization of the larger thrombi can occur at the entrance of the flow chamber, indicating possibly that the compound weakens interplatelet fibrinogen bridges and/or affects indirectly the formation or conversion of thrombin to fibrin, thereby decreasing the overall thrombus stability. It should be stressed that the released emboli are miniature in size and are not prone to occlude vascular beds. While diminishing platelet aggregation, when used in increasing concentration, the antiplatelet agent indirectly potentiates platelet adhesion on the collagen fibrils. Indeed, since the fluid layer near the reactive surface is only slowly depleted of platelets when large concentrations of the compound are used, it is most likely that, downstream, the number of platelet collisions, contacts, and attachments to the surface, leading to platelet spreading and adhesion on the surface, might be enhanced.

When compared with the inhibitory effect of the well-known reference compound chlorpromazine, GT-12's action on platelet responses is much stronger, possibly indicating a molecular structure more specific for interactions with anionic phospholipids (Quintana et al., 1981a) within the platelet plasma membrane's lipid
bilayer, and hence more suitable for hindrance of platelet aggregation. Indeed, by "fine-tuning" the chemical structure of several compounds for maximum inhibition of the aggregation process, Lasslo et al. (1983) suggested that compounds bearing two tertiary amino functions separated by an interatomic distance of about 8 Å, such as GT-12, have optimal configuration for interaction with two aggregation-inhibitory specific target sites in the plasma membrane. Although both compounds alter normal platelet function predominantly by the same mechanism (Lasslo and Quintana, 1984; Lasslo, 1984), chlorpromazine has been known also to have a stabilizing effect on RBC membranes, preventing hemolysis and release of the proaggregating ADP in situations of high shear rates (Born et al., 1970; Born and Wehrmeier, 1979). There is evidence, when comparing the results presented above with previous findings obtained in aggregometric studies with citrated PRP (Quintana et al., 1981b; Lasslo and White, 1984), that GT-12 interacts very little with red cell membranes or with most other plasma proteins. As shown in Figures 3.15 and 3.16, GT-12 at 50 μM inhibits ADP-induced aggregation by 66±1% and thrombin-induced aggregation by 82±2% in aggregometric studies (Lasslo et al., 1984). Similarly, the results of this investigation show that the compound, at this concentration, inhibits the average platelet density per unit area by 72±6% after 30 minutes incubation and by 83±8% after 5 minutes incubation, the number of platelets per thrombus by 87±2%, and the maximum number of aggregated platelets per thrombus by 62%, all of these results obtained after two minutes of perfusion over collagen-coated glass at 1000/sec. Undeniably, those results are in the same range as those published earlier by Lasslo et al. (1984). On the other hand, their data with chlorpromazine are significantly higher than those reported here.
Figure 3.15: Comparative inhibitory potencies in ADP-induced platelet aggregation in an aggregometer using PRP: X: α,α′-bis[3-(N,N-diethylcarbamoyl)piperidino]-p-xylene dihydrobromide (GT-12); II: N,N'-bis(1-decylnicotoyl)piperazine dihydriodide; and aspirin. Chlorpromazine registered 43 ± 1% inhibition at the same concentration and under the same conditions (from Lasslo et al, 1983).
Figure 3.15. (from Laslo et al., 1983).

Figure 3.16: Comparative inhibitory potencies in thrombin-induced platelet aggregation in an aggregometer with PRP. The notation for the two piperidine congeners is the same as that of
due to the presence of RBCs in the experiments performed in this study. Indeed, as also shown in Figures 3.15 and 3.16, chlorpromazine inhibits ADP-induced aggregation by 43±1% and thrombin-induced aggregation by 11±3% when used in 50 μM concentration in their experiments (Lasslo et al., 1984). On the other hand, the data of this study indicate that the drug at 100 μM only inhibits the average platelet density per unit area by 23±8% and the number of platelets per thrombus by 6±2%, and even slightly increases the number of aggregated platelets per thrombus by 1.4%.

The striking effect of GT-12 on the morphology of a single thrombus growing on a collagen-coated surface was demonstrated by the results of the microscopic measurements. As shown quantitatively in Figures 3.7 and 3.8, the thrombus rate of growth, when treated with 50 μM GT-12, practically vanishes after approximately 1 minute of flow, possibly indicating that an equilibrium is reached at that time between the rate of embolization of individual platelets and/or platelet aggregates from the thrombus and the rate of platelet attachment to the thrombus. Qualitatively, the effects of the compound are shown in Figures 3.10 and 3.11, respectively, in terms of three-dimensional structures of thrombi and in terms of contour diagrams. Three important pieces of information regarding the mechanism of thrombogenesis under the influence of an antiplatelet agent are obtained from those contour diagrams. First, there is evidence, by comparing the contour diagram (3) and the curve of the thrombus basal area as a function of time for the GT-12 case in Figure 3.8, that the upstream part of the thrombus has embolized from the surface between 60 and 90 seconds, since the basal area was measured to be the same at those times; second, after 30 or 60 seconds of flow, the thrombus grows mostly from its downstream edges; third, the thrombus is clearly
elongated in the direction of flow.

The first observation agrees with the results obtained from the macroscopic measurements which show that at 50 μM, GT-12 induces the embolization of parts of the larger thrombi near the inlet of the reactive surface. The second and third observations indicate that, although the compound considerably affects platelet to platelet interactions, the way platelets are recruited at the downstream ends of the growing thrombus is still the same as reported earlier (Hubbell and McIntire, 1986c, 1986a). Thrombin, generated enzymatically on the surface of the platelets in the presence of extracellular Ca$^{2+}$, and shown to be of vital importance in inducing platelet activation in this type of flow system (Hubbell and McIntire, 1986a, 1988), might be responsible for the bulk of platelet adhesion and aggregation observed, since the antiplatelet agent does not directly inhibit its production. Although apparently peculiar, the fact that increasing concentrations of GT-12 do not decrease thrombus size, or the number of aggregated platelets accordingly, as illustrated by the data in Table 3.2 and the contour diagrams (2) and (3) in Figure 3.11, shows that the final inhibitory effect of the compound is due to both the embolization rate of parts of the thrombus from the surface and the reaction rate between platelets at the surface. At 50 μM, platelet reactivity at the surface is the highest, but because the thrombus grows faster and bigger, the embolization rate is also the largest due to the combined effects of the drug action on thrombus stability and interplatelet bonds and the fluid drag forces at the high shear rate of 1000/sec. At 200 μM on the other hand, the reaction rate between platelets at the surface is so low that essentially no embolization occurs. At concentrations intermediate between 50 and 200 μM, it is very probable that both the
embolization rate and the reaction rate determine the inhibitory effect of the drug on thrombus growth at the end of the perfusion period.

Whereas several researchers, including Hubbell and McIntire (1988) and Baumgartner (1979), have shown that blockage of cyclo-oxygenase activity, and thereby TxA2 production, with aspirin did not inhibit or only moderately inhibited thrombus growth on collagen, the results of this study might indirectly confirm the findings of others that platelets might become activated, in response to high doses of collagen or thrombin, through an AA independent pathway (Vargaftig, 1977; Lapetina et al., 1978). Indeed, in the light of current interpretations (Kinlough-Rathbone and Mustard, 1987; Drummond and Maclntire, 1987), one may perceive from the results of this study that IP3 does play a meaningful role in inducing platelet activation - independently of any action in the AA metabolism - by mobilizing directly Ca2+ from intracellular storage sites, and generating sufficient internal calcium flux to cause platelet activation. This could explain why the inhibition of prostaglandin peroxide formation by aspirin barely affects thrombus growth on collagen. It is in contrast with the striking effect of GT-12. Indeed, current findings corroborate previously reported observations indicating that GT-12 acts (i) by interacting with and reducing there response sensitivity of anionic phospholipids (Quintana et al., 1981a; Lasslo, 1984), stabilizing membrane storage sites and impeding mobilization of Ca2+ in platelet cytosol (Lasslo and Quintana, 1984), (ii) by inhibiting activation of PLC through reinforcement of platelet membrane surfaces (Lasslo and White, 1984) and thereby precluding formation of IP3, as well as (iii) by blocking stimulation of PLA2 as a result of reduced Ca2+ flux, along with the pathway this generates.
The microscopic results of Figures 3.12 to 3.14 indicate that aurin tricarboxylic acid (ATA) considerably reduces individual thrombus size in response to collagen under flow conditions, in a concentration-dependent manner. Both concentrations of ATA used in this study significantly reduced the number of platelets per thrombus (≥ 80%) when compared to controls. This indicates that ATA inhibits platelet/platelet adhesion mediated by collagen in whole blood and is consistent with a major involvement of the largest vWF multimers found in plasma - and/or the vWF multimers released from platelets' granules upon activation - in the thrombogenesis process (Moake et al., 1986, 1988; Phillips et al., 1988). The results of this study also indicate that thrombus basal area does not decrease as rapidly as the number of platelets per thrombus with increasing drug concentration. In other words, platelet adhesion to collagen is not as readily affected by the drug as platelet adhesion to each other, when the compound is used in low concentration. In fact, it was reported (Packham and Mustard, 1984) that vWF might play a role in platelet adhesion to collagen only at shear rates that are typical of the microvasculature (1300-5200 sec⁻¹). At 1000 sec⁻¹, it is likely that vWF is not the only factor involved in that process. Other plasma proteins, such as fibrinogen, fibronectin, and thrombospondin, may be involved as well. Further experiments confirming that ATA does not bind to collagen fibrils or to other plasma proteins involved in the adhesion and aggregation process should to be done in order to further enlighten the mechanism of action of that antithrombotic agent in whole blood.
CHAPTER 4

ROLES OF COLLAGEN AND von WILLEBRAND FACTOR IN MURAL
THROMBOGENESIS UNDER DIFFERENT FLOW CONDITIONS

4.1 Introduction

The multimeric proteins collagen and von Willebrand factor are two of the most important proteins involved in the processes of platelet adhesion to and platelet aggregation on the subendothelium of injured blood vessels. Since most of these proteins are available today in purified forms, the main purpose of this study was to investigate, for each of them, their specific thrombogenicity, and/or the roles they might play in the thrombogenesis process under different flow conditions, spanning the physiological range. Because much work has been done by others in this area and in order to better understand the objectives of this study, a summary of what is actually known on the biology of those two proteins as well as on their role in the normal and abnormal hemostatic mechanisms is given below.

4.1.1 Collagen - a Review -
4.1.1.a Physiology and Biosynthesis

The connective tissue of most cells in multicellular organisms consists of the extracellular matrix and the cells found in it, such as fibroblasts, smooth muscle cells (SMC), and macrophages. The two major macromolecules found in the matrix are the collagens and the polysaccharide glycosaminoglycans. Also present in the matrix are the fibrous protein elastin and the two glycoproteins fibronectin and laminin. Collagen is by far the most abundant fibrous protein in mammals, constituting approximately twenty five percent of their total protein content. The collagens represent a family of closely related glycoproteins, each of which is genetically distinct, has unique structural features, and unique functions. Although the molecular structure of most collagens is well established today, incomplete information exists regarding their functions in the extracellular matrix (Alberts et al., 1983).

All collagen molecules, called tropocollagens, consist of three polypeptide chains (the \( \alpha \)-chains), wound around each other in a triple-stranded helical structure 300 nm long and 1.5 nm in diameter. Each chain contains approximately 1,000 amino acids. Although 18 genetically distinct \( \alpha \)-chains have been isolated, only 15 collagen molecules have been described until now. In a given collagen molecule, the \( \alpha \)-chains may or may not be identical. For example, type I collagen is a heterotrimer of two nonidentical chains, \( \alpha 1(I) \) and \( \alpha 2(I) \), and has the molecular composition \([\alpha 1(I)]_2 \alpha 2(I)\). In contrast, type II collagen is a homotrimer of three identical chains, \( \alpha 1(II) \), and forms molecules with the composition \([\alpha 1(II)]_3\). The collagen family is usually divided into two categories: first, the interstitial collagens (types I, II, and III), which are the most abundant in
connective tissues and which have been shown to form broad-banded fibrils of 67 nm periodicity in vivo and in vitro; second, the remaining collagens (types IV through IX), which do not form fibrils with a 67 nm periodicity in vivo, and, for the majority of them, have an uncertain quaternary structure in the extracellular matrix (Alberts et al., 1983; Uitto and Perejda, 1987).

Collagens are synthesized by several cells of the matrix, primarily endothelial cells (ECs), SMCs, and fibroblasts, depending upon the location and the collagen type. The biosynthesis of the α-chains occurs on membrane-bound ribosomes; those chains are then injected into the lumen of the endoplasmic reticulum (ER) as large precursors, called pro-α-chains. These precursors possess in addition to the α-chains, two globular peptides (propeptides) at both their amino- and carboxy-terminal ends. The three pro-α-chains in the procollagen molecule are covalently bound by interchain disulfide bonds within the carboxyl terminal globular domain which consists essentially of a large globular peptide. The amino terminal globular domain, on the other end, contains three separate regions. At the extreme amino end is a large globular region containing intrachain disulfide bonds, followed by a short triple-helical domain, and another but smaller globular domain. The role of those extension peptides is probably to guide triple-helix formation between three pro-α-chains in the lumen of the ER. Collagen has a much more regular amino acid sequence than most globular proteins, having a glycine residue (GLY) at every third residue and being rich in proline (PRO). Glycine, due to the smallness of its side chain, can easily occupy the interior of the collagen triple helix. Two important posttranslational modifications occur before secretion of the protein by exocytosis in the extracellular space. First, some of the proline and lysine
residues are hydroxylated in the ER prior to pro-α-chain association. It appears that the hydroxyl groups of the hydroxyproline residues form interchain hydrogen-bonds to stabilize the triple helix. Second, some of the hydroxylysine residues are glycosylated in the ER to form varying but unusually-short oligosaccharide side chains (galactose and glucose) whose function is currently unknown. Subsequently, procollagen molecules follow the classical secretory pathway, moving from the ER to the Golgi apparatus, where they are packaged into vesicles and then secreted into the extracellular space by fusion of the vesicle with the plasma membrane of the cell (Alberts et al., 1983; Uitto and Perejda, 1987).

During the secretion process, the terminal globular domains of the interstitial procollagen molecules are removed by specific proteolytic enzymes, namely the procollagen peptidases, to form tropocollagen molecules. The enzymatic sites for removal of those terminal peptides do not occur exactly at the transition between the triple helix and the propeptides, but rather several amino acids within the globular domain, leaving non-triple helical regions known as telopeptides. Those telopeptides are major sites for cross-links between molecules in fibers. Next, fibrillogenesis occurs between several collagen molecules near the cell surface, leading several investigators to think that the cell regulates the sites and the rates of fibril assembly. Most other procollagen molecules (types IV through IX) seem to remain totally or partially uncleaved during secretion, and thereby, do not form typical collagen fibrils. Type IV collagen, in particular, has been recently reported forming dimers and tetramers in vitro, but those unusual fibrils have not been detected yet in the extracellular matrix (Duncan et al., 1983; Furthmayr et al., 1983; Yurchenco and Furthmayr, 1984).
When fixed and stained, typical collagen fibrils exhibit cross-striations every 67 nm, reflecting the fact that the individual collagen molecules are staggered in the fibrils so that adjacent molecules are displaced longitudinally by 67 nm. A cylindrical fiber consists of several microfibrils, each of which is a group of five collagen molecules assembled into a long, thin helical subunit. These microfibrils then flatten as they assemble to form a thicker collagen fibril. Fibril diameter varies in living organism, ranging from 10 to 300 nm. Although the side chains of the amino acids in the tropocollagen molecules are largely responsible for fibril formation, these data suggest that other macromolecules present in the extracellular matrix might also regulate fibril formation by some undefined mechanism. Once formed, collagen fibrils are greatly strengthened by covalent intra- and intermolecular cross-links. If cross-linking is inhibited, collagenous tissues become fragile, and structures like skin, tendons, and blood vessels tend to tear. These covalent cross-links are unique to collagen and are formed in several steps, particularly in the non-helical telopeptide region. First, certain lysine and hydroxylysine residues are deaminated by the extracellular enzyme lysyl oxidase to yield highly reactive aldehyde groups. The aldehydes then form covalent bonds with each other or with other lysine or hydroxylysine residues. Some of these bonds are relatively unstable and are ultimately modified to form a variety of more stable cross-links. The extent and type of cross-linking varies from tissue to tissue depending on the tensile strength required. Interstitial collagens, however, in particular type I collagen, may also be present in the extracellular matrix as fibers, an assembly of several fibrils (Alberts et al., 1983; Uitto and Perejda, 1987). A schematic drawing of the various intracellular and extracellular events involved in the formation of a
collagen fibril is shown in Figure 4.1.

4.1.1.b The Collagens of the Vasculature

Although each collagen type has a unique structure and function, only those found in the human vasculature, that is, types I, III, IV, V, and VI, are described in some detail in this review (Alberts et al., 1983; Uitto and Perejda, 1987).

Type I collagen is a heterotrimer with chain composition $[\alpha 1(I)]_2 \alpha 2(I)$ which is broadly distributed throughout the connective tissues and is the major collagen found in bone, skin, and dentin. A homotrimer with chain composition $[\alpha 1(I)]_3$ has also been identified as a minor component of skin and as a biosynthetic product of embryonic and tumor cells.

Type III collagen is a homotrimer with chain composition $[\alpha 1(III)]_3$ which occurs in all connective tissues where type I is found. Although the exact function of type III collagen in those tissues is still uncertain, there appears to be a direct correlation between the relative proportion of type III to type I collagen and the extensibility of the connective tissue. For example, relatively high concentrations of type III collagen are found in highly extensible tissues such as skin and blood vessels.

As mentioned earlier, the interstitial procollagen molecules contain two types of structural domains, namely, a triple-helical domain and two globular domains at both terminal ends. The sizes and distributions of these domains in the interstitial procollagens are quite similar. Furthermore, their helical structure confers them a unique feature, namely, a resistance to proteolytic degradation by certain enzymes. Type III collagen, however, is a minor exception, in that it contains a single trypsin cleavage
Figure 4.1: Intracellular biosynthesis and extracellular processing of collagen fiber. (From Alberts et al., 1983).
site in the triple helix near the collagenase binding site. Type III collagen is also unique in that it contains disulfide-bonded cysteinylic residues within the triple helix near the carboxyl terminus.

The other collagens of the vasculature, that is, types IV, V and VI, differ significantly from the interstitial collagens mainly because they do not form broad-banded fibrils in vivo or in vitro, but also because they have different sizes and/or distributions of their structural domains.

**Type IV collagen** contains two α-chains, α1(IV) and α2(IV), and has the heterotrimeric structure [α1(IV)]_2 α2(IV). Type IV collagen is only found in the basal laminae of the basement membrane. The helical structure of that collagen differs somewhat from that of the interstitial collagens. First, it is 330 nm long instead of 300 nm; second, it contains cysteinylic residues that form disulfide bonds; third, it contains several regions in which the sequence GLY-X-Y is disrupted. The amino terminal domain is also different, containing a large triple-helical region separated from the α-chain by a small globular domain, named NC-2.

**Type V collagen** consists of three different α-chains: α1(V), α2(V), and α3(V). Although the exact number of molecules containing these chains is still ambiguous, at least four molecules have been reported with some confidence: [α1(V)]_2 α2(V), [α1(V)]_3, [α3(V)]_3, and α1(V)α2(V)α3(V). The exact location of type V collagen is another controversy. The most recent data indicate that it is found associated with basement membranes, with cell surfaces, and with the connective tissue stroma. The sizes and distributions of the structural domains of type V collagen and the interstitial collagens are quite similar. The most striking difference between those types is that only
a portion of the amino and carboxyl terminal globular domains is excised during secretion of type V collagen.

**Type VI collagen** contains two α-chains, α1(VI) and α2(VI), and has the heterotrimeric structure \([α1(VI)]_2 α2(VI)\). Although this collagen was first identified in the aortic intima, it is now thought to have a broad, but yet undefined, tissue distribution. Its main characteristics are a very short triple-helical domain (103 nm), a large globular domain at one end and a small globular domain at the other end of the triple-helical region, and the presence of disulfide bonds within both the helical and globular domains.

4.1.1.c **Collagen-Platelet Interaction and Objectives**

Collagen is the most abundant fibrous protein in the vasculature (Alberts et al., 1983) and has been recognized to be a primary surface on which blood platelets adhere, and subsequently aggregate, following removal of the luminal endothelium by hemodynamic factors and/or chemical agents (Packham and Mustard, 1984; Santoro and Cunningham, 1981). In addition to its role in the induction of platelet aggregation, collagen induces the secretion of platelet-bound proaggregatory substances, including ADP, the coagulation factors, factor V and factor VIII-vWF, and fibrinogen, and directly initiates several steps of the intrinsic coagulation pathway, whose main purpose is to form fibrin and thereby consolidate the platelet plug (Barnes, 1982). In particular, collagen may activate factor XII to XIIa and the platelet-associated factor XI to Xla, and specifically increase platelet-associated factor V activity. Activation of platelets by collagen also leads to the production of thrombin and TxA₂, two of the most powerful
platelet-aggregating agents yet described. Despite its vital role in the normal hemostatic mechanism, collagen is also associated with the pathological complications of hemostasis, namely, thrombosis and atherosclerosis, leading to the formation of mural thrombi and emboli, and, in the most severe cases, to the obstruction of the blood vessel and to tissue death.

Complications arise, when trying to determine the thrombogenicity of collagen in vivo, because blood vessel walls contain different collagen subtypes, in varying amounts, with changing quaternary structures, and thus with different responses with respect to platelet adhesion and thrombus formation (Packham and Mustard, 1984; Barnes, 1982). Collagen types I, III, IV, V and VI have been demonstrated to be present in the vessel wall by several chemical and immunohistological methods (Chung and Miller, 1974; Madri et al., 1980; McCullagh et al., 1980; Furthmayr et al., 1983; Burgess and Morris, 1987). Type IV is the predominant collagen form found in the basement membrane beneath the endothelium, although in vitro culture of vascular endothelial cells (ECs) has indicated that those cells can secrete types III and V as well (Sage et al., 1979; Sankey and Barnes, 1980; Sankey et al., 1981; Sage and Bornstein, 1982). More recently, other studies have suggested that type V collagen might also be located on the EC surface, possibly making those cells nonthrombogenic (Parsons et al., 1983 and 1986). The interstitial collagens (types I and III) are the most abundant collagen types found in the subendothelium of the vessel wall. In the young vessel wall, where there is little if any intimal thickening, type III collagen is the predominant collagenous species in the space immediately between the basement membrane and the internal elastic lamina. As the vessel wall becomes older, the intima thickens and several biochemical
analyses have demonstrated that type I collagen, synthesized there by migrating smooth muscle cells, is the predominant species in the thickened intima, as well as in the media and the adventitia (McCullagh et al., 1980). Type V collagen has been found in the basement membrane, the subendothelium, and the media, without specific location in the extracellular matrix (Mayne, 1987). It has been suggested, however, that type V may be closely associated with the interstitial collagens and possibly involved in the organization or formation of interstitial collagen fibrils (Mayne, 1987; Fessler and Fessler, 1987). From one study using immunofluorescent staining of blood vessels with antibodies prepared against type VI collagen, it was reported that this collagen type had a broad distribution throughout the matrix with the exception of the basement membrane (Jander at al., 1981). Although the interstitial collagens are known to form native-type fibrils (67 nm periodicity) in vivo, the exact molecular arrangement of the other collagens of the vasculature remains unresolved and somewhat controversial (Burgeson and Morris, 1987; Mayne, 1987; Fessler and Fessler, 1987).

By measuring changes in light transmittance with an aggregometer, many research groups have investigated the thrombogenicity of individual collagen subtypes, in purified forms, in the last few years (Barnes, 1982). In that system, most of them have concluded that monomeric collagen (tropocollagen) is unable to induce platelet aggregation and that some degree of polymerization into fibrillar collagen is required to induce platelet aggregation (Barnes, 1982). In other words, all collagen types found in the blood vessel wall have been shown to induce platelet aggregation when exposed to platelet in a suitable fibrillar structure. Although the interstitial collagens in their native-type quaternary structure (67 nm periodicity) have been shown to induce
platelet aggregation in vivo and in vitro, other ordered molecular assemblies such as the segment-long-spacing (SLS) polymers, in which the collagen molecules align alongside in a "head-to-head" and "tail-to-tail" arrangement, or alternatively, the fibrous-long-spacing (FLS) polymer, in which there is end-to-end aggregation of molecules laterally associated in a "head-to-tail" alignment, can also induce platelet aggregation in vitro. The native-type fibril can be formed in vitro from tropocollagen solution by incubation at 37°C or by dialysis against 0.002 M Na₂HPO₄. More recently, a few studies have been conducted to assess the thrombogenicity of several collagen types under physiological flow conditions in vitro (Parsons et al., 1983 and 1986; Baumgartner, 1977; Houdijk et al., 1985). In some instances, platelet/platelet adhesion was blocked with aspirin, apyrase, thrombin inhibitors, etc., preventing thrombus formation, and only platelet/collagen adhesion was measured. Furthermore, the effect of wall shear rate, a fundamental flow parameter controlling platelet transport to the wall, on collagen thrombogenicity was often addressed incompletely.

For those reasons, the purpose of this study was to investigate the effect of wall shear rate on platelet adherence to and thrombus formation on several purified collagen types (types I, III, IV, V and VI) found in the vasculature. For collagen types IV, V and VI, we also examined the effects of molecular configuration (monomeric vs. fibrillar) and method of fibrillogenesis (thermal gelation vs. dialysis) on their reactivity with human blood platelets. In this context, heparinized whole blood with fluorescent-labelled platelets was perfused in a parallel plate flow chamber coated with a purified collagen for two minutes at different wall shear rates, ranging from 100 sec⁻¹ (a typical venous wall shear rate) to 1500 sec⁻¹ (a typical arterial wall shear rate). Platelet
surface deposition was continuously monitored by an epifluorescence video microscopy system, coupled with digital image processing techniques. This experimental set-up allowed us to visualize qualitatively and analyze quantitatively the size and shape of individual platelet aggregates forming locally in the field of view, as well as to estimate the local platelet surface coverage. Macroscopic analysis of the end-point platelet accumulation along the coated surface was performed with the computerized microphotometric measurement system described earlier. With this method, we are able to determine the axial dependence of platelet deposition along the reactive surface. The video microscopy equipment, the digital image processing techniques and the microphotometric measurement system were described in details in Chapter 2.

4.1.2 von Willebrand Factor - a Review -

Most of the information presented here was obtained from three excellent reviews on the subject (Meyer and Baumgartner, 1983; Girma et al., 1987; Moake, 1989).

4.1.2.a Characteristics and Functions

von Willebrand Factor (vWF) is a high molecular weight glycoprotein, absent in von Willebrand’s disease, and which has been shown to play an important role in the interaction between blood platelets and the subendothelium of injured blood vessels. vWF is a multimeric protein composed of identical subunits. Its concentration in plasma varies between 7 to 10 μg/ml and its molecular weight between 0.5 to 20 x10^6 daltons.

It has two major functions in vivo: first, it serves as a cofactor of platelet adhesion
to the subendothelium, especially at high shear rates; second, it serves as the carrier protein for the coagulation Factor VIII, absent in hemophilia A. Those two functions are also illustrated in Figure 4.2.

In vitro, vWF is estimated by its ristocetin-cofactor activity and by its antigenic determinants that bind very specifically polyclonal or monoclonal antibodies. vWF is synthesized by the endothelial cells (ECs), secreted either in the blood stream or in the subendothelial matrix, and by the megakaryocytes. There are three sources of vWF involved in platelet adhesion to the subendothelium. First, there is the vWF circulating in plasma which has been shown to bind to the subendothelium, to support platelet initial contact, spreading, and even thrombus formation, and to be necessary for maximum platelet adhesion. Second, there is the vWF present in the vessel wall which could support up to 50% of the total platelet adhesion. Finally, there is the vWF released from α-granules of platelets, whose role in the thrombogenesis process remains unclear.

vWF is a multimeric protein of various size. In particular, unusually large vWF forms, absent from normal plasma, are present, together with the normal plasma forms, within ECs. In vitro, those unusually large forms and the the normal forms are secreted by the ECs in the culture medium and can stick to the extracellular cell matrix. In vivo, on the other hand, it is suspected that those unusually large forms may become depolymerized as they are secreted by ECs into the blood stream, resulting in smaller vWF multimers. The interesting point, however, is that those unusually large forms may be secreted, without being depolymerized, into the subendothelial matrix, therefore being a potential important modulator of platelet adhesion.
Figure 4.2: Functions of von Willebrand Factor: carrier protein for the coagulation Factor VIII (top) and cofactor of platelet adhesion to the subendothelium (bottom). (From Hirsh and Brain, 1983).
4.1.2.b Interaction of vWF with Platelets

vWF interacts with two binding sites on the platelet plasma membrane. vWF binds to the glycoprotein Ib (Gp Ib), absent in Bernard-Soulier syndrome, during platelet/vessel wall interaction, and to glycoprotein IIb/IIIa (Gp IIb/IIIa), absent in Glanzmann's thrombasthenia, during platelet/platelet interactions. Those receptors on the plasma membrane become energized only when platelets are activated by some stimulus. In fact, it has been shown that native human VWF only binds its platelet receptors in the presence of ristocetin, ADP, or thrombin. More recently, the carbohydrate side chains (sialic acid and penultimate galactose residues) have been proposed as being the structural moieties responsible for vWF binding to the platelet membrane. Indeed, it was found that modified vWF (asialo-vWF), on which the penultimate galactose residues were exposed, bound directly to the platelet membrane and allowed platelet aggregation even in the absence of ristocetin.

4.1.2.c Molecular Structure and Function Relationship of vWF

Recent data have shown that all the essential information regarding the function of vWF resides in its basic subunit, that is in a single polypeptide chain, and is not brought about by the conformational changes caused be the polymerization into large multimers. In fact, this was shown in perfusion studies by the fact that platelet adhesion to collagen was supported in a large extent by a dimeric proteolytic fragment of vWF, instead of the complete multimeric protein. This topic remains controversial, however, because Moake and coworkers (1986 and 1988) have also shown that the unusually large vWF multimers secreted by ECs and the largest forms found in plasma are more effective in
inducing shear-induced platelet aggregation in high shear stress environments than the normal plasma forms. Other studies using molecular cloning of cDNA of vWF and amino acid sequencing of vWF have been very helpful in localizing the different functional domains on the molecule. Those studies have revealed that the vWF precursor protein is made of the normal "mature" vWF polypeptide bound to another protein called vW antigen II, which is split off from vWF in the ECs. The functional role of vW antigen II is unknown at the present time, but it contains an arginine-glycine-aspartic acid (RGD) sequence, also found in other adhesive proteins binding Gp Iib/IIIa, such as vWF, fibrinogen, and fibronectin. Binding sites on the vWF subunit are known for collagen, heparin, Gp Ib, and GP Iib/IIIa. Little is known, however, concerning the binding sites for factor VIII and the subendothelial matrix. The repeating domains of amino acids in the vWF precursor protein and the functional domains in a single "mature" vWF subunit are illustrated in Figure 4.3.

4.1.2.d Interaction of vWF with Components of the Subendothelium and Objectives

The precise binding site for vWF in the vessel wall is still unknown. It was shown that vWF binds to monomeric and fibrillar human collagen types I and III found in the vessel wall and that platelet adhesion to those two collagen types is vWF-dependent at high wall shear rates. However, collagen is probably not the only binding site for vWF in the subendothelium, as shown by the fact that monoclonal antibodies against vWF, inhibiting binding of vWF to collagen, had no influence on the binding of vWF to the matrix of cultured ECs and smooth muscle cells.

Tchopp, Weiss, and Baumgartner (1974) were the first to demonstrate the role
Figure 4.3: Schematic representation of the VWF precursor (left), showing the signal peptide residues 1-25, the VWF A1 domain (residues 26-763), and the mature VWF (residues 764 to 1270). The hatched areas indicate internal homologous regions in the amino acid sequence of the precursor. The hatched area (from Gilmour et al., 1997).
played by vWF in supporting platelet adhesion to the subendothelium. For that purpose, they perfused human blood through a flow chamber containing deendotheliazed segments of everted rabbit aorta. They showed that both platelet adhesion and thrombus formation were decreased in von Willebrand's disease, either in citrated or native blood. Since then, other models (Weiss et al., 1978a; Weiss et al., 1978a; Baumgartner et al., 1980b; Sixma et al., 1984), in some cases using human arteries or polyclonal and monoclonal antibodies to vWF, have given similar results. In those studies, the wall shear rate was the critical parameter determining the role of vWF in the process of platelet adhesion to the subendothelial matrix. At low wall shear rates ($\leq 200$ sec$^{-1}$), platelet adhesion was reported to be normal in von Willebrand's disease. At intermediate wall shear rates ($500$ - $1000$ sec$^{-1}$), adhesion defects occurred only in citrated blood, but not in native blood. At higher wall shear rates ($\geq 1,300$ sec$^{-1}$), adhesion defects also occurred in native blood. Those results led some investigators to think that there might be two different platelet adhesion mechanisms to the subendothelium: one that involves vWF in high shear environments and another that does not require vWF in low shear environments.

Based on what is known on vWF and its crucial role in the thrombogenesis process, preliminary experiments were performed in this study with the following objectives in mind: (1) to further investigate platelet adhesion and thrombus formation defects in von Willebrand's disease as a function of wall shear rate, using heparinized whole blood perfused in a parallel plate flow chamber coated with collagen fibrils. (2) To study the importance of platelet-released vWF in platelet adhesion and thrombus formation as a function of wall shear rate, and (3) to determine the potential role of the unusually
large vWF multimeric forms secreted by ECs into the extracellular matrix in augmenting platelet adhesion and thrombus formation on collagen-coated surfaces.

4.2 Experimental Technique

4.2.1 Perfusion Studies

For the experiments involving collagen, heparin-anticoagulated whole blood (10 U/ml), drawn from a large pool of healthy donors, with fluorescent-labelled platelets (10 μM mepacrine) was aspirated by a syringe pump (Harvard Apparatus, South Natick, MA, Model 935) for two minutes at a controlled wall shear rate of 100 sec⁻¹, 800 sec⁻¹, or 1500 sec⁻¹ from a test tube, kept at 37°C in a water bath, through a polycarbonate parallel-plate flow chamber, as described in depth in Chapter 2. Before being inserted in the flow chamber, 7.4 cm² of a glass cover slip (Corning Glass Works, Corning, NY; No.1, 24 x 50 mm) were coated with the purified collagen of interest, the remaining portion of the slide being covered with parafilm.

For the experiments involving vWF, blood was obtained from three normal individuals and one patient with severe von Willebrand's disease (vWD). In one set of experiments, blood was drawn from the patient with vWD into 10 U/ml of heparin and 10 μM mepacrine as before. In another set of experiments, the vWF-deficient platelets were removed from platelet-rich plasma (PRP) by centrifugation and normal washed platelets (NP) added back to make a final platelet count of approximately 120,000/μl. The protocol used to wash platelets was the following. Blood from a healthy donor was
drawn into acid-citrate-dextrose, and PRP obtained by centrifugation at 250 g for 15 minutes. The pH was adjusted to 6.5 and platelets were pelleted by centrifugation. Those platelets were washed once in HEPES buffer (10 mM, pH=7) containing 145 mM NaCl, 5 mM KCl, 0.5 mM Na₂HPO₄, 1 mM MgSO₄, 0.1 mM CaCl₂, 5.5 mM glucose, 3.5 g/L bovine serum albumin, 50 U/ml sodium heparin, and 2.5 U/ml apyrase, and resuspended in the heparinized vWD platelet-poor plasma (PPP). Finally, RBCs from the vWD patient were added to this reconstituted PRP in a ratio 3:2 to yield an hematocrit of approximately 40%. In those experiments, blood was perfused for 1.5 minutes into a parallel plate flow chamber coated with type I bovine collagen fibrils at three different wall shear rates, 100 sec⁻¹, 800 sec⁻¹, and 1500 sec⁻¹. In a third set of experiments, we eliminated plasma vWF from the test medium by washing normal platelets in the same way and resuspending them in HEPES buffer (HEPES-NaCl-KCl-Na₂HPO₄-MgSO₄-glucose-BSA), pH=7.4, containing an increased concentration of CaCl₂ (1 mM). The final platelet count was approximately 300,000/μl in this case. Concurrently, RBCs were washed a number of times in phosphate buffer and saline containing albumin (3.5 mg/ml) and dextrose (1 mg/ml) at a pH of 7.4. Again, the platelet suspension was mixed with packed washed RBCs 3:2 to yield an hematocrit of approximately 40%. In those experiments, a glass cover slip was first coated with type I human collagen fibrils, formed by thermal gelation as described below. The slide was then rinsed with 10 ml of isotonic saline and coated with 200 μl of prewarmed endothelial cell supernatants containing unusually large vWF multimers, generously provided by Dr. J.L. Moake. The vWF antigen levels in those samples, quantified by solid-phase immunoradiometric assay (Moake et al., 1982), varied between 9.9 and
11.9 U/dl. The supernatant was allowed to evaporate completely at room temperature, at which time the slide was rinsed with 10 ml of PBS (pH=7.4) and placed inside the parallel plate flow chamber. The reconstituted blood was perfused for 1.5 minutes at 800 sec\(^{-1}\) or 1 minute at 2000 sec\(^{-1}\).

4.2.2 Collagen Preparation and Glass Cover Slip Coating

- **Type I Bovine Fibrils**: type I acid-insoluble collagen (Sigma Chemical Co., St. Louis, MO; C9879) from bovine achille's tendon was prepared as previously reported in Chapter 3. 200 µl of the fibrillar collagen suspension were contacted with a glass cover slip under static conditions for 45 to 60 minutes before the supernatant was rinsed off with 10 ml of sterile isotonic saline. The final average collagen density on the surface was estimated to be around 3.50 µg/cm\(^2\).

- **Types I, III, IV and V Human Fibrils (by thermal gelation)**: types I, III, IV and V acid-soluble collagen were purified by pepsin extraction and/or salt fractionation methods (Sigma Chemical Co.; C7774, C4407, C7521 and C3657). 2.5 mg of each collagen subtype were mixed with 1 ml of 0.012 N HCl (pH=2) cooled at 4°C in a polypropylene test tube. The test tube was then placed on a low-speed nutator and the collagen allowed to dissolve completely for 1 hour at room temperature. This monomeric collagen (tropocollagen) solution was kept at 4°C until further utilization. A neutralized, isotonic collagen solution was then prepared by mixing 1 ml of chilled tropocollagen solution with 104.2 µl of 0.1 M NaOH and 104.2 µl of 10X phosphate-buffered saline solution (0.2 M Na\(_2\)HPO\(_4\), 1.3 M NaCl, pH=7.4) to make a final collagen concentration of 2.07 mg/ml. The pH of that solution was adjusted to 7.4 ±
0.2 by addition of a few drops of 0.01 M HCl or 0.01 NaOH. 71.5 μl of this neutralized collagen solution was then placed on a glass cover slip laid in the bottom of a petri dish (Becton Dickinson Labware, Oxnard, CA; Falcon 1001, 100 x 15mm). Collagen fibrillogenesis was then initiated by allowing the dish to float for 15 minutes in a water bath preheated at 37°C. Next, the glass cover slip was placed under a ventilated hood and the collagen gel supernatant was allowed to evaporate completely during 30 to 60 minutes. The collagen-coated glass was kept in a humid petri dish to prevent protein denaturation until assembling of the flow chamber. By this technique, the glass cover slip is coated with a uniform and continuous layer of human collagen fibrils, with an average platelet density on the surface of approximately 20 μg/cm².

- **Type VI Human Fibrils (by thermal gelation):** type VI acid-soluble collagen was extracted by digestion of human placenta with pepsin and was purified by differential salt precipitation and column chromatography (Heyltx Corporation, Houston, TX). 100 μg lyophilized collagen samples were provided solubilized in 1 M acetic acid. The purity (≥95%) of each preparation was documented by SDS-polyacrylamid-gel-electrophoresis. A neutralized, isotonic collagen solution was prepared as before by adding to each sample 100 μl of 0.012 N HCl, 10.4 μl of 0.1 M NaOH and 10.4 μl of 10X phosphate-buffered saline solution. That collagen solution was then placed on a glass cover slip and thermal gelation initiated as before. The final collagen surface density on the surface was approximately 13.5 μg/cm² in this case.

- **Types IV, V and VI Human Monomers:** monomeric type IV and V collagen solutions were prepared by dissolving 2.07 mg of each purified collagenous species in 1 ml of 0.012 N HCl on a low speed nutator for 30 minutes. The type VI collagen provided was
already dissolved in 1 M acetic acid. Each collagen solution was then placed directly on
the glass cover slip and allowed to evaporate completely under the hood. Again, the final
collagen density on the surface is about 20 μg/cm² for types IV and V collagens, and
13.5 μg/cm² for type VI.

- **Types IV and V Human Fibrils (by dialysis):** 5 mg of purified collagen types IV and
V were dissolved to 1 mg/ml in 50 mM acetic acid. Each collagen solution was then placed
in sulfur-free EDTA-treated cellulose dialysis tubing preincubated in 1% sodium
benzoate (Spectrum Medical Industries, Inc., Los Angeles, CA; M.W. cutoff: 50,000).
Dialysis was performed against 1000 ml of 20 mM Na₂HPO₄ at 4°C for 48 hours, in an
attempt to form fibrils. Next, the collagen suspension was removed from the tubing and
centrifuged at 12000 rpm for two minutes to remove particulate material. The
suspension supernatant was diluted with an equal volume of phosphate buffered saline
(PBS; pH=7.4). A modified Lowry assay (Protein Assay Kit No. P5656, Sigma chemical
Co) was used to determine spectrophotometrically the final collagen concentration. The
appropriate collagen suspension volume was added to a glass cover slip to make the final
collagen surface density equal to 20 μg/cm².

4.2.3 Aggregometric Studies

Aggregometric quantitations of platelet adhesion to collagen types IV, V and VI, in
monomeric and fibrillar forms, were carried out in a dual sample aggregation meter
(Sienco, Inc., Morrison, CO; Model DP-247-E). In this system, 500 μl of citrated
platelet-rich plasma (PRP) were mixed with a test volume containing varying amounts
of purified collagen in a glass aggregometer cuvette. Those experiments were performed
at room temperature.

4.2.4 Statistical Analysis

Most results were expressed, when appropriate, as mean ± standard deviation (number of experiments). Tests of significance of an observed difference between k sample means were used on the results of the macroscopic measurements, expressed in terms of average platelet density accumulated on the reactive surface after two minutes of flow. t-statistic (Student's t-test) for comparison between two means (k = 2) and F-statistic (analysis of variance, one way, no replication) for comparison between k means (k > 2) were performed and the significance level, the p- or α-value, respectively, calculated.

4.3 Results

4.3.1 Collagen

4.3.1.a Macroscopic Measurements

The results of the macroscopic measurements are shown in Figures 4.4 to 4.9 and Table 4.1. Figures 4.4 to 4.7 show platelet density (PD), in terms of platelets per 1000 μm², versus axial position along the collagen-coated surface. Those figures illustrate the effect of wall shear rate on platelet surface deposition after 2 minutes of flow on different fibrillar collagenous species, prepared by thermal gelation. For type I bovine collagen (Figure 4.4), it is clear that platelet surface deposition increases significantly
Figure 4.4: Platelet accumulation on type I bovine collagen-coated glass as a function of wall shear rate. The abscissa represents the axial position along the reactive surface. Each curve represents an average of n experiments performed with different donors.
Figure 4.5: Platelet accumulation on type I human collagen-coated glass as a function of wall shear rate. Collagen fibrils were formed by thermal gelation. The abscissa represents the axial position along the reactive surface. Each curve represents an average of n experiments performed with different donors.
Figure 4.6: Platelet accumulation on type III human collagen-coated glass as a function of wall shear rate. The same comments as those in Figure 4.5 apply.
Figure 4.7: Platelet accumulation on type IV human collagen-coated glass as a function of wall shear rate. The same comments as those in Figure 4.5 apply.
Figure 4.8: Bar graphs representing average platelet density (APD) after 2 minutes of flow on type IV, V and VI collagen fibrils formed by thermal gelation as a function of wall shear rate. At each wall shear rate, the APDs are expressed in terms of percentages of the APD value for type IV collagen. The error bars represent half the values of the standard deviations.
Figure 4.9: Bar graphs representing average platelet density (APD) after 2 minutes of flow on type IV, V and VI collagen monomers as a function of wall shear rate. For a given collagen subtype, the APD value is expressed in terms of percentage of the APD value for the same collagen in fibrillar form.
<table>
<thead>
<tr>
<th>collagen type</th>
<th>wall shear rate (sec(^{-1}))</th>
<th>average platelet density (platelets / 1000 (\mu)m(^2)) mean ± s.d (n)</th>
<th>Student - t - test 95% confidence interval</th>
<th>Statistical significance ((\alpha)) of difference between means*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine</td>
<td>100</td>
<td>11.54 ± 2.22 (4)</td>
<td>± 4.08</td>
<td>(\alpha &lt; 0.01)</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>66.25 ± 17.6 (2)</td>
<td>± 223.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>159.22 ± 30.13 (5)</td>
<td>± 41.88</td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>100</td>
<td>4.4 ± 1.27 (2)</td>
<td>± 16.14</td>
<td>(\alpha &lt; 0.01)</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>40.65 ± 15.3 (6)</td>
<td>± 17.60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>62.45 ± 11.85 (4)</td>
<td>± 21.76</td>
<td></td>
</tr>
<tr>
<td>Type III</td>
<td>100</td>
<td>23.11 ± 3.52 (3)</td>
<td>± 10.70</td>
<td>(\alpha &lt; 0.01)</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>69.90 ± 15.1 (8)</td>
<td>± 13.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>48.85 ± 20.35 (9)</td>
<td>± 16.62</td>
<td></td>
</tr>
<tr>
<td>Type IV</td>
<td>100</td>
<td>4.75 ± 0.64 (2)</td>
<td>± 5.75</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>21.8 ± 1.32 (3)</td>
<td>± 3.28</td>
<td>(\alpha &lt; 0.01)</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>12.85 ± 0.35 (2)</td>
<td>± 3.15</td>
<td></td>
</tr>
</tbody>
</table>

* \(\alpha\) is calculated from analysis of variance (ANOVA, one way, no replication) for each collage type. It is used to determine if, for a given collagen subtype, the APD means at the three different wall shear rates are statistically different from each other with 99% confidence.
with increasing wall shear rate and that most of the aggregation occurs near the inlet of the collagen coating. Figure 4.5 shows that, in the case of type I human collagen, platelet surface deposition is very low at 100 sec\(^{-1}\) with practically no axial dependence in this case, while platelet aggregation increases dramatically at 800 sec\(^{-1}\) with large aggregates forming near the inlet. At 1500 sec\(^{-1}\), however, the PD peak has moved further downstream, possibly indicating some embolization of the larger thrombi near the inlet. In the cases of types III and IV collagen, PD along the reactive surface increases from 100 sec\(^{-1}\) to 800 sec\(^{-1}\), but then decreases from 800 sec\(^{-1}\) to 1500 sec\(^{-1}\), as illustrated in Figures 4.6 and 4.7, respectively. The PD peak is located at 4 µm from the inlet at all wall shear rates in the case of type III collagen, possibly indicating a lack of immediate platelet reactivity with the surface, whereas the location of maximum PD is at or near the inlet for type IV collagen, as expected. Again, at the lowest wall shear rate, PD on types III and IV collagen is relatively independent of axial position along the reactive surface.

The effect of wall shear rate and collagen type on the average platelet density (APD), estimated by dividing the total number of platelets accumulated on the surface during the perfusion period by the total area of the coated surface (26.3 x 12.7 mm\(^2\)), is shown in Table 4.1. The APD increases with increasing wall shear rate for type I bovine and type I human collagen, whereas it reaches a maximum value at 800 sec\(^{-1}\) on types III and IV human collagen, as mentioned above. The levels of significance of the observed differences between the APD means at the different wall shear rates were estimated by analysis of variance for each collagen subtype. As indicated in Table 4.1, the differences in APD due to wall shear rate are all statistically significant with 99% confidence (\(\alpha <\)
0.01). The basement membrane collagen (type IV) appears to be less thrombogenic than the interstitial collagens at all wall shear rates, except at 100 sec\(^{-1}\), where there is no significant difference with type I collagen (p < 0.3). Comparisons between the APDs of the interstitial collagens show that type III is the most thrombogenic at low and intermediate wall shear rates, while type I is the most reactive at high wall shear rate. Again, the significance level of the observed difference between the APD means of those two collagens were calculated by the Student's t-test to be p < 0.01, p < 0.01, and p < 0.1 at 100 sec\(^{-1}\), 800 sec\(^{-1}\) and 1500 sec\(^{-1}\), respectively.

Comparisons, in terms of APD, between type IV collagen fibrils and, types V and VI collagen fibrils, prepared by thermal gelation as before, are shown in Figure 4.8 at different wall shear rates. The APDs for types V and VI were expressed as percentages of the APD value for type IV at each wall shear rate. Those data indicate that type V is clearly less thrombogenic than either type IV or VI at all wall shear rates, except at 100 sec\(^{-1}\), at which type VI is slightly less reactive than type V. Although type VI is less potent than type IV at 100 sec\(^{-1}\), it has similar reactivity at 800 sec\(^{-1}\) and is roughly twice as much thrombogenic at 1500 sec\(^{-1}\). Finally, Figure 4.9 illustrates the differences in thrombogenicity between monomeric and fibrillar collagen types IV, V and VI at different wall shear rates. Those data represent the APD for each collagen subtype in monomeric form, expressed in terms of percentage of the APD for the same collagen in fibrillar form. Figure 4.9 indicates that, although type IV and VI collagen monomers are less reactive than their respective fibrils, type V monomers are somewhat more potent towards platelet adhesion and thrombus formation than type V fibrils, especially at 800 sec\(^{-1}\), where there is almost a two and a half-fold increase in APD.
4.3.1.b Microscopic Measurements

In order to analyze in detail the effect of wall shear rate on platelet adhesion and aggregation on various types of collagens, it is very informative to look at the size and shape of individual thrombi, as well as at the local platelet surface coverage. The results of this microscopic study are presented quantitatively in Tables 4.2 to 4.4, and qualitatively in Figures 4.10 to 4.15.

Quantitative information on individual thrombus size, in terms of average height per thrombus (AHPT) and average volume per thrombus (AVPT), are presented in Table 4.2 for the different collagens of the vasculature as a function of wall shear rate. Those data were obtained again with collagen fibrils formed by thermal gelation. Also presented there, is the local percentage platelet surface coverage (%SC), estimated in the field of view. For type I human collagen, all three parameters, AHPT, AVPT and %SC, increase dramatically with increasing wall shear rate, as expected from the results of the macroscopic measurements. This increase in thrombus size and platelet surface deposition with wall shear rate can also be observed qualitatively in Figure 4.10, which represents three digitized images of growing platelet aggregates forming in the field of view on type I human collagen fibrils. Those images were digitized individually after 2 minutes of flow at three different wall shear rates, 100 sec\(^{-1}\), 800 sec\(^{-1}\) and 1500 sec\(^{-1}\). The data for AVPT and %SC in Table 4.2, as well as the images of Figure 4.11, clearly indicate that type III human collagen is most thrombogenic at 800 sec\(^{-1}\), which is again consistent with the data reported earlier from the macroscopic measurements. In addition, when comparing the thrombogenicity of those two collagens, type III appears
Table 4.2: Effect of Wall Shear Rate and Collagen Type on Individual Thrombus Size and Local Platelet Surface Coverage

<table>
<thead>
<tr>
<th>Collagen Type</th>
<th>Wall Shear Rate (sec⁻¹)</th>
<th>Average Height per Thrombus (µm) mean ± s.d (n)</th>
<th>Average Volume per Thrombus (µm³) mean ± s. d. (n)</th>
<th>% Surface Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>100</td>
<td>2.68 ± 0.28 (129)</td>
<td>63.7 ± 20.5 (129)</td>
<td>3.40</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>2.94 ± 0.18 (150)</td>
<td>387.8 ± 108.2 (150)</td>
<td>21.90</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>5.41 ± 0.80 (28)</td>
<td>4575.1 ± 1642.9 (28)</td>
<td>26.06</td>
</tr>
<tr>
<td>Type III</td>
<td>100</td>
<td>3.58 ± 0.40 (129)</td>
<td>345.7 ± 153.0 (129)</td>
<td>13.71</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>4.19 ± 0.44 (48)</td>
<td>2966.8 ± 915.4 (48)</td>
<td>37.41</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>4.71 ± 0.42 (113)</td>
<td>839.9 ± 480.7 (113)</td>
<td>22.18</td>
</tr>
<tr>
<td>Type IV</td>
<td>100</td>
<td>2.95 ± 0.85 (103)</td>
<td>200.0 ± 106.3 (103)</td>
<td>7.68</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>4.68 ± 0.74 (50)</td>
<td>1420.8 ± 221.9 (50)</td>
<td>16.71</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>4.63 ± 0.70 (27)</td>
<td>1894.4 ± 248.6 (27)</td>
<td>12.16</td>
</tr>
<tr>
<td>Type V</td>
<td>100</td>
<td>2.39 ± 0.51 (116)</td>
<td>157.1 ± 38.1 (116)</td>
<td>8.40</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>3.30 ± 0.28 (146)</td>
<td>175.8 ± 25.7 (146)</td>
<td>8.56</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>3.97 ± 0.50 (41)</td>
<td>270.1 ± 76.0 (41)</td>
<td>3.07</td>
</tr>
<tr>
<td>Type VI</td>
<td>100</td>
<td>3.29 ± 0.29 (259)</td>
<td>208.1 ± 58.9 (259)</td>
<td>18.03</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>3.92 ± 0.45 (139)</td>
<td>557.1 ± 250.7 (139)</td>
<td>21.74</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>3.90 ± 0.21 (120)</td>
<td>8985.3 ± 9505.3 (6)</td>
<td>20.31</td>
</tr>
</tbody>
</table>
Table 4.3: Effect of Collagen Molecular Structure and Technique of Fibrillogenesis on Individual Thrombus Size and Local Platelet Surface Coverage at 800 sec\(^{-1}\).

<table>
<thead>
<tr>
<th>collagen type</th>
<th>collagen molecular structure</th>
<th>average height per thrombus (µm) mean ± s.d. (n)</th>
<th>average volume per thrombus (µm(^3)) mean ± s.d. (n)</th>
<th>% surface coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type IV</td>
<td>fibrils (heat)</td>
<td>4.68 ± 0.74 (50)</td>
<td>1420 ± 221.9 (50)</td>
<td>16.71</td>
</tr>
<tr>
<td></td>
<td>fibrils (dialysis)</td>
<td>3.77 ± 0.24 (90)</td>
<td>52.0 ± 4.5 (90)</td>
<td>1.37</td>
</tr>
<tr>
<td></td>
<td>monomers</td>
<td>3.52 ± 0.57 (198)</td>
<td>975.7 ± 773.1 (42)</td>
<td>18.26</td>
</tr>
<tr>
<td>Type V</td>
<td>fibrils (heat)</td>
<td>3.30 ± 0.28 (146)</td>
<td>175.8 ± 25.7 (146)</td>
<td>8.56</td>
</tr>
<tr>
<td></td>
<td>fibrils (dialysis)</td>
<td>3.25 ± 0.28 (96)</td>
<td>37.9 ± 6.2 (96)</td>
<td>1.23</td>
</tr>
<tr>
<td></td>
<td>monomers</td>
<td>6.72 ± 1.13 (44)</td>
<td>2473.1 ± 671.0 (44)</td>
<td>17.10</td>
</tr>
<tr>
<td>Type VI</td>
<td>fibrils (heat)</td>
<td>3.92 ± 0.45 (139)</td>
<td>557.1 ± 250.7 (139)</td>
<td>21.74</td>
</tr>
<tr>
<td></td>
<td>monomers</td>
<td>1.80 ± 0.23 (199)</td>
<td>115.8 ± 24.9 (199)</td>
<td>14.10</td>
</tr>
</tbody>
</table>
Table 4.4: Effect of Wall Shear Rate and Collagen Type on Individual Thrombus Shape and Orientation.

<table>
<thead>
<tr>
<th>collagen type</th>
<th>wall shear rate (sec$^{-1}$)</th>
<th>circularity factor (CIRC)</th>
<th>aspect ratio (ASPR)</th>
<th>angle of orientation$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>100</td>
<td>1.17</td>
<td>0.65</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>2.19</td>
<td>0.50</td>
<td>2.93</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>1.71</td>
<td>0.47</td>
<td>-2.31</td>
</tr>
<tr>
<td>Type III</td>
<td>100</td>
<td>1.05</td>
<td>0.68</td>
<td>3.60</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>1.95</td>
<td>0.41</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>1.81</td>
<td>0.55</td>
<td>2.16</td>
</tr>
<tr>
<td>Type IV</td>
<td>100</td>
<td>0.42</td>
<td>0.81</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>0.62</td>
<td>0.60</td>
<td>4.13</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>0.44</td>
<td>0.66</td>
<td>-2.64</td>
</tr>
<tr>
<td>Type V</td>
<td>100</td>
<td>0.29</td>
<td>0.86</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>0.67</td>
<td>0.67</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>0.52</td>
<td>0.65</td>
<td>-5.45</td>
</tr>
<tr>
<td>Type VI</td>
<td>100</td>
<td>1.35</td>
<td>0.61</td>
<td>2.21</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>1.33</td>
<td>0.55</td>
<td>-2.44</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>0.89</td>
<td>0.63</td>
<td>0.14</td>
</tr>
</tbody>
</table>

$^*$ The angle of orientation is given in degrees. It is zero for horizontal objects; increases counterclockwise and decreases clockwise.
Figure 4.10: Images of mural thrombi digitized after 120 seconds of blood flow over type I human collagen-coated glass at three different wall shear rates: 100 sec\(^{-1}\) (A), 800 sec\(^{-1}\) (B), and 1500 sec\(^{-1}\) (C). Collagen fibrils were formed by thermal gelation. The fluorescence intensity in those monochrome video images ranges from black to white, where black represents the surface background and white the regions of intense platelet accumulation.
Figure 4.11: Images of mural thrombi digitized after 120 seconds of blood flow over type III human collagen-coated glass at three different wall shear rates: 100 sec\(^{-1}\) (A), 800 sec\(^{-1}\) (B), and 1500 sec\(^{-1}\) (C). The same comments as those in Figure 4.10 apply.
Figure 4.12: Images of mural thrombi digitized after 120 seconds of blood flow over type IV human collagen-coated glass at three different wall shear rates: 100 sec\(^{-1}\) (A), 800 sec\(^{-1}\) (B), and 1500 sec\(^{-1}\) (C). The same comments as those in Figure 4.10 apply.
Figure 4.13: Images of mural thrombi digitized after 120 seconds of blood flow over type V human collagen-coated glass at three different wall shear rates: 100 sec\(^{-1}\) (A), 800 sec\(^{-1}\) (B), and 1500 sec\(^{-1}\) (C). The same comments as those in Figure 4.10 apply.
Figure 4.14: Images of mural thrombi digitized after 120 seconds of blood flow over type VI human collagen-coated glass at three different wall shear rates: 100 sec⁻¹ (A), 800 sec⁻¹ (B), and 1500 sec⁻¹ (C). The same comments as those in Figure 4.10 apply.
Figure 4.15: Images of mural thrombi digitized after 120 seconds of blood flow at 800 sec⁻¹ over three different monomeric collagen-coated glass: type IV (A), type V (B), and type VI (C).
to be the most thrombogenic at low and intermediate wall shear rates, while type I is the most reactive at high wall shear rate, as reported earlier. As expected, average thrombus size and %SC increase from 100 sec\(^{-1}\) to 800 sec\(^{-1}\) on type IV human collagen fibrils (see Table 4.2). Although the average thrombus size is similar at 800 sec\(^{-1}\) and 1500 sec\(^{-1}\) for that collagen, there is a 37.4% increase in %SC at 800 sec\(^{-1}\) compared to 1500 sec\(^{-1}\). This can also be visualized by comparing Figures 4.12(B) and 4.12(C). Whereas the average thrombus size on type IV collagen is comparable to those on the interstitial collagens, the %SC on the former one is considerably less than on the latter two in most cases. This agrees well with the data reported earlier in terms of PD and APD. The average size of the platelet aggregates forming on type V human collagen, in terms of AHPT and AVPT, increase slowly with increasing wall shear rate, as indicated in Table 4.2. The %SC drops from about 8.5% at low and intermediate wall shear rates to 3.07% at 1500 sec\(^{-1}\). In fact, both the size of individual thrombi and the local platelet surface deposition are considerably less for type V than for the other types of collagen encountered in this study, consistent with the macroscopic observations. This is also illustrated clearly by Figure 4.13. Table 4.2 indicates that average thrombus size and %SC increase from 100 sec\(^{-1}\) to 800 sec\(^{-1}\) on type VI human collagen. Although both AHPT and %SC remain nearly constant as the wall shear rate is further increased to 1500 sec\(^{-1}\), the AVPT increases dramatically, which could justify the large increase in APD reported earlier in Figure 4.8 at 1500 sec\(^{-1}\). Those observations can also be visualized from Figure 4.14.

The effects of collagen molecular structure (fibrillar vs. monomeric) and technique of fibrillogenesis (thermal gelation vs. dialysis) on average thrombus size and local
platelet surface coverage are shown quantitatively in Table 4.3 and qualitatively in Figure 4.15 for collagen types IV, V and VI at 800 sec\(^{-1}\). As indicated in Table 4.3, the fibrils prepared by dialysis, either for type IV or type V collagen, were largely unreactive with platelets. Indeed, when compared to the fibrillar collagens prepared by thermal gelation, %SC diminished by 91.8% and 85.6%, and AVPT by 96.3% and 78.4%, on types IV and V collagen fibrils prepared by dialysis, respectively. Those collagens in monomeric form, on the other hand, supported platelet adhesion and aggregation quite well, as shown in Table 4.3. AHPT and AVPT decreased moderately in the case of type IV monomers, compared to type IV fibrils formed by thermal gelation, while the %SC were quite similar. This can also be observed qualitatively by comparing Figure 4.12(B) to Figure 4.15(A). In the case of type V monomers, there is a considerable increase in thrombus size and %SC, compared to type V fibrils formed by thermal gelation (see Table 4.3). This increase can also be visualized by comparing Figure 4.13(B) to Figure 4.15(B). Type VI monomers appear to be less thrombogenic than type VI fibrils prepared by thermal gelation, although small aggregates still form on the reactive surface as indicated in Table 4.3 and Figure 4.15(C). Those microscopic data are in good agreement with the macroscopic results presented in Figure 4.9.

The three parameters indicative of thrombus shape and orientation in the flow field, namely, the circularity factor (CIRC), the aspect ratio (ASPR), and the angle of orientation, are shown in Table 4.4 as a function of wall shear rate for the various collagens of the vasculature. The angle of orientation is relatively small, ranging from 0.08° for type IV at 100 sec\(^{-1}\) to -5.45° for type V at 1500 sec\(^{-1}\). This means that, on the average, platelet aggregates are aligned closely with the horizontal flow field. Also
shown in Table 4.4 is the fact that ASPR is smaller than 1.0 in all cases, suggesting that most thrombi are not only aligned with the flow field but also elongated in the flow direction. Values of ASPR for type IV and V collagens, however, are slightly larger than those for the other collagens, which indicates that thrombi forming on type IV and V collagens have, in general, more uniform dimensions, and are less slender and more rounded. Those observations are confirmed by the lower CIRC values for those two collagen types compared to the same values for the other types of collagen. This can also be visualized by comparing Figure 4.12(B) or 4.12(C) to Figure 4.10(C).

4.3.1.c Aggregometric Measurements

Aggregometric studies were performed on types IV, V and VI collagen monomers and fibrils prepared by dialysis, by measuring changes in light transmittance in an aggregometer. Unlike the flow system, the aggregometer allows evaluation of the aggregatory properties of collagens in the bulk of a stirred suspension. Adding 25 μg, 50 μg or 100 μg of Type IV and 10 μg or 50 μg of type VI collagen in monomeric form to 0.5 ml of citrated PRP had virtually no effect on platelets. Whereas no platelet response occurred after addition of 50 μg or less of type V monomeric collagen, 53% and 54% aggregation were immediately recorded after addition of, respectively, 100 μg and 200 μg of that collagen at room temperature. Our data also indicate that types IV and V collagen fibrils formed by dialysis did not aggregate platelets to any significant extent in this system.

4.3.2 von Willebrand Factor - Macroscopic Results -
The results of the macroscopic measurements concerning vWF are shown in Figures 4.16 to 4.23. Figures 4.16 to 4.18 show platelet surface density (PD), in terms of platelets per 1000 μm², versus axial position along a type I bovine collagen-coated surface. Those figures illustrate the effect of wall shear rate on platelet surface deposition after 90 seconds of flow using severe von Willebrand's disease blood, in other words, blood containing no vWF in plasma or in the platelets' α-granules. Figures 4.16 and 4.17 represent the raw data as obtained from the computerized microphotometric measurement system, while the curve-fitted data are shown in Figure 4.18. Although the control curves shown in Figure 4.4 were obtained after 120 seconds of flow, the results of Figures 4.16 to 4.18 show that there are significant platelet adhesion and aggregation defects on collagen in absence of vWF at all wall shear rates studied, but that those defects are also more pronounced at the highest wall shear rate (1500 sec⁻¹). Although, at 100 sec⁻¹ and 800 sec⁻¹, PD decreases with increasing axial position as in the controls, the PD profile at 1500 sec⁻¹ is considerably flatter than the control, with a minimum platelet surface deposition near the inlet and a maximum approximately 8 mm downstream. Figures 4.19 to 4.21 show measured fluorescence intensity as a function of axial position along the bovine collagen-coated surface at three different wall shear rates. Those figures illustrate the effect of platelet-released vWF on platelet surface deposition. As described earlier in section 4.2, normal platelets (NP) were added back to severe vWD plasma in those experiments. As shown in Figure 4.19, the presence of normal platelets causes a slight increase in PD at practically all axial positions at 100 sec⁻¹. At higher wall shear rates, on the other hand, the effect of
Figure 4.16: Effect of wall shear rate on platelet surface deposition on type I bovine collagen fibrils, using blood of a patient with severe von Willebrand's disease.
Figure 4.17: Effect of wall shear rate on platelet surface deposition on type I bovine collagen fibrils, using blood of a patient with severe von Willebrand's disease.
Figure 4.18: Effect of wall shear rate on platelet surface deposition on type I bovine collagen fibrils, using blood of a patient with severe von Willebrand's disease. The data of Figures 4.16 and 4.17 were curve-fitted with cubic polynomials.
Figure 4.19: Effect of platelet-released vWF on platelet surface deposition on type I bovine collagen fibrils at 100 sec⁻¹. In those experiments, normal washed platelets (NP) were resuspended in vWD-PPP, and RBCs were added back to yield a final hematocrit of approximately 40%. The measured fluorescence intensity is proportional to the number of platelets reacted on the surface.
Figure 4.20: Effect of platelet-released vWF on platelet surface deposition on type I bovine collagen fibrils at 800 sec⁻¹. In those experiments, normal washed platelets (NP) were resuspended in vWD-PPP, and RBCs were added back to yield a final hematocrit of approximately 40%. The measured fluorescence intensity is proportional to the number of platelets reacted on the surface.
Figure 4.21: Effect of platelet-released vWF on platelet surface deposition on type I bovine collagen fibrils at 1500 sec⁻¹. In those experiments, normal washed platelets (NP) were resuspended in vWD-PPP, and RBCs were added back to yield a final hematocrit of approximately 40%. The measured fluorescence intensity is proportional to the number of platelets reacted on the surface.
Figure 4.22: Effect of the unusually large von Willebrand Factor multimers secreted by endothelial cells on platelet surface deposition on type I human collagen fibrils. Blood was perfused for 1.5 minutes at 800 sec$^{-1}$. The absolute fluorescence intensity is proportional to the number of platelets reacted on the surface.
Figure 4.23: Effect of the unusually large von Willebrand Factor multimers secreted by endothelial cells on platelet surface deposition on type I human collagen fibrils. Blood was perfused for 1 minute at 2000 sec\(^{-1}\). The absolute fluorescence intensity is proportional to the number of platelets reacted on the surface.
platelet-released vWF on PD is relatively insignificant, as shown in Figures 4.20 and 4.21.

Shown in Figures 4.22 and 4.23 are the effects of the unusually large vWF multimers (ULF), secreted by ECs in vitro, on platelet surface deposition, respectively, at 800 sec$^{-1}$ and 2000 sec$^{-1}$. Those figures show the curve-fitted absolute fluorescence intensity data, calculated by subtracting the background intensity from the measured intensity, versus axial position along a type I human collagen-coated glass, with and without ULF. Those data indicate only a slight increase in platelet surface deposition in presence of the unusually large vWF forms at practically all axial positions. In addition, it should be pointed out that the shapes of those curves are quite unusual, with the minimum platelet surface deposition at the inlet.

4.4 Discussion and Conclusions

4.4.1 Collagen

With the recognition of collagen polymorphism in the blood vessel wall, the relative platelet reactivity of the different collagen types towards platelet adhesion and aggregation has become a question of importance in hemostasis and vascular disorders. Furthermore, collagen molecular structure has been shown to determine, in many instances, the ability of collagen to react with platelets, this subject remaining controversial today (Packham and Mustard, 1984; Santoro and Cunningham, 1981; Barnes, 1982). The results of the present study indicate that the magnitude of the shear
forces, developed by any viscous fluid in motion, can also play a significant role with respect to collagen reactivity towards human blood platelets.

The shape of the curves in Figures 4.4 to 4.7 represents the balance between platelet transport to the surface, platelet reaction at the surface, and in some instances, platelet embolization from the surface (Folie and McIntire, 1988; Hubbell and McIntire, 1986c). Therefore, both mass transfer to and from the surface, which depends upon the local shear rate and the presence of red blood cells (RBCs), as well as rate of reaction at the surface, which in turn depends upon the thrombogenicity of the coated surface, must be taken into consideration when analyzing those macroscopic results in this model blood vessel. In the case of type I bovine collagen, Figure 4.4 clearly indicates that most platelet aggregation occurs near the inlet of the collagen coating, indicating that the process of mural thrombogenesis is limited downstream by platelet diffusion from the bulk fluid to the reactive surface. Indeed, after most platelets present in the fluid layer adjacent to the reactive surface have reacted, this layer becomes depleted of platelets. At low wall shear rate, this platelet-free layer is only slowly replenished with platelets due to the fact that this is a transport-limited process. As the wall shear rate increases, the effective platelet diffusivity near the surface also increases through a RBC-induced rotation mechanism (Keller, 1971), leading to faster platelet transport from the bulk to the surface and to more platelet accumulation on the surface, as can be seen from Figure 4.4. Bovine collagen forms large native-type fibers which are very reactive towards platelets even at high wall shear rates when platelet contact with the surface occurs within a relatively short time and surface drag forces are relatively large. This is certainly one of the main reasons why bovine collagen is more thrombogenic than any
of the various human collagens investigated in this study (see table 4.1), which form fibers or fibrils of smaller diameters. This observation fits reasonably well in a theory proposed by several investigators (Santoro and Cunningham, 1980; Barnes et al., 1980), according to which the collagen-platelet interaction involves the participation of multiple sites on the platelet membrane interacting simultaneously with an equivalent number of sites on the collagen surface. Therefore, with regard to platelet adhesion and aggregation, the physical geometry of the collagen surface, and consequently the size of the fibrils or fibers, certainly are important factors to consider.

Figures 4.5 to 4.7 and Table 4.1 clearly demonstrate the importance of wall shear rate concerning platelet accumulation on human collagen fibrils formed by thermal gelation. Indeed, while platelet surface deposition on type I collagen increases monotonically with increasing wall shear rate as expected, it reaches a maximum at 800 sec\(^{-1}\) and then decreases as the wall shear rate is further increased to 1500 sec\(^{-1}\), on types III and IV collagen. As the wall shear rate increases, platelet transport to the surface increases, but there are also decreasing amounts of proaggregatory substances present at or near the surface, as shown in Chapter 5 (Folie and McIntire, 1989a). In other words, the amounts of platelet-released proaggregatory substances - such as ADP and von Willebrand factor - and of substances metabolically produced by platelets - such as TxA\(_2\) and thrombin - vary greatly depending upon the local wall shear rate (Folie and McIntire, 1989a). If the collagen of interest does not activate platelets to release or produce platelet-activating agents in sufficient amounts to compensate for their removal from the local vicinity of the thrombus by increasing convective forces, a point may be reached at which the concentration of one of several agents present in the vicinity of the
reactive surface will drop below the minimum threshold required for platelet aggregation. In that case, more of the platelets which flow near the surface will not be activated, thereby decreasing the overall mural thrombogenesis process. It could be imagined that different collagen types might induce the release of those proaggregatory substances in varying amounts, which would also explain the collagens' different reactivity towards platelets with increasing wall shear rate. We have shown that thrombin, for instance, is one of the most important proaggregating agents present in the vicinity of a growing thrombus (Folie and McIntire, 1989a). Any sharp decrease in thrombin concentration due to flow in combination with a lack of production by platelets could have significant consequences on the activation of additional platelets at the site of thrombosis.

In contrast to bovine collagen, platelet accumulation on any of those three human collagens is practically independent of axial position at the low wall shear rate of 100 sec\(^{-1}\), as also shown in Figures 4.5 to 4.7. At higher wall shear rates, platelet aggregation and thrombus formation occur more extensively, especially at or near the inlet; the process of mural thrombogenesis changes from a reaction-limited to a transport-limited process as one moves further downstream. In Figure 4.5, the nonmonotonic shape of the curve at 1500 sec\(^{-1}\) probably indicates some embolization of the larger thrombi near the inlet, as reported before (Folie and McIntire, 1988). When comparing the thrombogenicity of the interstitial collagens, both Table 4.1, in terms of APD, and the curves of Figures 4.5 and 4.6, indicate that type III collagen is the most thrombogenic at the low and intermediate wall shear rates, while type I is the most reactive at the high wall shear rate. This contrasts the results obtained from
aggregometric studies (Barnes et al., 1976; Santoro and Cunningham, 1977), according to which the aggregatory potency of both collagen types in fibrillar form is relatively similar. On the other hand, the results of this study agree relatively well with those obtained by Parsons et al. (1986), who showed that platelet adherence to type III is greater than to type I at 1000 sec\(^{-1}\) in a similar flow system. As shown in Figure 4.7 and Table 4.1, the basement membrane collagen (type IV) in fibrillar form is clearly less thrombogenic than either of the interstitial collagens, as reported by others (Parsons et al., 1986).

The results of Figure 4.8 indicate that type V collagen in fibrillar form is the least thrombogenic among the collagens of the vasculature at all wall shear rates considered here, as was also reported by Parsons et al. (1983 and 1986). Those data also indicate that the thrombogenicity of type VI collagen increases much faster with increasing wall shear rate than that of either type IV or type V collagen. Comparisons with the data of Table 4.1 show that, on a macroscopic level, the interstitial collagens are more reactive with platelets than the other collagen types. One likely explanation for that observation is that the different collagens stimulate platelets to release or produce platelet-activating agents in varying extent. Different fibril or fiber size, chemical composition, and receptors involved in platelet binding, between the different collagen types, are possible causes. When investigating the difference in thrombogenicity between monomeric and fibrillar collagens, the results of Figure 4.9 clearly indicate that type IV and VI monomers are less potent than their fibrils while, peculiarly, type V monomers are more reactive than type V fibrils. Whereas the results of most investigators, including those of Barnes and MacIntire (1979a and 1979b) and Chang et
al. (1980), agree on the fact that most collagens in monomeric form do not aggregate platelets significantly in an aggregometer, the results of this study somewhat superficially appear to contradict those findings. This possibly indicates that a glass substrate with a large collagen surface density may provide a better environment for enhancement of platelet adhesion and/or aggregation on monomeric collagen. In this context, the aggregometric results of this study with monomeric collagen types IV and VI do compare with others' data (Barnes and MacIntire, 1979a and 1979b), while the observation that type V monomers cause some platelet aggregation when large collagen concentrations are used disagree with the results of Chang et al. (1980).

The effects of wall shear rate and collagen type on individual thrombus size and shape, as well as on local platelet surface coverage were demonstrated by the results of the microscopic measurements. As shown quantitatively in Table 4.2 and qualitatively in Figures 4.10 to 4.14, for each wall shear rate considered in this study, the interstitial collagens (type I and/or type III) in fibrillar form are the most thrombogenic among the collagens of the vasculature. At 1500 sec\(^{-1}\), type I is clearly the most reactive, while at the lower wall shear rates type III is the most potent. However, the results of this study also suggest that, locally at the place of maximum platelet accumulation on the coated surface, the adhesive and aggregatory capacity of type IV and VI collagen fibrils can not be denied. For instance, type IV, in terms of AVPT and AHPT, appears more reactive than type I at 100 sec\(^{-1}\) and 800 sec\(^{-1}\) (see Table 4.2). Only type V collagen in fibrillar form is clearly less reactive than any of the other collagen subtypes, in accordance with the macroscopic measurements and the data reported in the literature (Parsons et al., 1983 and 1986).
In contrast to what has been observed in most aggregometric studies (Barnes and Maclntire, 1979a and 1979b), but in agreement with the macroscopic data, thrombus formation does occur, in varying extent, on type IV, V and VI collagens dissolved in solution as monomers, as shown in Table 4.6 and Figure 4.15. Those preliminary experiments were performed at a wall shear rate of 800 sec\(^{-1}\) and with a relatively high surface collagen density of 20 µg/cm\(^2\) for type IV and V, and 13.5 µg/cm\(^2\) for type VI. Those microscopic data indicate that type V monomers are more reactive than type V fibrils prepared by thermal gelation, while type IV monomers have similar reactivity towards platelets when compared to type IV fibrils formed by the same process. Type VI monomers, on the other hand, are considerably less thrombogenic than type VI fibrils, as would be expected. It is possible, as noticed elsewhere (Shade and Barondes, 1982), that small microfibrils, dimers, trimers, tetramers, etc., form when the tropocollagen solution is placed on the glass cover slip and allowed to evaporate at room temperature. Within this context, Mugli & Baumgartner (1973) have reported that aggregation can occur with very tiny fibrils, such as those found early in the course of fibrillogenesis. This usually occurs before any increase in opacity in the collagen solution can be detected. Nevertheless, it should be pointed out that, although type IV, V and VI, have not been found as large fibrils or fibers "in vivo" (Burgeson and Morris, 1987; Rhodes, 1982), one can not rule out the possibility that they might be present as microfibrils in the vessel wall. In fact, Duncan et al. (1983) and Yurchenco and Furthmayr (1984) have reported that the structure of the NH\(_2\) terminal of procollagen IV molecules alone supports self-assembly into tetramers "in vitro", while Furthmayr et al. (1983) have reported that type VI collagen may originate from a microfibrillar component of
connective tissues similar to those found at the interface between elastic and collagenous fibrils. Further study on the exact molecular arrangement of the collagens fixed at the surface needs to be done before any definitive conclusions can be drawn on this matter.

Also shown in Table 4.3 is the fact that type IV and V fibrils formed by dialysis against 20 mM disodium hydrogen phosphate at 4°C are quite unreactive in this flow system. This was also confirmed by the aggregometric studies. This technique has been used successfully before by several investigators to form fibrils "in vitro". Houdijk et al. (1985) were able to form native-type fibrils from type I and III human collagen dissolved in solution by this technique and have reported that those fibrils could induce some thrombus formation in their flow system. Similarly, Barnes et al. (1980) reported to have formed non-striated (amorphous) fibrils from type IV collagen purified from human placenta with this technique. Those fibrils also proved to be potent inducers of platelet aggregation in their aggregometer. A likely explanation for our observations is that amorphous collagen fibrils were probably formed by this technique and that those fibrils support neither platelet adhesion nor aggregation in a flow system such as the one used here. In this context, Chang et al. (1980) have noticed that type V collagen can induce platelet aggregation and the release reaction when added to PRP in an aggregometer in the native-type fibrillar form but not in the monomeric or amorphous fibrillar forms, in contrast to what Barnes et al. (1980) found with type IV amorphous fibrils. Barnes (1982) has also reported that dialysis at 4°C against a low ionic strength phosphate buffer, as the one used in these experiments, only forms fibrils of 67 nm periodicity with the interstitial collagens, but not with either type IV or type V human collagen.
The results of Table 4.4 concerning individual thrombus shape and the way they grow with respect to the flow field, confirm the observations made independently by Adams et al. (1983) and Hubbell and McIntire (1986), as well as in the study of Chapter 3 (Folie and McIntire, 1988), that platelet aggregates are in general elongated in the flow direction. Our results also show that thrombi forming on type IV and V collagens are not as stretched in the flow direction as those growing on the other collagens of the vasculature. In addition, in the former cases, thrombi grow as individual entities without connections between them, as is the case with the interstitial collagens at intermediate and high wall shear rates. This can be visualized by comparing Figures 4.10(C) and 4.11(C) with Figure 4.12(C) for example.

In conclusion, it can be said from the results of this study that both interstitial collagens in their native-type fibrillar forms do support platelet adhesion as well as thrombus formation to varying extent depending on the local wall shear rate. Type I collagen is the predominant collagenous species in the thickened intima of aging blood vessels, as well as in the intimal atherosclerotic plaque (Morton and Barnes, 1982). For those reasons and also because the data of this study indicate that its thrombogenicity increases with increasing wall shear rate, type I collagen appears as the most thrombogenic and therefore the most dangerous among the collagens of the vasculature. This would be particularly true in presence of severe high grade stenoses, in which cases local shear stresses are considerably elevated above normal levels. If the injury is limited to the basement membrane, the results of this study indicate that small to medium size thrombi could form on type IV collagen fibrils or "microfibrils", the predominant collagenous species there, at low and intermediate wall shear rates, with no
further increase at higher wall shear rates. In this context, the main purpose of type IV collagen might be to maintain vascular integrity, as was proposed by others (Meyer, 1980), with no major involvement in thrombosis or atherosclerosis. The pathophysiological role of type V collagen is more ambiguous to determine because of conflicting results and yet undefined molecular structure in the vessel wall. Although the results presented here and others’ data (Parsons et al., 1983 and 1986) seem to agree on the fact that type V fibrils are quite nonthrombogenic in a flow system, the results of this study also indicate that type V monomers or possibly “microfibrils” can support some platelet aggregation. Even if this were verified, it should be remembered that type V is only a minor component of the basement membrane and subendothelium, the two areas most likely to be exposed to blood upon tissue injury. Finally, the results of this study indicate that type VI collagen can also promote some thrombus formation especially at high shear rates, at which it becomes more thrombogenic than either type IV or V collagen. More information concerning its exact molecular structure and location in the vessel wall is needed before a function can be unambiguously assigned to this collagen type.

4.4.2. von Willebrand Factor

The data presented in Figures 4.16 to 4.18, using heparinized whole blood of a patient with severe von Willebrand’s disease (vWD), confirm previously reported data (Tchopp et al., 1974; Weiss et al., 1978a; Weiss et al., 1978b; Baumgartner et al., 1980) using citrated and native blood, concerning the importance of vWF in the
processes of platelet adhesion and aggregation on collagenous surfaces and the subendothelium under flow. Indeed, the data shown here and others' indicate that vWF is especially important at high shear rates (≥ 1300 sec⁻¹), as those found in the microcirculation, when the residence time of platelets near the reactive surface and therefore the time available for the establishment of a stable bond between platelets and the collagen fibrils is short. Nevertheless, at the intermediate wall shear rate of 800 sec⁻¹, the data of this study also suggest that there is a significant platelet deposition defect on collagen-coated glass with vWD-blood, as reported by Houdijk and coworkers (1985). Having established the crucial importance of vWF in the thrombogenesis process in this flow system, an attempt was made to investigate the role of platelet-released vWF in that process by replacing the vWF-deficient platelets by normal washed platelets, a topic not well documented in the literature. The data presented in Figures 4.19 to 4.21 indicate that only at low shear rate could endogenous released platelet vWF multimers augment platelet surface deposition on collagen. In fact, this result somewhat confirms the numerical data presented in Chapter 5, that indicate that platelet-released vWF may support platelet aggregation, but not necessarily platelet adhesion, at low and intermediate wall shear rates (< 1000 sec⁻¹). It should be pointed out, however, that relatively small platelet concentrations were used in those experiments, and that similar experiments with normal platelet counts should be done before any definitive conclusions can be drawn on this subject.

Because the unusually large vWF multimers secreted by ECs in the extracellular matrix of injured blood vessel walls could be important modulators of the thrombogenesis process, an attempt was made to investigate their potential role in
inducing platelet adhesion and aggregation in vitro inside a parallel plate flow chamber coated with type I human collagen fibrils. Prior to perfusion, the collagen-coated surface was incubated with EC supernatant containing the unusually large vWF multimers. The results of Figures 4.22 and 4.23 show a slight increase in platelet surface deposition in presence of the ULF compared to the controls. In addition, that increase was relatively similar at both wall shear rates studied (800 sec\(^{-1}\) and 2000 sec\(^{-1}\)). Those results are rather surprising especially because Moake and coworkers (1986 and 1988) have shown that the largest plasma vWF multimers as well as the ULF derived from ECs are very effective in inducing shear-induced platelet aggregation at high shear rates in a cone and plate viscometer. Although binding of plasma FVIII-vWF complex to human collagen fibrils has been shown to occur in vitro by several investigators (Scott et al., 1981; Morton et al., 1983; Kessler et al., 1984), Scott and coworkers (1981) have reported that binding to collagen-coated surfaces occurred at a much slower pace than binding in the bulk of a collagenous suspension. In fact, they showed that it takes several hours (24-48 hours) for a significant amount of binding to occur on collagen-coated wells, which is a much longer period of time than the incubation periods of 1 to 2 hours used in this study. On the other hand, it was shown recently (Moake and Gardner, 1989) that collagen binds preferentially and perhaps more rapidly the largest vWF multimers. Nevertheless, it is possible that the results of this study might be due to experimental artifacts. In addition to FVIII-vWF, fibronectin may also be required in the test medium for optimal platelet adhesion and thrombus formation on fibrillar collagen, as reported by others (Houdijk et al., 1985; Bastida et al., 1987).
CHAPTER 5

MATHEMATICAL MODELING OF MURAL THROMBOGENESIS

- A MICROSCOPIC APPROACH -

5.1 Background and Objectives

When blood platelets flow near the subendothelium of an injured blood vessel (Hirsh and Brain, 1983), an artificial polymeric surface used in a prosthetic blood-contacting device (Anderson and Kottke-Marchant, 1985), or an atherosclerotic lesion (Turitto and Baumgartner, 1982) a number of hemorheological and biochemical events take place very rapidly near that site to form thrombi (solid platelet aggregates) on the reactive surface. Antecedent to the aggregation process and immediately following initial platelet adhesion and activation on the reactive surface, biochemical proaggregatory agents are released extracellularly from the platelet granules or produced metabolically on the platelet membrane. Accumulating in high concentration locally at the site of aggregate formation, these platelet-activating substances act synergistically to activate additional platelets and accelerate the process of thrombus growth (Kinlough-Rathbone and
Mustard, 1986). Releasable materials are important in normal thrombogenesis, for platelets lacking these agents (storage pool disease) have been shown to have reduced thrombus formation on subendothelium and fibrillar collagen (Baumgartner and Mugli, 1976).

Because the relative importance of most proaggregatory substances in inducing mural thrombogenesis is still uncertain and because direct measurement of very low concentrations of chemicals in a dynamic system is difficult, a two-dimensional theoretical model, involving the simulation of blood flow between two parallel plates over a model thrombus attached to the bottom plate, was developed in this study. Four potential platelet-activating agents were investigated. Adenosine diphosphate (ADP) and von Willebrand factor (vWF) are released, respectively, from the dense and α-granules of activated platelets, while TxA2 and thrombin are enzymatically generated on or near the platelet membrane (Report of the National Heart, Lung, and Blood Institute Working Group, 1985). For each agent, the estimated maximum concentration in the neighborhood of one thrombus or two adjacent thrombi generating and releasing that compound, was compared with the concentration known to affect platelet function in various static in vitro measurement systems.

Because the local concentration profiles of releasable substances depend primarily on the rate of thrombus growth or the thrombus size, and on the rate of transport of those substances from the aggregate site, concentration profiles were computed as a function of wall shear rate. This is the main fluid mechanical variable controlling surface access. Indeed, blood flow greatly increases the effective diffusivity of cells and proteins, through a red blood cell (RBC) rotation and/or an interparticle collision
mechanism, in a shear rate dependent manner (Eckstein, 1982). Because the thrombogenesis process occurs near the surface where the local concentration of RBCs is low, the increased diffusivity is predominantly due to the rotation of RBCs in a shear field, and Keller's mixing model (Keller, 1971; Wang and Keller, 1985) was used to estimate the enhanced diffusivities of platelet-activating agents in whole blood.

An epifluorescence video microscopy system, coupled with digital image processing techniques, described in detail in Chapter 2, was utilized to determine experimentally the rates of growth and sizes of individual thrombi at different wall shear rates, ranging from 100 sec$^{-1}$ (a typical venous shear rate) to 1500 sec$^{-1}$ (an arterial shear rate). Whole human blood was perfused in a parallel-plate flow chamber coated with human collagen type I fibrils, a predominant thrombogenic protein found in the subendothelium of injured blood vessels (Morton and Barnes, 1982). The data obtained in those experiments were then used in the mathematical model to estimate the wall fluxes of platelet-activating agents at various wall shear rates.

Only two papers using similar microscopic modeling have been reported in the literature. The first one used only a qualitative approach to determine the approximate shapes of the concentration contours generated (Butruille et al., 1975). The second one, from Hubbell and McIntire (1986a), did investigate the role of several platelet-activating substances in promoting thrombus growth, but using a one-dimensional model of a flat thrombus and ignoring the presence of RBCs. Consequently, neither the flow disturbances caused by the finite size of the growing platelet aggregates nor the effect of shear rate on the magnitude of the wall flux of platelet-activating agents were considered.
The purpose of the present study, also reported elsewhere (Folie and McIntire, 1989), was, therefore, to estimate computationally the concentration profiles of four proaggregatory compounds (ADP, TxA₂, thrombin, and vWF) released from a more realistic model thrombus, thereby gaining some insight into which platelet-activating substances affect the mechanism of thrombus growth on a microscopic scale. The effect of flow, aggregate size, aggregate geometry, and number of aggregates on those concentration profiles was also investigated. Finally, while some effects of disturbed flow and vortices in thrombogenesis have been measured experimentally by others (Karino and Goldsmith, 1979a, 1979b), the size of the recirculating regions developing on both sides of a thrombus, as well as the magnitude of the embolizing stresses and the torque, acting at the thrombus surface, were estimated in the simulation, and their physiological relevance discussed.

5.2 Experimental Technique

5.2.1 Collagen Preparation

2.5 mg of type I acid-soluble human collagen (Sigma Chemical Co., St. Louis, MO) purified by a pepsin extraction and salt fractionation method from human placenta (Niyibizi et al., 1984) were mixed with 1 ml of 0.012 N HCl (pH=2) cooled at 4°C in a polypropylene test tube. The test tube was then placed on a low-speed nutator and the collagen allowed to dissolve completely for 1 hour at ambient temperature. This monomeric collagen (tropocollagen) solution was kept at 4°C until further utilization. A neutralized, isotonic collagen solution was then prepared by mixing 1 ml of chilled
tropocollagen solution with 104.2 μl of 0.1 M NaOH and 104.2 μl of 10 X phosphate-buffered saline solution (0.2 M Na₂HPO₄, 1.3 M NaCl, pH=7.4) to make a final collagen concentration of 2.07 mg/ml. The pH of that solution was adjusted to 7.4 ± 0.2 by addition of a few drops of 0.01 M HCl or 0.01 NaOH.

5.2.2 Flow Studies and Collagen Coating

Heparin-anticoagulated whole blood with fluorescent-labelled platelets was aspirated by a syringe pump for two and a half minutes at a controlled shear rate of 100 sec⁻¹, 800 sec⁻¹, or 1500 sec⁻¹ from a test tube into a polycarbonate parallel-plate flow chamber, as described in detail in Chapter 2. Before being assembled as part of the flow chamber, 7.4 cm² of a glass cover slip were coated with 71.5 μl of the neutralized tropocollagen solution (2.07 mg/ml), the remaining portion of the slide being covered with parafilm. The slide was then placed in the bottom of a petri dish (Becton Dickinson Labware, Oxnard, CA; Falcon 1001, 100 x 15 mm) and collagen fibrillogenesis initiated by allowing the dish to float for 12 minutes in a water bath preheated at 37°C. This technique, as reported by others (Gelman et al., 1979; Barnes, 1982), allows the monomeric collagen solution to polymerize into native-type collagen fibrils of 67 nm periodicity, shown as the optimum molecular configuration for platelet aggregation (Barnes, 1982). The glass cover slip was then placed under a ventilated hood and the collagen gel supernatant allowed to evaporate completely during 30 to 60 minutes. The collagen-coated glass was kept in a humid petri dish to prevent the protein from denaturating until assembling of the flow chamber. By this method, the glass cover slip is coated with a continuous layer of type I (by classification) human
collagen in fibrillar form, with an average collagen density of approximately 20 
µg/cm², which is within the range of densities required for maximum platelet 
aggregation (Houdijk et al., 1985).

5.3 Theory

Consider whole blood flowing at low Reynolds number between the two parallel plates
of a rectangular channel 200 µm-thick, over a two-dimensional model thrombus
attached to the bottom plate. The fluid approaches the thrombus as a Newtonian fluid in a
fully-developed laminar flow regime with a typical parabolic velocity profile. A species
of interest A is released from the thrombus surface at a constant specified flux, Nₐ. Some
of the species analyzed will also react by a first order bulk phase reaction, A → B, where
B is some inactive form of A. The physical situation is depicted in Figure 5.1. The
different cases treated in this simulation, including the various object geometries and
sizes, as well as the situation where two aggregates, located near or far away from each
other, are growing on the same surface, are shown in Figure 5.2.

5.3.1 Chemical Reactions

Thrombin is generated enzymatically on the surface of the platelet, where factor Xₐ,
activated by both the intrinsic and extrinsic pathways, factor Vₐ, and Ca²⁺ combine with
platelet membrane phospholipids to form the prothrombinase complex. This complex can
rapidly convert circulating prothrombin to thrombin (Report of the National Heart,
Lung, and Blood Institute Working Group, 1985). Because this membrane reaction goes
Figure 5.1: Geometry of the parallel-plate flow chamber, used both for the experiments and in the computer simulation. Blood flows from left to right over a semicircular model thrombus of diameter L. A substance A is released from the thrombus surface at a constant flux $N_A$, and then, may become inactivated by a first order chemical reaction in the bulk. The fluid enters as a Newtonian fluid in a fully-developed laminar flow regime with a typical parabolic velocity profile.
Figure 5.2: Model thrombus sizes and geometries. The six following cases were analysed:
(i) rectangular model thrombus: (a) L=10μm, D_1=4L, D_2=10L ; (b) L=20μm, D_1=4L, D_2=6L
(ii) semicircular model thrombus: (a) L=10μm, D_1=4L, D_2=10L ; (b) L=20μm, D_1=4L, D_2=6L
(iii) two rectangular thrombi: L=20μm, D_1=1L, D_2=6L ; (iv) two rectangular thrombi: L=20μm, D_1=1L, D_2=4L.
to completion immediately, the rate of release of thrombin from the thrombus surface was assumed equal to the rate of arrival of prothrombin to the thrombus surface. Here we also assumed that the amount of thrombin produced from prothrombin diffusing inside the thrombus is small compared to the amount produced on the thrombus surface. Thrombin released is inactivated in the bulk phase by the circulating inhibitor antithrombin III (AT III). This reaction is greatly accelerated in the presence of heparin, a commonly-used antithrombotic agent (Griffith, 1982). Mathematically, the reactions can be written as:

\[ \text{A} \overset{k}{\rightarrow} \text{B} \quad \text{a rapid first order reaction at the thrombus surface where A is assumed to be completely consumed.} \]

\[ \text{B} + \text{C} \overset{k'}{\rightarrow} \text{D} \quad \text{a second order bulk phase reaction (the rate-limiting step) for which } k' \text{, the reaction rate constant, is a strong function of heparin concentration.} \]

where \( A = \text{prothrombin}; B = \text{thrombin}; C = \text{AT III}; D = \text{inactive complex.} \)

**Thromboxane } A_2**, an arachidonic acid metabolite, has a short half-life (43 sec) in aqueous media and undergoes rapid nonenzymatic hydrolysis to thromboxane \( B_2 \), which is inactive biologically (Hammarstrom et al., 1979). This reaction is as following:

\[ \text{E} \overset{k}{\rightarrow} \text{F} \quad \text{a rapid first order reaction.} \]

where \( E = \text{TXA}_2; F = \text{TXB}_2. \)

Because ADP and vWF (with the notation: \( G = \text{ADP}; H = \text{vWF} \)) have relatively long half-lives, compared with the time required to activate platelets, they were assumed not
to react in the bulk phase. Indeed, although ADP is eventually metabolized to AMP by the
ADP-ases, studies have indicated half-lives of the order of 20 to 45 minutes for ADP
in plasma (Rozenberg and Holmsen, 1968). Similarly, studies with plasma vWF have
revealed half-lives of several hours for that protein (Williams, 1983). Disappearance
of vWF by binding to platelet membrane glycoproteins was also not considered in this
study.

5.3.2 Equations

The convection-diffusion equation describing the physical situation is given by:

\[ V_x \frac{\partial C_j}{\partial x} + V_y \frac{\partial C_j}{\partial y} = D_{j,c} \left[ \frac{\partial^2 C_j}{\partial x^2} + \frac{\partial^2 C_j}{\partial y^2} \right] - k C_r \]  

\[(5.1)\]

where \( V_x \) and \( V_y \) represent the \( x \) and \( y \) components of the velocity vector; \( C_j \) is the
concentration of the species of interest \( j \); \( D_{j,c} \) is the effective diffusion coefficient of
species \( j \) in whole blood; \( k \) is a bulk phase reaction rate constant; and \( C_r \) is the product of
the reactant concentrations. For species \( E \), \( C_r = C_e \), while for species \( B \), \( C_r = C_b \times C_c \),
where the subscripts refer to the species.

Equation (1) was solved with the following imposed boundary conditions:

(i) on the thrombus surface:

\[ C_a = 0; D_{b,c} \frac{\partial C_b}{\partial n} = -D_{a,c} \frac{\partial C_a}{\partial n}; D_{j,c} \frac{\partial C_j}{\partial n} = N_j \text{ for } j = e, g, \text{ or } h \]  

\[(5.2)\]
(ii) **upstream from the thrombus** \( x = x_0 \) and **on the top plate** \( y = B \):

\[
C_j = C_{j_0} \quad \text{for } j = a, c, h; \quad C_i = 0 \quad \text{for } i = b, e, g
\]  

(5.3)

(iii) **downstream from the thrombus** \( x = x_e \):

\[
\frac{\partial C_j}{\partial x} = 0 \quad \forall j
\]  

(5.4)

where \( n \) is the direction normal to the boundary. For a given wall shear rate, the flux \( N_j \) is constant for ADP, TxA₂, and vWF, and was calculated from experimental data, as explained later. The thrombin flux, on the other hand, was estimated by first solving equation (5.1) for prothrombin, assuming a concentration of prothrombin equal to zero at the thrombus surface and then calculating the flux of prothrombin over the surface area of the object. That value, corrected for the difference in effective diffusion coefficients between the two compounds, was used as the thrombin flux. \( C_{j0} \) represents the initial concentration of substance \( j \) in whole blood (see Table 5.1).

In order to solve equation (5.1), the equation of motion for an incompressible Newtonian fluid with constant physical properties (the Navier-Stokes equation), together with the continuity equation, had to be solved for the velocity field. Those equations, in vector notation, assuming steady state, are given by:

\[
\nabla \cdot \vec{V} = 0
\]  

(5.5)
\[ \rho \nabla \cdot \mathbf{V} = -\nabla P + \mu \nabla^2 \mathbf{V} + \rho g \]  

(5.6)

with the following boundary conditions:

(i) **upstream** \((x = x_0)\):

\[ V_y = 0; \ V_x = V_{\text{max}} \left[ 1 - \left( \frac{y^2}{B^2} \right) \right] \text{ (parabolic profile)} \]  

(5.7)

(ii) **along top and bottom plate** \((y = \pm B)\) and on the thrombus surface:

\[ V_x = V_y = 0 \text{ (no slip)} \]  

(5.8)

(iii) **downstream** \((x = x_e)\):

\[ t_n = t_t = 0 \]  

(5.9)

where \(V_x\) and \(V_y\) are, respectively, the \(x\) and \(y\) components of the velocity vector \(\mathbf{V}\); \(V_{\text{max}}\) is the maximum velocity at the center of the channel; \(P\) is the pressure; \(g\) is the gravity field; \(\rho\) and \(\mu\) are, respectively, the fluid density and viscosity; \(t_n\) and \(t_t\) are the normal and tangential components of the stress vector \(\mathbf{t}\); and \(\nabla\) is the Laplace operator. At the outflow boundary \((x = x_e)\) the assumption of fully-developed flow is made; the velocities are left free so that the boundary condition of zero stress in the normal and
tangential directions is obtained.

Equations (1) to (9) were then dedimensionalized by introducing the following dimensionless quantities:

\[
C_j^* = \frac{C_j D_j \rho}{N_j B} \quad P^* = \frac{P}{\rho V_{\text{max}}^2} \quad N^* = \frac{N_j}{\text{Pe} N_{j,\text{rf}}} \quad L^* = \frac{L}{B}
\]

\[
k^* = \frac{k B}{V_{\text{max}}} \quad g^* = \frac{g B}{V_{\text{max}}^2} \quad V^* = \frac{V}{V_{\text{max}}}
\]

\(L\) representing any length \(x, y,\) or \(n.\)

This yields, dropping the asterisks, the following system of weakly-coupled partial differential equations (PDEs):

\[
\nabla \cdot \vec{V} = 0 \quad (5.10)
\]

\[
\nabla \cdot \vec{V} = -\nabla P + \left( \frac{1}{Re} \right) \nabla^2 \vec{V} + \vec{g} \quad (5.11)
\]

\[
\nabla \cdot \nabla C_j = \left( \frac{1}{Pe} \right) \left( \nabla^2 C_j \right) - k C_r \quad (5.12)
\]

with the following dimensionless boundary conditions:

(i) on the thrombus surface:

\[
C_a = 0; \quad \frac{\partial C_b}{\partial n} = \frac{\partial C_e}{\partial n} = \frac{\partial C_j}{\partial n} = 1.0 \quad \text{for } j = e, g, \text{ or } h \quad (5.13)
\]
\[ V_x = V_y = 0 \]

(5.14)

(ii) at \( x = x_0 \) and \( y = B \):

\[ C_j = 0 \quad \text{for } j = b, c, \text{or } g; \quad C_i = \frac{D_{\text{ix}} C_i}{N_i B} \quad \text{for } i = a, c, \text{or } h \]  

(5.15)

(iii) at \( x = x_e: \frac{\partial C_j}{\partial x} = 0 \quad \forall j; t_e = t = 0 \)

(iv) at \( x = x_0: \quad V_y = 0; V_x = 1 - y^2 \)

(5.17)

(v) at \( y = \pm B: \quad V_x = V_y = 0 \)

(5.18)

The dimensionless parameter \( \text{Re} \) is the Reynolds number \((\rho V_{\text{max}} B / \mu)\), ranging from 0.17 to 3.40, which represents the ratio of inertial forces to viscous forces, and \( \text{Pe} \) is the Peclet number \((V_{\text{max}} B / D_{i,e})\), ranging from 1,200 to 3,700, which is the ratio of mass transport by convection to mass transport by diffusion (Bird, 1960).

Those equations were solved numerically using a finite element method, as explained in depth in Appendix C.

5.3.3 Biodynamical Properties

The sizes of the standing vortices developing upstream and downstream of the model thrombi because of the disturbed flow, were estimated by calculating the distribution of the shear stress \( t_\tau \), the tangential component of the stress vector, along the bottom plate and the thrombus surface. The \( i \)-component of the stress vector \( \tau \) is given by:
\[ t_i = \sigma_{ij} n_j; \sigma_{ij} = -P \delta_{ij} + \mu \left( V_{i,j} + V_{j,i} \right) \] (5.19)

where \( \sigma_{ij} \) is the stress tensor, \( n_j \) is the unit normal vector at the boundary, and \( \delta_{ij} \) is the Kronecker delta. Here the comma represents partial differentiation with respect to the following index, and repeated indices imply summation. The separation and reattachment points, as shown in Figure 5.3, are defined as the points where the shear stress changes sign. The size of those recirculating regions was also estimated graphically by plotting streamlines around the object. For two-dimensional incompressible flow, the stream function \( \psi \) is the remaining non-zero component of a vector potential which satisfies the continuity equation identically. Therefore, by definition:

\[ V_x = \frac{\partial \psi}{\partial y}; V_y = -\frac{\partial \psi}{\partial x} \] (5.20)

The forces acting on the thrombus surface, possibly leading to embolization of the aggregate, were estimated as a function of Re, model thrombus geometry and size. By definition, the total force acting on the object is the surface integral of all normal and shearing stresses acting on it. The component of the resultant force parallel to the undisturbed initial velocity is called the drag \( D \), while the component perpendicular to that direction is called the lift \( L \) (Schlichting, 1979). The dimensionless coefficients for lift (\( C_L \)) and drag (\( C_D \)) were calculated according to:
Figure 5.3: Definition sketch of the standing vortices developing around a semicircular model thrombus. S, separation point; R, reattachment point. The normal and tangential components of the stress vector, $t_n$ and $t_t$, are also shown.
\[ C_D = \frac{\int t_x \, d\eta}{0.5 \rho U^2 h} \quad ; \quad C_L = \frac{\int t_y \, d\eta}{0.5 \rho U^2 h} \quad (5.21) \]

where \( U \) is the velocity of the undisturbed flow at the top of the object; \( h \) is the object height; \( t_x \) and \( t_y \) are, respectively, the \( x \) and \( y \) components of the stress vector; and \( \eta \) represents the path along the object boundary. The moment coefficient \( (C_M) \), defined as the dimensionless torque acting on the object, was also computed according to:

\[ C_M = \frac{\int r t_n \sin \phi \, d\eta + \int r t_s \sin \theta \, d\eta}{0.5 \rho U^2 h^2} \quad (5.22) \]

where \( r \) is the distance between the origin and the point of action of the force; \( \theta \) is the angle between \( t_x \) and \( r \); and \( \phi \) is the angle between \( t_n \) and \( r \) (see Figure 5.3). It should be pointed out that, for geometrically similar objects, those dimensionless coefficients are functions of one parameter only, the Reynolds number. In other words, for a given thrombus geometry, the thrombus size is not a variable (Schlichting, 1979).

The average basal shear stress \( (\bar{\tau}_s) \) and average basal tensile or normal stress \( (\bar{\tau}_n) \) were also computed according to:

\[ \bar{\tau}_s = C_D \left( 0.5 \rho \frac{U^2 h}{L} \right) \quad \text{and} \quad \bar{\tau}_n = C_L \left( 0.5 \rho \frac{U^2 h}{L} \right) \quad (5.23) \]

where \( L \) represents the object diameter in the case of a semicircular model thrombus,
and the object length in the case of a rectangular one.

5.4 Estimation of the Model Parameters

The model parameters for the platelet-activating agents and the chemical reactions, as well as the shear rate dependent physical parameters are summarized in Tables 5.1 and 5.2, respectively, while the experimental data on thrombus growth rates and sizes, as well as the estimated platelet-activating agent wall fluxes are shown, as a function of wall shear rate and thrombus size, for the semicircular and rectangular model thrombi, in Tables 5.3 and 5.4, respectively. Those parameters were either obtained from the literature or estimated as shown below.

5.4.1 Flux

The wall fluxes of all platelet-activating substances, with the exception of thrombin, were estimated, as a function of wall shear rate, from a knowledge of their generation rates or amounts per platelet, and the growth rates or sizes of the model thrombi. Although values for those generation rates or amounts per platelet were obtained from other sources (Adams and Feuerstein, 1983; De Caterina et al., 1984) (see Table 5.1), the number of platelets constituting an average single thrombus at discrete times was measured in this study at different wall shear rates, as shown in Figure 5.4. Next, by assuming an average platelet volume of 10 μm³, the average thrombus volume was also estimated, as indicated in Figure 5.5. Both data sets were then curve-fitted with cubic polynomials using the least square regression technique.
Table 5.1: Model Parameters for the Platelet-Activating Agents and the Chemical Reactions.

<table>
<thead>
<tr>
<th>Chemical Species</th>
<th>Molecular Weight (daltons)</th>
<th>Brownian Diffusion Coefficient*, Db (cm² / sec)</th>
<th>Pseudo 1st Order Reaction Rate Constant, k (sec⁻¹)</th>
<th>Amount Per Plateletf</th>
<th>Initial Whole Blood Concentrationg</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>424.2</td>
<td>2.57 x 10⁻⁶</td>
<td>--</td>
<td>2.4 x 10⁻¹⁷ mole</td>
<td>0.000</td>
</tr>
<tr>
<td>TxA2</td>
<td>352.5</td>
<td>2.14 x 10⁻⁶</td>
<td>0.0161†</td>
<td>.95 x 10⁻²⁰ mole / sec</td>
<td>0.000</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>72000</td>
<td>3.32 x 10⁻⁷</td>
<td>--</td>
<td>--</td>
<td>0.869 µM</td>
</tr>
<tr>
<td>Thrombin</td>
<td>36600</td>
<td>4.16 x 10⁻⁷</td>
<td>--</td>
<td>--</td>
<td>0.000</td>
</tr>
<tr>
<td>Antithrombin III</td>
<td>62000</td>
<td>3.49 x 10⁻⁷</td>
<td>0.0201§</td>
<td>--</td>
<td>2.844 µM</td>
</tr>
<tr>
<td>Heparin</td>
<td>16000</td>
<td>--</td>
<td>11.850‖ 127.0‖</td>
<td>--</td>
<td>2.844 µM</td>
</tr>
<tr>
<td>vWF</td>
<td>--</td>
<td>1.20 x 10⁻⁷</td>
<td>--</td>
<td>0.2 x 10⁻⁹ units</td>
<td>0.608 units / ml</td>
</tr>
</tbody>
</table>

* see Hubbell and McIntire (1986a); Adams and Feuerstein (1983).
† for the nonenzymatic hydrolysis of TxA₂ to TxB₂ (see Hubbell and McIntire, 1986a).
§ for the inhibition of thrombin by 2.844 µM AT III alone.
‖ for the inhibition of thrombin by 2.844 µM AT III accelerated by 2.0833 µM heparin (300 USP units / mg), the experimentally-used concentration.
¶ for the inhibition of thrombin by 2.844 µM AT III accelerated by 0.09 µM heparin, the concentration for maximum rate of inhibition.
f see Adams and Feuerstein (1983); De Catarina et al. (1984).
g assuming an average hematocrit of 39.2 %.
Table 5.2: Shear Rate Dependent Parameters.

<table>
<thead>
<tr>
<th>Wall Shear Rate (sec(^{-1}))</th>
<th>Apparent Viscosity*, (\mu) (cp)</th>
<th>Enhanced Diffusion Coefficient(#), (D_i) (cm(^2) / sec)</th>
<th>Reynolds Number(&amp;) (Re)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>4.223</td>
<td>1.36 x 10(^{-6})</td>
<td>0.178</td>
</tr>
<tr>
<td>800</td>
<td>3.405</td>
<td>1.09 x 10(^{-5})</td>
<td>1.770</td>
</tr>
<tr>
<td>1500</td>
<td>3.288</td>
<td>2.04 x 10(^{-5})</td>
<td>3.436</td>
</tr>
</tbody>
</table>

* see Cokelet (1987).
\(\#\) calculated from equation (5.28).
\(\&\) \(Re = \rho V_{\text{max}} B / \mu\)
<table>
<thead>
<tr>
<th>Diameter (μm)</th>
<th>Wall Shear Rate (sec(^{-1}))</th>
<th>Time (sec)</th>
<th>Thrombus Growth Rate (platelet/sec)</th>
<th>Thrombus Size (platelet)</th>
<th>ADP Flux (mol/μm(^2) s)</th>
<th>TxA2 Flux (mol/μm(^2) s)</th>
<th>vWF Flux (units/μm(^2) s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>100</td>
<td>63.68</td>
<td>2.27</td>
<td>44.9</td>
<td>34.7</td>
<td>2.71</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>28.2</td>
<td>3.55</td>
<td>26.1</td>
<td>34.3</td>
<td>1.71</td>
<td>1.58</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>26.1</td>
<td>3.04</td>
<td>46.5</td>
<td>46.5</td>
<td>1.58</td>
<td>3.9</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>1303.32</td>
<td>2.87</td>
<td>11.0</td>
<td>130.32</td>
<td>2.87</td>
<td>3.16</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>209.95</td>
<td>2.63</td>
<td>10.1</td>
<td>209.95</td>
<td>2.63</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>55500</td>
<td>6.78</td>
<td>25.8</td>
<td>55500</td>
<td>6.78</td>
<td>25.8</td>
</tr>
<tr>
<td>Length (µm)</td>
<td>Height (µm)</td>
<td>Width (µm)</td>
<td>Wall Shear Rate (sec⁻¹)</td>
<td>Time (sec)</td>
<td>Thrombus Growth Rate (platelet / sec)</td>
<td>Thrombus Size (platelet)</td>
<td>ADP Flux (mol / µm² s) x 10²⁰</td>
</tr>
<tr>
<td>------------</td>
<td>-------------</td>
<td>------------</td>
<td>--------------------------</td>
<td>------------</td>
<td>--------------------------------------</td>
<td>-------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>7.5</td>
<td>100</td>
<td>89.07</td>
<td>2.41</td>
<td>57.5</td>
<td>23.2</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td></td>
<td></td>
<td>15.48</td>
<td>3.28</td>
<td>39.5</td>
<td>31.5</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td></td>
<td></td>
<td>22.63</td>
<td>3.47</td>
<td>37.5</td>
<td>33.2</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>15</td>
<td>100</td>
<td>143.00</td>
<td>2.79</td>
<td>206.3</td>
<td>6.70</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td></td>
<td></td>
<td>135.56</td>
<td>4.84</td>
<td>302.7</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td></td>
<td></td>
<td>68.030</td>
<td>7.66</td>
<td>300.0</td>
<td>18.4</td>
</tr>
</tbody>
</table>
Figure 5.4: Number of platelets constituting an average single growing thrombus as a function of time and wall shear rate. The experimental data were curve-fitted with the following cubic polynomials: \( P_{1500} = -7.62 + 0.40t + 7.50 \times 10^{-2}t^2 - 2.11 \times 10^{-4}t^3 \), \( R^2 = 0.99 \); \( P_{800} = -21.80 + 4.71t - 5.22 \times 10^{-2}t^2 + 2.59 \times 10^{-4}t^3 \), \( R^2 = 0.90 \); \( P_{100} = 17.50 - 2.38t + 4.15 \times 10^{-2}t^2 - 1.10 \times 10^{-4}t^3 \), \( R^2 = 0.91 \), where \( P \) and \( t \) represent, respectively, the number of platelets per thrombus and the time in seconds, and \( R^2 \) is the coefficient of determination, defined as \( R^2 = (S_t - S_r)/S_t \), where \( S_t \) is the total sum of the squares around the mean, and \( S_r \) is the sum of the squares of the residuals around the regression line.
Figure 5.5: Average thrombus volume as a function of time and wall shear rate. The experimental data were curve-fitted with the following cubic polynomials: $P_{1500} = -76.2 + 4.0t + 0.75t^2 - 2.11\times10^{-3}t^3$, $R^2 = 0.99$; $P_{800} = -218.0 + 47.1t - 0.522t^2 + 2.59\times10^{-3}t^3$, $R^2 = 0.90$; $P_{100} = 32.62 - 0.81t - 0.11t^2 + 1.83\times10^{-3}t^3$, $R^2 = 0.98$. The variables are the same as those in Figure 5.4.
The strategy used was to match the model thrombus volume to the measured average thrombus volume, and solve the resulting polynomial for a feasible time root \( 0 < t(\text{sec}) < 150 \). The number of platelets per thrombus or the thrombus growth rate could then be estimated, at that specific time, from the polynomials (or their time-derivatives) curve-fitting the data of Figure 5.4. The details of the flux computations are shown below for the 20-μm-long hemispherical thrombus at 1500 sec\(^{-1}\).

**ADP Flux**

80% of the ADP content of the dense granules in each platelet is assumed to be instantaneously released as the platelet attaches to the growing thrombus (Adams and Feuerstein, 1983). The ADP flux is, therefore, essentially dependent upon the thrombus growth rate. From the work of Adams and Feuerstein (1983), a single platelet contains \( 3.0 \times 10^{-17} \) moles of ADP inside its dense granules. The growth rate of the model thrombus is estimated from the experimental data as follows. The volume of a hemisphere, with a diameter of 20 μm, is \( (2/3)(\pi)(10)^3 \) or 2,094.4 μm\(^3\). The polynomial curve-fitting the data of Figure 5.5 at 1500 sec\(^{-1}\) is then set equal to that value:

\[
2,094.40 = -76.20 + 4.00(t) + 0.75(t)^2 - 2.11 \times 10^{-3} (t)^3
\]

with \( t \) representing time in seconds. That equation is solved numerically for a feasible time root, and \( t \), in this case, is found to be 55.5 seconds. The thrombus growth rate, at that specific time, may now be estimated by evaluating the time-derivative of the
corresponding polynomial representing the data of Figure 5.4 at 1500 sec⁻¹, as follows:

\[
\frac{d(plt)}{dt} = 0.40 + 2(7.50 \times 10^{-2})t - 3(2.11 \times 10^{-4})t^2
\]

\[= 55.5\]

\[= 6.80 \text{ plt/sec}.
\]

One may now calculate the ADP flux over a thrombus surface area of \((2)(\pi)(10)^2\) or 628.3 \(\mu\text{m}^2\), as:

\[
\text{FLUX (ADP)} = \frac{(6.80 \text{ plt/sec})(3 \times 10^{-17} \text{ mole/plt})(0.80)}{628.3 \mu\text{m}^2}
\]

\[= 25.80 \times 10^{-20} \text{ mole/\mu m}^2\text{ sec}
\]

**vWF FLUX**

It was assumed, in this case, that the combination of collagen fibrils and shear stress is sufficient to force platelets to release all their vWF content, this needing further experimental confirmation, however. A single platelet contains \(0.2 \times 10^{-9}\) Units of vWF inside its \(\alpha\)-granules (Adams and Feuerstein, 1983). Using a similar procedure than the one used to calculate the ADP flux, the vWF flux over a thrombus surface area of 628.3 \(\mu\text{m}^2\) is given by:

\[
\text{FLUX(vWF)} = \frac{(6.80 \text{ plt/sec})(0.2 \times 10^{-9})(1.0)}{628.3 \mu\text{m}^2}
\]

\[= 2.15 \times 10^{-12} \text{ Units/\mu m}^2\text{ sec}
\]
**TxA₂ Flux**

Because TxA₂ is generated enzymatically within the platelet, its flux depends primarily on the number of platelets composing the thrombus, rather than on the thrombus growth rate. It is also assumed that its intracellular precursor, arachidonic acid, is present in excess, therefore not limiting TxA₂ metabolism. Furthermore, since the internal diffusion of TxA₂ from the thrombus core to the outside fluid was not taken into account when estimating the wall fluxes of that compound, the calculated concentration profiles represent upper bounds on those observed experimentally. From measurements made by De Caterina et al. (1984) and calculations similar to those of Hubbell and McIntire (1986a), TxA₂ generation rate per platelet is estimated to be $0.95 \times 10^{-20}$ mole/sec. The model thrombus size at $t = 55.5$ sec is computed by evaluating the polynomial curve-fitting the data of Figure 5.4 at 1500 sec⁻¹, as following:

$$\left[ \text{# platelets} \right]_{t=55.5} = -7.62 + 0.40(t) + 7.50 \times 10^{-2}(t)^2 - 2.11 \times 10^{-4}(t)^3$$

$$= 209.40 \text{ plt}$$

The TxA₂ flux may now be calculated as:

$$\text{FLUX (TxA₂)} = \frac{(209.40 \text{ plt})(0.95 \times 10^{-20} \text{ mole/plt sec})}{628.3 \mu \text{m}^2}$$

$$= 3.20 \times 10^{-21} \text{ mole/\mu m}^2 \text{ sec}$$
Table 5.3, for circular model thrombi, and Table 5.4, for rectangular ones, summarize all experimentally-derived data, including platelet-activating agent fluxes, the sizes and growth rates of model thrombi, and the time roots as a function of wall shear rate and model thrombus size.

5.4.2 Reaction Rate Constants

The detailed calculations of the chemical reaction rate constant for the nonenzymatic hydrolysis of TxA2 to TxB2 in aqueous medium can be found in Hubbell (1986). The value used is given in Table 5.1.

Inactivation of thrombin by AT III

Thrombin is inactivated, in the bulk phase, by the circulating AT III, assumed to be present in excess around the thrombus ( [AT III] >> [T], where [AT III] is the AT III molar concentration and [T] is the thrombin molar concentration ). The whole blood concentration of AT III is approximately 2.844 $\mu$M, assuming a plasma concentration of 29 mg/dl and an average hematocrit of 39.2%. The observed second order rate constant, $k_{\text{obs}}''$, was measured by Rozenberg (1982) to be $4.25 \times 10^5$ M$^{-1}$min$^{-1}$, with a reaction rate equation of the form: $-d[T]/dt = k_{\text{obs}}''[\text{AT III}][T]$. An observed pseudo first order reaction rate constant, $k_{\text{obs}}'$, may now be estimated according to $k_{\text{obs}}' = k_{\text{obs}}''[\text{AT III}]$, and in the present case, turns out to be 0.0201 sec$^{-1}$. 
Inactivation of thrombin by AT III in presence of heparin

Inhibition of thrombin by AT III is greatly accelerated in presence of heparin. In order to fully investigate the inhibitory effect of heparin, calculations were performed with two different heparin concentrations, the experimentally-used concentration and the concentration for maximum rate of inhibition, as determined by the data of Griffith (1982). Heparin, with a specific activity of 300 USP units/mg, is used in concentration of 10 units/ml of blood in the experiments, which corresponds to a whole blood concentration of 2.0833 μM. From Figure 1 in Griffith (1982), the observed second order rate constant $k''_{obs}$ is about $0.25 \times 10^9$ M$^{-1}$min$^{-1}$ at that heparin concentration. The observed pseudo first order rate constant, $k'_{obs}$, may be estimated as reported above and is found to be 11.85 sec$^{-1}$. From the same figure in Griffith (1982), the maximum rate of inhibition for that reaction occurs at $[\text{heparin}] = 9.0 \times 10^{-8}$ M, and with $k''_{obs} = 2.68 \times 10^9$ M$^{-1}$min$^{-1}$. $k'_{obs}$ is 127.0 sec$^{-1}$ in this case. All reaction rate constants are summarized in Table 5.1.

5.4.3 Physical Parameters

All physical parameters were estimated as constant average quantities, for a given wall shear rate, in this model. Assuming a homogeneous fluid, most parameters were calculated on the basis of an average shear rate $\langle \dot{\gamma} \rangle$ across the channel, which, for a parabolic velocity profile, is given by:

$$\langle \dot{\gamma} \rangle = \frac{1}{B} \int_0^B \dot{\gamma}(x) \, dx = \frac{\dot{\gamma}_{wall}}{2}$$

(5.24)
Those assumptions are justified, for the present model, by the facts that the crowding of RBCs in the center of the vessel (rouleaux formation), leading to a large plasmatic layer near the wall surface, and thereby to a non-uniform cell distribution across the channel, occurs predominantly in vessels of very small diameter. Also, the non-Newtonian behavior of blood, another consequence of the non-uniform RBC distribution, which can lead to a non-parabolic velocity profile, is important primarily at very low shear rates (< 100 sec⁻¹) (Lih, 1975).

Because it has been shown (Cokelet, 1987) that, in vessels with diameters smaller than approximately 500 μm, the average hematocrit in the tube, \( h_t \), is less than the hematocrit of the entering fluid, \( h_e \) (chosen arbitrarily as 40% here), a phenomenon referred to as the Fåhraeus effect, the magnitude of that effect was estimated theoretically by the authors for the present model. Based on Lih's model derived for a tube with a stepwise RBC distribution, and using, as a first approximation, Einstein's model for the hematocrit-dependency of viscosity (\( \eta_r = \mu/\mu_0 = 1 + Ah_k \)), where \( \mu \) and \( \mu_0 \) are the viscosities of whole blood and plasma respectively; \( h_k \) is the hematocrit of the uniform cell concentration region in the central core; and \( A \) is a constant equal to 2.5), the following relationship was derived:

\[
\frac{h_t^2 + h_e}{1.5 A \left( \frac{1}{k^2} - 1 \right)} - \frac{h_e}{1.5 A \left( \frac{1}{k^2} - k \right)} = 0
\]  \( (5.25) \)
where \( h_e \) is 0.40, and \( \kappa \) is the dimensionless thickness of the plasmatic layer. Thomas (1962) has shown this to be related to the relative particle size by:

\[
1 - \kappa = 0.76 \left( \frac{\delta}{B} \right)
\] (5.26)

where \( \delta \) is the equivalent RBC radius (2.75 \( \mu \)m), and \( B \) is half the thickness of the channel (100 \( \mu \)m). It should be pointed out that the thickness of the plasmatic layer is only 2.1 \( \mu \)m using the above relationship, which is small compared to the sizes of the model thrombi, thereby validating the original assumption of nearly uniform cell concentration across the channel. From equation (5.25), \( h_1 \) is calculated to be 39.2%, indicating that the Fåhraeus effect is relatively weak in this model. That value was used, however, in all subsequent calculations involving hematocrit.

Because blood viscosity is known to be a weakly decreasing function of shear rate (\( \dot{\gamma} > 100 \text{ sec}^{-1} \)) (Karino and Goldsmith, 1985), an average viscosity was estimated at each shear rate (see Table 5.2), using an equation derived by Quemada (Cokelet, 1987), that represents the rheological properties of blood and related red cell suspensions over a wide range of conditions.

Since the problem treated is essentially an isothermal one, with a constant temperature of 37° C, the density of blood was considered constant at 1.1064 gr/cm³ in all calculations.

When RBCs are present in a non-uniform velocity field, such as exists in parallel plate flow, the cells rotate, entraining the small fluid layers surrounding the cells
to rotate also. Those local secondary flows greatly augment the effective diffusivities of cells and large molecular weight species, particularly vWF multimers and thrombin, when compared to Brownian diffusion alone. Keller (1971) has developed a model which gives the effective diffusion coefficient, $D_e$, as:

$$D_e = D_b + D_i$$

(5.27)

where $D_b$ is the Brownian molecular diffusion coefficient (see Table 5.1), and $D_i$ is the rotation-induced diffusion coefficient (see Table 5.2), given by:

$$D_i = 0.18 \delta^2 \dot{\gamma}_{\text{wall}}$$

(5.28)

Because the thrombogenesis process occurs predominantly near the wall surface, where RBC concentration is low, it is postulated that the enhanced diffusivity is due primarily to RBC rotation rather than to interparticle collision, which may be the prevailing mechanism in the bulk. For the same reason, the wall shear rate $\dot{\gamma}_{\text{wall}}$, rather than the average shear rate $\langle \dot{\gamma} \rangle$ is used in equation (5.28). The parameters for the platelet-activating agents and the chemical reactions, and the shear rate-dependent physical parameters are summarized in Tables 5.1 and 5.2, respectively.

5.5 Results and Discussion
The effects of viscous shear flow on the mechanics of thrombus growth and embolization, as well as the concentration profiles of four potential platelet-activating substances generated in the vicinity of various model thrombi, were estimated at various wall shear rates, spanning the physiological range. The reasons for taking the effect of wall shear rate into account and for choosing two particular model thrombus geometries in this study, were obtained from the following experimental observations. A pseudo-color enhanced representation of three-dimensional reconstructions of thrombi formed after 120 seconds of blood flow at 1500 sec\(^{-1}\) over a human collagen-coated surface is shown in Figure 5.6. As can be observed from that figure, the actual thrombi have steep edges, are irregularly-shaped on the top and elongated in the flow direction. These thrombi could well be imagined as having contours shaped between that of a perfect semicircle and that of a perfect rectangle. Similarly, Figure 5.7 shows three-dimensional reconstructions of thrombi formed after 120 seconds of flow at three different wall shear rates, 1500 sec\(^{-1}\), 800 sec\(^{-1}\), and 100 sec\(^{-1}\). This figure illustrates, in a qualitative manner, the fact, observed by others (Turitto et al., 1987), that the rate of platelet accumulation on a thrombogenic surface increases with increasing wall shear rate, resulting in thrombi of different sizes. Because the wall fluxes of all platelet-activating agents depend primarily on the rate of thrombus growth or the thrombus size itself, it was therefore necessary to consider the effect of wall shear rate when estimating those fluxes in this study.

5.5.1 Effects of Viscous Shear Flow

Although the problems of a circular cylinder and a sphere exposed to a uniform shear
Figure 5.6: Estimation of the three-dimensional structures of thrombi formed after 120 seconds of blood flow at 1500 sec$^{-1}$ over type I human collagen-coated glass cover slip in a parallel plate flow chamber. Flow is from left to right. The length bar applies to both the length and the height of the thrombi. Light blue represents the bottom of the thrombi, while red represents the top.
Figure 5.7: Effect of wall shear rate on the kinetics of thrombus growth. The same comments as those in Figure 5.6 apply.
flow are well-known ones in fluid mechanics (Schlichting, 1979; Dennis and Chang, 1970), the situation of a small object attached to a plane wall in a uniform viscous shear flow has been considered by relatively few investigators (Kiya and Arie, 1975; Hyman, 1972; Mills, 1968). Therefore, the objective of this work was to study quantitatively the effects of viscous shear flow on a growing thrombus as a function of wall shear rate and model thrombus size and geometry, and to discuss the implications of those effects with respect to the thrombogenesis mechanism.

The schematic definition of the standing vortices developing upstream and downstream of a growing thrombus as a result of viscous shear flow is given in Figure 5.3. The lengths \( L_u \) and \( L_d \) of the upstream and downstream vortices, respectively, as well as the reattachment angle \( \beta_R \) and the separation angle \( \beta_S \), are shown in Table 5.5 as a function of the particle Reynolds number \( R_h \) and model thrombus. In general, the results of this study confirm the predictions made by Kiya & Arie (1975) and, also shown by Mills (1968) for creeping flow (\( R_h \leq 0.1 \)) over a square-edged orifice plate in a circular pipe, that standing vortices do develop upstream and downstream of the obstacle even in the limit \( R_h \to 0 \). This is in contrast to the case of a circular cylinder or a sphere in a uniform shear flow, for which a separated region with back-flow appears behind the object when the Reynolds number approaches 3.5 for the former (Kiya and Arie, 1975) and 20 for the latter (Schlichting, 1979). The data presented in Table 5.5 indicate that \( L_u \) and \( \beta_R \) decrease slightly with increasing \( R_h \), while \( L_d \) and \( \beta_S \) increase slightly with increasing \( R_h \), which is consistent with the data presented in Figures 7 and 8 by Kiya & Arie (1975). Whereas their data show that for large \( R_h \)
Table 5.5: Sizes of the Standing Vortices as a Function of Reynolds Number and Model Thrombus.

<table>
<thead>
<tr>
<th>$R_h$</th>
<th>Semicircle L=10 µm</th>
<th>Rectangle L=10 µm</th>
<th>$R_h$</th>
<th>Semicircle L=20 µm</th>
<th>Rectangle L=20 µm</th>
<th>A</th>
<th>B</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.016</td>
<td>0.980</td>
<td>3.640</td>
<td>0.064</td>
<td>1.780</td>
<td>3.470</td>
<td>4.860</td>
<td>20.000</td>
<td>4.880</td>
<td>2.830</td>
</tr>
<tr>
<td>0.008</td>
<td>0.990</td>
<td>3.670</td>
<td>0.033</td>
<td>1.820</td>
<td>3.500</td>
<td>4.870</td>
<td>20.000</td>
<td>4.890</td>
<td>2.940</td>
</tr>
<tr>
<td>0.001</td>
<td>1.060</td>
<td>3.760</td>
<td>0.03</td>
<td>1.920</td>
<td>3.600</td>
<td>4.880</td>
<td>20.000</td>
<td>4.900</td>
<td>3.260</td>
</tr>
<tr>
<td>0.016</td>
<td>1.020</td>
<td>4.470</td>
<td>0.064</td>
<td>1.950</td>
<td>3.630</td>
<td>20.000</td>
<td>7.830</td>
<td>3.200</td>
<td>7.900</td>
</tr>
<tr>
<td>0.008</td>
<td>1.010</td>
<td>4.450</td>
<td>0.033</td>
<td>1.910</td>
<td>3.570</td>
<td>20.000</td>
<td>7.750</td>
<td>3.120</td>
<td>7.720</td>
</tr>
<tr>
<td>0.001</td>
<td>0.990</td>
<td>4.410</td>
<td>0.003</td>
<td>1.850</td>
<td>3.490</td>
<td>20.000</td>
<td>7.610</td>
<td>3.060</td>
<td>5.960</td>
</tr>
<tr>
<td>0.016</td>
<td>4.900</td>
<td>35.700</td>
<td>0.064</td>
<td>4.120</td>
<td>34.900</td>
<td>28.410</td>
<td>41.700</td>
<td>28.620</td>
<td>36.500</td>
</tr>
<tr>
<td>0.008</td>
<td>5.000</td>
<td>35.800</td>
<td>0.033</td>
<td>4.260</td>
<td>35.070</td>
<td>28.530</td>
<td>41.740</td>
<td>28.990</td>
<td>36.750</td>
</tr>
<tr>
<td>0.001</td>
<td>5.660</td>
<td>36.100</td>
<td>0.003</td>
<td>4.740</td>
<td>35.460</td>
<td>28.670</td>
<td>41.800</td>
<td>29.280</td>
<td>38.050</td>
</tr>
<tr>
<td>0.016</td>
<td>5.350</td>
<td>29.700</td>
<td>0.064</td>
<td>4.900</td>
<td>30.850</td>
<td>38.800</td>
<td>30.000</td>
<td>32.200</td>
<td>29.820</td>
</tr>
<tr>
<td>0.008</td>
<td>5.210</td>
<td>29.670</td>
<td>0.033</td>
<td>4.630</td>
<td>30.720</td>
<td>38.760</td>
<td>29.890</td>
<td>32.640</td>
<td>29.670</td>
</tr>
<tr>
<td>0.001</td>
<td>4.900</td>
<td>29.600</td>
<td>0.003</td>
<td>4.320</td>
<td>30.470</td>
<td>38.800</td>
<td>29.760</td>
<td>32.420</td>
<td>29.720</td>
</tr>
</tbody>
</table>

Notes:  
- $L_u$ and $L_d$ are given in µm, while $\beta_R$ and $\beta_S$ in degrees.  
- For comparison, the Reynolds number is defined as in Khy and Arie (1975), namely, $R_h = U h_p / \mu$, where $U$ is the undisturbed velocity at the top of the object, and $h$ is the height of the object.  
- Two 20 µm-long rectangular model thrombi separated by a distance of 20 µm.  
- Two 20 µm-long rectangular model thrombi separated by a distance of 200 µm.
Figure 5.8: Velocity vector plot (a) and streamlines (b) in the case of two nearby-growing rectangular model thrombi 20 μm-long, at Re = 0.178. In (a), the direction and length of the arrows represent, respectively, the direction and magnitude of the velocity vectors, plotted at each nodal point. In (b), the values for the dimensionless stream function $\psi^*$ (where $\psi^* = \psi/V_{max}$) are:

- A = 0.16 x 10^{-4}
- B = 0.0
- C = 0.10 x 10^{-3}
- D = 0.10 x 10^{-2}
- E = 0.50 x 10^{-2}
- F = 0.10 x 10^{-1}
- G = 0.20 x 10^{-1}
- H = 0.30 x 10^{-1}
- I = 0.50 x 10^{-1}
the downstream vortex is always much larger than the upstream one, the data of Table 5.5 reveal that both standing vortices become geometrically similar as $R_h \to 0$. The size of both recirculation regions is strongly dependent upon the model thrombus geometry, as indicated by the relatively large standing vortices formed with rectangular model thrombi compared to those formed with semicircular ones. The most interesting result, however, appears in the case of two rectangular thrombi growing near each other and separated by a distance equal to one thrombus length or 20 $\mu$m. Indeed, as shown by the streamlines in Figure 5.8(b) and the data in Table 5.5, a large standing vortex, 20 $\mu$m-long and 8 to 9 $\mu$m-high, develops between the two thrombi at all wall shear rates investigated. On the other hand, this large vortex is not present when the thrombi are separated by 10 times a thrombus length or 200 $\mu$m (see Table 5.5).

Another effect of viscous shear flow is the nonuniform fluid velocity distribution across the channel, and in particular, the considerably reduced velocities in and near the recirculating regions. This effect is shown graphically by the velocity vector plot of Figure 5.8(a), where the direction and the length of the arrows, represent, respectively, the direction and magnitude of the velocity vectors, plotted at each nodal point. From that figure, one can see that the velocity components in the recirculating wake regions and their surroundings, as well as in the fluid layer directly above the thrombi, are very small when compared to those in the bulk. In fact, at 1500 sec$^{-1}$, the smallest velocity vector estimated has a magnitude of 0.0375 cm/sec, while the speed of the entering fluid at the top of the objects, $U$, is 1.425 cm/sec. Similarly, at 100 sec$^{-1}$, the smallest calculated speed in the vortex region is about $2.5 \times 10^{-3}$ cm/sec, while $U$ is $95 \times 10^{-3}$ cm/sec.
The drag coefficient $C_D$, the lift coefficient $C_L$, and the moment coefficient $C_M$, decrease more or less linearly with the Reynolds number $R_h$, when plotted on a logarithmic scale, as shown in Figure 5.9. Most noteworthy is that $C_D$ and $C_M$ are always larger for the rectangular model thrombus than for the semicircular one, whereas $C_L$ is independent of thrombus geometry for $R_h > 0.003$. The difference in $C_D$ values between the semicircular and the rectangular protrusions are relatively small, however. This was also reported by Basmadjian (1984), who noticed that $C_D$ values for small protrusions of different shapes differed little over the full range of viscous flow. When extrapolating the values of $C_D$ and $C_M$ obtained for the semicircular projection to $R_h = 0.1$, the results of this study agree reasonably well with those obtained by Kiya & Arie (1975). Indeed, from the data of Figure 5.9, $C_D$ and $C_M$ are 160 and 80, respectively, at $R_h = 0.1$, compared to 170 and 100 from Figures 12 and 13 of Kiya & Arie (1975). Also shown in Figure 5.9, is the case of two nearby-growing thrombi. For that particular situation, $C_D$ and $C_M$ are always larger for the upstream thrombus (A) than for the downstream one (B), while the same values are found for $C_L$.

The average stresses and the torque acting on the aggregate as it grows under the effects of viscous shear flow, eventually leading to its embolization from the wall surface, are summarized in Table 5.6, as a function of wall shear rate and model thrombus. Both, shear stress and normal stress, as well as the torque, increase with increasing wall shear rate, as normally expected. On the other hand, the embolizing stresses are insensitive to thrombus dimension. This was also shown empirically by
Figure 5.9: Drag coefficient $C_D$, lift coefficient $C_L$, and moment coefficient $C_M$ as a function of the Reynolds number $R_h$, defined in terms of the undisturbed velocity at the top of the object $U$ and the height of the object $h$ as $R_h = \frac{U h}{\mu}$. Solid lines are used for single rectangular or semicircular model thrombi, while dashed lines are used for two nearby-growing rectangular model thrombi.
Table 5.6: Average Shear and Normal Stresses, and the Torque, as a Function of Wall Shear Rate and Model Thrombus.

<table>
<thead>
<tr>
<th>Stress*</th>
<th>Wall Shear Rate (sec(^{-1}))</th>
<th>Semicircle L=10 (\mu)m</th>
<th>Semicircle L=20 (\mu)m</th>
<th>Rectangle L=10 (\mu)m</th>
<th>Rectangle L=20 (\mu)m</th>
<th>(D=20 \mu)m</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shear Stress (\tau_s)</td>
<td>100</td>
<td>15.0</td>
<td>15.5</td>
<td>20.0</td>
<td>19.5</td>
<td>21.0</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>95.0</td>
<td>94.5</td>
<td>117.0</td>
<td>118.5</td>
<td>137.0</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>171.0</td>
<td>170.5</td>
<td>213.0</td>
<td>214.5</td>
<td>250.5</td>
</tr>
<tr>
<td>Normal Stress (\tau_n)</td>
<td>100</td>
<td>25.5</td>
<td>20.0</td>
<td>20.0</td>
<td>20.0</td>
<td>20.5</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>43.0</td>
<td>49.5</td>
<td>43.0</td>
<td>49.0</td>
<td>55.0</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>65.0</td>
<td>74.5</td>
<td>71.0</td>
<td>73.0</td>
<td>86.5</td>
</tr>
<tr>
<td>Torque (x 10^3)</td>
<td>100</td>
<td>.003</td>
<td>.017</td>
<td>.009</td>
<td>.033</td>
<td>.035</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>.027</td>
<td>.106</td>
<td>.051</td>
<td>.204</td>
<td>.228</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>.049</td>
<td>.192</td>
<td>.092</td>
<td>.412</td>
<td>.417</td>
</tr>
</tbody>
</table>

* All stresses are given in dynes/cm\(^2\) and the torque in dyne-cm.
Basmadjian (1984), who found that, in creeping flow regime \((R_h \leq 0.1)\) and for sufficiently small protrusions \((h/2B \leq 0.05)\), both, shear stress and tensile stress, are independent of object height. The numerical results of Table 5.6 for the average shear stress \(\tau_s\) agree within 25-30\% with the values predicted by a semi-empirical equation derived by Basmadjian (1984) \((\tau_s = 30\mu<V>^2/2B,\) where \(<V>\) equals \(2/3 \, V_{max}\) and represents the average fluid velocity across the channel). Also shown in Table 5.6, is the slightly larger average shear stress acting on the rectangular object compared to the one acting on the semicircular object, whereas the average normal stresses are similar for both geometries. In the case of two nearby-growing thrombi, the average shear stress and the torque acting on the downstream thrombus \((B)\) are reduced by approximately 25\% when compared to those acting on the upstream one \((A)\), which could favor the growth of the former aggregate. Again, the average normal stresses are similar for both thrombi. It is interesting to notice that at the low wall shear rate of 100 sec\(^{-1}\) both shear stress and normal stress have relatively similar values, while, as the wall shear rate increases, the shear stress becomes the predominant embolizing stress. As distinct from the stresses, the torque is not only a function of thrombus geometry but also a function of thrombus size.

Figure 5.10 shows four pseudo-color enhanced images of growing thrombi, digitized after, respectively, 30, 75, 105, and 120 seconds of blood flow over a collagen-coated surface. This photograph explicitly shows that the large zones of recirculation and back-flow (indicated by blue crosses) developing between nearby-growing thrombi at particular stages in the thrombosis process are also the sites of massive platelet aggregation at later stages. The region of recirculation present near the center of Figure
Figure 5.10: Pseudocolor enhanced images of mural thrombi digitized after 30 sec (A), 75 sec (B), 105 sec (C), and 120 sec (D) of blood flow over a collagen-coated surface at 1000 sec⁻¹. The fluorescence intensity ranging from black to white in the original monochrome video image is mapped into a transition from blue to red, where blue represents the surface background and red the regions of intense platelet accumulation. The blue crosses, in that figure, indicate the location of possible standing vortices developing between growing thrombi.
5.10(A), for instance, is completely covered with platelets 45 seconds later, as indicated at the same location in Figure 5.10(B).

5.5.2 Concentration Profiles of Platelet-Activating Agents

The concentration profiles of released platelet-activating agents are presented as three-dimensional plots in this paper (see Figure 5.12 for example), and those require a few comments. The x-y plane in those figures represents the actual channel geometry as drawn in Figure 5.1, while the concentration variation of the substance of interest over that two-dimensional space is represented by the third dimension (c axis). In other words, the height at any nodal point in the x-y plane corresponds to the magnitude of the concentration of the substance of interest at that point. Blood is flowing in the positive x-direction, that is, from right to left in those figures. All those figures also exhibit two local maxima (labelled 1 and 2) and one local minimum, upstream and downstream from the thrombus and near the top of the thrombus, respectively. The presence of local maxima is related to the standing vortices which develop on both sides of the aggregate, as mentioned earlier. Indeed, because those recirculating regions are areas of low fluid velocities, the retention time of any released material, and thereby its concentration, is correspondingly larger in those regions. Although the fluid velocities near the top of the aggregate are considerably smaller than those in the bulk, as shown previously, they are, however, larger than those within the vortices, which explains the presence of a local minimum in that region. In fact, as one moves away from the wall or the aggregate surface into the bulk, the convective dispersion rapidly becomes important, resulting in steep decreasing concentration gradients. Therefore, only
platelets that flow in close proximity to the thrombus surface or the wall surface have a chance to become activated, and subsequently to aggregate, either by being trapped in the vortex regions or by flowing directly within the zone of high concentration of platelet-activating agents present near the top of the thrombus. It should be pointed out that the downstream maximum is always larger than the upstream one, which can be explained as follows: some of the molecules released from the upstream part or the top of the thrombus are carried along by convection in the direction of the downstream vortex, increasing the local concentration there. This theoretical result correlates well with previous experimental observations reported in Chapter 3 and independently by other investigators (Adams et al., 1983; Hubbell and McIntire, 1986c). Indeed, it was postulated that "fresh" platelets arriving at the site of aggregation become activated, as they pass through a "cloud" of platelet-activating agents surrounding the thrombus, and therefore adhere preferentially at the downstream edge of the aggregate. In addition, as will be shown below, the concentrations of platelet-activating agents accumulating in the vortices present between nearby growing thrombi, are significantly larger than those found in the vicinity of isolated thrombi. This result may also explain the experimental observation made earlier in this report, that platelets aggregate massively in the spaces between adjacent thrombi.

5.5.2.a Adenosine Diphosphate

Shown in Figure 5.11 is the maximum ADP concentration (in μmolar) as a function of wall shear rate and model thrombus. As can be seen from that figure, the maximum ADP concentration increases with decreasing wall shear rate. This can also be visualized
by comparing the three-dimensional plots of Figures 5.12 and 5.13 for the 10 μm-long semicircular model thrombus at 100 sec\(^{-1}\) and 1500 sec\(^{-1}\), respectively. The curves in the upper left-hand corners of those figures represent the variation of the ADP concentration at the wall as a function of axial position in the channel. The points labelled 1 and 2 correspond to the two maxima on the 3-D plots, and are located, respectively, at the upstream and downstream intersections between the bottom plate of the channel and the model thrombus. At 100 sec\(^{-1}\) the larger maximum has a value slightly above 4.5 μM, while at 1500 sec\(^{-1}\) it is only about 0.75 μM, which corresponds approximately to a decrease of 83 % due to the increase in wall shear rate. This decrease in ADP concentration with increasing shear rate is due to both larger convective and diffusive forces at the high shear rate, which tend to reduce the residence time of ADP molecules around the aggregate despite the increase in flux (see Tables 5.3 and 5.4). The results of this study are considerably larger than those found in the case of a flat model thrombus (Hubbell and McIntire, 1986a). Indeed, values of 0.7μM and 0.32 μM were reported for flow over a flat 10 μm-long thrombus at 100 sec\(^{-1}\) and 1500 sec\(^{-1}\), respectively. These differences are due first to the presence of large vortices in this simulation and second, to the fact that ADP fluxes were underestimated in the previous analysis.

Whereas thrombus size or geometry does not seem to affect, in any significant extent, the maximum ADP concentration at high wall shear rates, it certainly does at low wall shear rates, as shown in Figure 5.11. In general, the maximum ADP concentration is larger for a semicircular model thrombus than for a rectangular one, and it decreases with increasing size. At 100 sec\(^{-1}\), for instance, the maximum ADP concentration for
the 10 μm-long semicircular model thrombus is slightly above 4.5 μM as mentioned earlier, while it is only around 2.5 μM for the 20 μm-long one. It should be stressed, however, that this value is still considerably larger than the 0.90 μM reported for a 20 μm-long flat thrombus (Hubbell and McIntire, 1986a). The reason for that decrease is that the ADP flux from the aggregate surface depends upon the rate of platelet arrival to that surface. Although the rate of platelet arrival, as determined experimentally, is greater for rectangular model thrombi than for semicircular ones, and increases slightly with increasing thrombus size, the rate of arrival per unit area is smaller for large thrombi and/or rectangular ones because of their larger surface area, especially at low shear rates (see Tables 5.3 and 5.4).

As shown in Figure 5.14(b), the region of recirculation and back-flow developing between two nearby-growing 20 μm-long rectangular thrombi is also a zone of local high ADP concentration. This is not present, however, in the case of two similar thrombi separated by ten times the same distance, as shown in Figure 5.14(a). In fact, at 100 sec⁻¹, the maximum ADP concentration increases by 22 %, when compared to the case of a single growing thrombus or of two distant thrombi of the same size. As shown quantitatively in Figure 5.11, this effect increases with decreasing wall shear rate.

As reported by Weiss (1982), the level of ADP required to cause platelet shape change and irreversible aggregation in platelet-rich-plasma (PRP) is 0.2 μM and 1.0 μM, respectively. The results of this study lie in the region of the minimum threshold for irreversible platelet aggregation. It is likely, however, that, as the wall shear rate and thrombus size increase, the maximum ADP concentration could decrease below that minimum. In other words, ADP is likely to play its most important role in the
Figure 5.11: Maximum ADP concentration as a function of wall shear rate and model thrombus. Solid lines are used for single thrombi, while dashed lines for two thrombi. A similar notation to the one of Figure 5.2 is used for designating the different model thrombi: (i) rectangular model thrombus, (a) $L=20 \ \mu m$, (b) $L=10 \ \mu m$; (ii) semicircular model thrombus, (a) $L=20 \ \mu m$, (b) $L=10 \ \mu m$; (iii) two rectangular model thrombi, $L=20 \ \mu m$, $D=20 \ \mu m$; (iv) two rectangular model thrombi, $L=20 \ \mu m$, $D=200 \ \mu m$. $L$ represents the thrombus length or diameter, and $D$ the distance separating the thrombi.
Figure 5.12: ADP concentration profile over a semicircular model thrombus 10 µm in diameter. Whole blood flows from right to left at a wall shear rate of 100 sec⁻¹. The x-y plane represents the actual channel geometry, while the height at any nodal point in the x-y plane corresponds to the magnitude of the ADP concentration at that point.
Figure 5.13: ADP concentration profile over a semicircular model thrombus 10 μm in diameter. Whole blood flows from right to left at a wall shear rate of 1500 sec⁻¹. The same comments as those of Figure 5.12 apply.
Figure 5.14: ADP concentration profile over (a) two 20 μm-long rectangular model thrombi 200 μm apart, (b) two 20 μm-long rectangular model thrombi 20 μm apart. Whole blood flows from right to left at a wall shear rate of 100 sec⁻¹.
thrombogenesis process at low shear rates, typical of the venous system, and during the early stages of the process, when aggregate sizes are relatively small.

5.5.2.b Thromboxane A₂

The variation of the maximum TxA₂ concentration (in nmolar) with wall shear rate is shown in Figure 5.15 for different model thrombi. As for ADP, the maximum TxA₂ concentration increases with decreasing wall shear rate. This is also shown qualitatively in Figure 5.16 for the 20 μm-long rectangular model thrombus, for which the maximum TxA₂ concentration passes from 12.9 nM at 1500 sec⁻¹, to 20.9 nM at 800 sec⁻¹, and to 61.2 nM at 100 sec⁻¹, corresponding to a total increase of 79 %. The maximum TxA₂ concentration increases with increasing thrombus size, and does not vary much with thrombus geometry (see Figure 5.15). The former observation is basically due to the fact that the TxA₂ flux from the thrombus surface depends on the total number of platelets constituting that thrombus, and not on the rate of platelet arrival to the thrombus, as is the case for ADP. The latter observation is a result of the relatively similar fluxes estimated for the rectangular and semicircular model thrombi, as shown in Tables 5.3 and 5.4. Also shown in Figure 5.15 is the significant increase in maximum TxA₂ concentration in the case of two nearby-growing thrombi when compared to the case of a single thrombus or of two distant thrombi of the same size, especially at low shear rate. At 100 sec⁻¹, for instance, that increase is around 21.2 %.
Figure 5.15: Maximum TxA₂ concentration as a function of wall shear rate and model thrombus. The notation is similar to the one used in Figure 5.11.
Figure 5.16: Effect of wall shear rate on TxA2 concentration profile over a 20 μm-long rectangular model thrombus: (a) 1500 sec⁻¹; (b) 800 sec⁻¹; (c) 100 sec⁻¹.
In general, the values estimated in this work for the maximum TxA$_2$ concentrations are smaller than those reported earlier for the flow over a flat thrombus (Hubbell and McIntire, 1986a). For example, the maximum value found in the case of a 20 µm-long semicircular model thrombus at 100 sec$^{-1}$ is slightly above 60 nM, whereas the same value was reported to be around 200 µM in the case of a flat thrombus. This apparent discrepancy is mostly due to overestimation of the TxA$_2$ fluxes, especially for the large aggregates, reported in the case of a flat thrombus.

Because TxA$_2$ has a short half-life in aqueous solution, it is difficult to determine what TxA$_2$ concentrations are needed to stimulate platelet activity in vitro. Therefore, it is assumed, as mentioned by others (Hubbell and McIntire, 1986a), that the biological behavior of TxA$_2$ is similar to the one of its close analogue, U-46619 (11,9-epoxy-methano analogue of PGH$_2$, Upjohn Company), which causes shape change at concentrations of 6-60 nM, reversible aggregation at 110-300 nM, and irreversible aggregation at concentrations greater than 600 nM (Jones et al., 1979). According to those numbers, the concentrations calculated for TxA$_2$ are only sufficient to cause platelet shape change. It is possible that, as the thrombus grows larger, enough TxA$_2$ will be released to cause reversible platelet aggregation. On the other hand, it is very unlikely that TxA$_2$, alone, could induce irreversible platelet aggregation. Those results confirm the experimental data reported by Baumgartner (1979) who showed that, preventing TxA$_2$ generation by blocking cyclooxygenase activity with aspirin, caused little change in thrombus formation in heparin anticoagulated or native rabbit blood on
subendothelium.

5.5.2.2 Thrombin

The maximum thrombin concentration is presented in Table 5.7 as a function of wall shear rate, heparin concentration, and model thrombus. Thrombin is known to cause irreversible platelet aggregation at levels of 0.1 to 0.3 units/ml in PRP (Weiss, 1982). All the values shown in Table 5.7 are significantly larger than the minimum threshold required for irreversible platelet aggregation. Indeed, the calculated maximum thrombin concentration values range from 279.2 units/ml to 21.8 units/ml, both found in the case of two nearby-growing thrombi, respectively, at 1500 sec\(^{-1}\) with no heparin and at 100 sec\(^{-1}\) with 0.09 μM heparin (see Table 5.7).

The maximum thrombin concentration drops very drastically when thrombin is inhibited by AT III in presence of heparin. This is shown both quantitatively in Table 5.7, and qualitatively in Figure 5.17 for the 20 μm-long rectangular model thrombus at 800 sec\(^{-1}\). In that figure, the maximum thrombin concentration drops from 227.3 units/ml in absence of heparin, as would be found in an unaltered physiological environment (Figure 5.17c), to 86.1 units/ml in presence of 0.09 μM heparin, the concentration at which the enzymatic inactivation of the released thrombin by AT III is maximum (Figure 5.17a) (Griffith, 1982). An intermediate value of 181.1 units/ml is found in presence of 2.0833 μM heparin, the experimentally-used concentration (Figure 5.17b). Most noteworthy in Figure 5.17a is the lack of a concentration tail downstream of the aggregate, as well as the small peaks on both sides of the thrombus, when compared to Figure 5.17c. Those results are a consequence of the rapid inactivation
Table 5.7: Maximum Thrombin Concentration\(^\text{§}\) as a function of Wall Shear Rate, Heparin Concentration, and Model Thrombus.

<table>
<thead>
<tr>
<th>wall shear rate (sec(^{-1}))</th>
<th>heparin concentration ((\mu)M)</th>
<th>semicircle L=10 (\mu)m</th>
<th>semicircle L=20 (\mu)m</th>
<th>rectangle L=10 (\mu)m</th>
<th>rectangle L=20 (\mu)m</th>
<th>A</th>
<th>B</th>
<th>A</th>
<th>B</th>
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</thead>
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<tr>
<td>1500</td>
<td>0.0000</td>
<td>178.0</td>
<td>210.3</td>
<td>192.7</td>
<td>230.3</td>
<td>279.2</td>
<td>252.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.0833(^*)</td>
<td>170.7</td>
<td>194.3</td>
<td>181.6</td>
<td>201.9</td>
<td>225.6</td>
<td>222.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0900(^\d)</td>
<td>124.1</td>
<td>115.5</td>
<td>119.7</td>
<td>87.8</td>
<td>155.4</td>
<td>144.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>0.0000</td>
<td>176.4</td>
<td>208.0</td>
<td>190.9</td>
<td>227.3</td>
<td>276.5</td>
<td>249.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.0833</td>
<td>163.5</td>
<td>180.6</td>
<td>171.6</td>
<td>181.1</td>
<td>194.3</td>
<td>199.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0900</td>
<td>95.3</td>
<td>102.6</td>
<td>114.1</td>
<td>86.1</td>
<td>72.0</td>
<td>81.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.0000</td>
<td>144.8</td>
<td>140.1</td>
<td>156.3</td>
<td>184.1</td>
<td>224.9</td>
<td>202.3</td>
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</tr>
<tr>
<td></td>
<td>2.0833</td>
<td>96.5</td>
<td>92.6</td>
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<td>57.4</td>
<td>22.7</td>
<td>21.8</td>
<td>49.2</td>
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</tr>
</tbody>
</table>

\(^*\) the experimentally-used heparin concentration.
\(^\d\) the heparin concentration for maximum rate of inhibition (see Griffith, 1982).
\(^\text{§}\) All thrombin concentrations are given in units / ml.
Figure 5.17: Effect of heparin on thrombin inhibition by circulating AT III. The thrombin concentration profiles are shown over a 20-μm-long rectangular model thrombus at 800 sec⁻¹. The heparin concentrations in whole blood are as following: (a) 0.09 μM heparin, the concentration for maximum rate of inhibition, (b) 2.0533 μM heparin, the experimentally used concentration, and (c) no heparin.
of thrombin molecules, as they are released from the aggregate, by the circulating AT III molecules when heparin is present. In fact, the AT III molecules present in the standing vortices keep large quantities of thrombin molecules from accumulating in those regions, thereby reducing considerably the size of the two concentration maxima. Furthermore, because of this rapid inactivation reaction, thrombin molecules have no chance to diffuse away from the wall or to be carried by convection downstream from the aggregate. At 3 μm from the wall, for instance, the maximum thrombin concentration drops to 0.051 units/ml and 0.018 units/ml in Figures 5.17c and 5.17a, respectively, which correspond to a 4,457- and 4,783-fold decrease when compared to the maximum values at the wall.

The effect of wall shear rate on the maximum thrombin concentration is also shown in Table 5.7. In general, that value decreases with decreasing shear rate, and this effect is particularly noticeable when the rate of thrombin inhibition is accelerated by heparin. For instance, in the case of a 20 μm-long rectangular model thrombus, the maximum thrombin concentration decreases by 20.1% when the wall shear rate decreases from 1500 sec\(^{-1}\) to 100 sec\(^{-1}\) with no heparin present, while that decrease is 74.1% with 0.09 μM heparin. This result can be explained by the fact that the rate of thrombin production increases while its rate of inhibition decreases, with increasing wall shear rate. Indeed, at low wall shear rates, the convective forces near the thrombus surface are relatively small, and the potential residence time of the released thrombin molecules comparatively long. In those conditions, AT III molecules become more effective in inhibiting a large number of thrombin molecules, particularly when the rate of reaction is enhanced by heparin. In addition, the rate of thrombin production
increases with increasing wall shear rate because of the increasing diffusive and convective forces carrying prothrombin, the precursor of thrombin, from the bulk to the aggregate surface. This is also shown in Tables 5.3 and 5.4 as an increase in thrombin flux with wall shear rate.

The calculated thrombin concentration values here are considerably larger than the values reported for planar thrombi, mainly because the estimated thrombin fluxes were underestimated in the previous analysis (Hubbell and McIntire, 1986a). In addition, contrary to those previously reported data, the results of this study predict a decrease in thrombin concentration with increasing thrombus size and decreasing wall shear rate, in presence of heparin.

It is clear from those results, that thrombin is likely to be the most potent platelet-activating substance, and therefore, should play a profound role in the mechanism of mural thrombogenesis on collagen, even in the presence of heparin. These findings agree well with the very recent data reported by other groups (Heras et al., 1988; Jang et al., 1988; Schneider et al., 1988), which indicate that thrombus formation in vivo on damaged blood vessels is a thrombin-mediated, but heparin-resistant, process.

5.5.2.d Platelet-Released von Willebrand Factor

The maximum vWF multimer concentrations developing in the vicinity of various model thrombi are shown in Figure 5.18 at two extreme wall shear rates, 100 sec\(^{-1}\) and 1500 sec\(^{-1}\). For the same reasons as mentioned earlier for ADP, the maximum vWF concentration values increase with decreasing shear rate and thrombus size, are larger
Figure 5.18: Maximum platelet-released vWF multimer concentration as a function of wall shear rate and model thrombus. The notation is similar to the one used in Figure 5.11.
for semicircular model thrombi than for rectangular ones, and increase in the case of two nearby-growing thrombi, when compared to the case of a single thrombus or of two distant thrombi of the same size. At 100 sec\(^{-1}\), for instance, that increase is approximately 25.0%.

The endogenous human platelet vWF multimers include unusually large vWF multimeric forms similar to those released by endothelial cells (ECs) and larger than the largest multimeric forms found in normal human plasma (Moake et al., 1988). Moake et al. (1986, 1988) showed that both vWF multimers secreted by ECs and those released by platelets are more effective in inducing shear-induced platelet aggregation than the largest plasma forms. Also, the antigenic levels of those ultralarge vWF multimers required to cause platelet aggregation are considerably smaller than those found in normal human plasma (1 unit/ml). In fact, they showed that antigenic levels of platelet-released vWF < 0.015 units/ml can cause massive platelet aggregation at relatively high shear stress (120 dynes/cm\(^2\)), in the presence of a small amount of ADP serving as a cofactor. The same group showed that 0.05 units/ml of EC-secreted vWF multimers is sufficient to cause massive platelet aggregation when platelets are sheared at 180 dynes/cm\(^2\).

With those numbers in mind, the data of Figure 5.18 clearly indicate that platelet-released vWF multimers may be important in platelet/platelet adhesion in flowing systems and/or in thrombus consolidation due to bound vWF multimers on adjacent platelet membranes, especially at low and intermediate wall shear rates (< 1000 sec\(^{-1}\)). This finding is particularly important because, while the role of vWF in inducing platelet-vessel wall and platelet/platelet interaction has been reported as
essential mostly at high shear rates (Baumgartner et al., 1980b; Houdijk et al., 1985), these results and the recently reported experimental data by Badimon et al. (1988) indicate that vWF may also modulate those effects at low wall shear rates.

5.6 Conclusions

When interpreting the data of this study in the context of thrombus formation, it should be remembered that platelet-activating substances are known to act synergistically with each other. Therefore subtreshold concentrations of one agent present with subtreshold concentrations of another agent may cause platelet activation. It has been found, for instance, that only nanomolar quantities of ADP are sufficient to cause massive platelet aggregation, when combined with large or unusually large vWF multimers from plasma, platelets, or ECs (Moake et al., 1988).

Even in the presence of the anticoagulant heparin, thrombin appears to be the most important of the platelet-activating substances analyzed here. The roles played by ADP and platelet-released vWF may be significant, especially at low shear rates and during the early stages of thrombus growth. The role of TxA2 appears to be secondary, but for the reasons mentioned above, this low concentration of compound could stimulate platelets to respond with greater sensitivity to other activating agents. Furthermore, the results also suggest that TxA2 may become more important in the later stages of the thrombogenesis process, when the aggregates become larger and the concentrations of other agents such as ADP or thrombin diminish.

Standing vortices developing on both sides of individual thrombi and between
nearby-growing thrombi are responsible for the observed rises in concentrations of platelet-activating agents within those regions, and give theoretical support to the experimentally-observed mechanisms of thrombus growth. Indeed, it has been reported that thrombi grow primarily from their downstream edges, and that massive platelet aggregation occurs in the spaces between nearby-growing thrombi. In other words, platelets accumulate preferentially in the regions of large platelet-activating agent concentrations and low fluid velocities. Finally, it was observed that the average shear and tensile stresses, acting on the thrombi, increase with increasing blood flow rate, but are independent of thrombus size and only weakly dependent on thrombus geometry. In other words, embolization of such small protrusions may occur only at relatively high wall shear rate. In fact, this correlates well with the previous experimental observations reported in Chapter 3 and independently by Hubbell (1986), that occasional embolization of the larger thrombi (10-20 μm-high) formed on collagen-coated glass occurred at wall shear rates ≥ 1500 sec⁻¹. It is likely, however, that, as the size of the aggregate increases, the embolizing stresses may become functions of both the wall shear rate and the thrombus size. In this context, it is probable that emboli forming in high flow rate vessels such as the arterioles, are much smaller than those developing in the venous system for instance. The latter ones, because of their large sizes, could be potentially more dangerous for they are prone to occlude small blood vessels distally.
CHAPTER 6
MATHEMATICAL MODEL OF PLATELET TRANSPORT IN FLOWING
WHOLE BLOOD
- A MACROSCOPIC APPROACH -

6.1 Introduction

The thrombogenesis process is a very complex problem in which both biochemical and hemorheological factors have considerable importance. Probably the most crucial hemodynamic phenomena occurring at the onset of thrombus formation are platelet transport to the blood vessel wall and platelet reaction at the wall surface. The latter event is mediated by exposure of blood to foreign surfaces, including the subendothelium of an injured blood vessel (Turitto and Baumgartner, 1982) and polymeric surfaces used for artificial organs (Anderson and Kottke-Marchant, 1985), and depends to a large extent on the type of surface exposed. The former one, on the other hand, has been shown experimentally by several groups (Karino and Goldsmith, 1985; Eckstein, 1982) to depend primarily on the wall shear rate, the vessel diameter, and the blood hematocrit. Although those macroscopic variables are often known in a given physiological or pathological situation, on a microscopic scale they directly influence the
behavior of RBCs under flow conditions and indirectly the dynamic behavior of platelets, in particular near the vessel walls. Indeed, it has been reported (Baumgartner, 1973; Turitto and Weiss, 1980; Eckstein, 1982; Beck and Eckstein, 1980; Tangelder et al., 1985) that RBCs affect the effective diffusivity of platelets in flowing whole blood and are also responsible for the non-uniform distribution of those cells across the blood vessels. In addition, in presence of RBCs, blood may behave as a non-Newtonian fluid with a non-parabolic, blunt velocity profile, especially in low shear environments (Karino and Goldsmith, 1985). For those reasons, the results obtained from mathematical models using classical transport theory often failed to agree accurately with the experimental observations (Turitto and Baumgartner, 1975; Hubbell, 1986).

Therefore, the purpose of this study was to develop and solve a new mathematical model of platelet transport in flowing whole blood, coupled with platelet reaction at the wall. A two dimensional parallel plate flow chamber was chosen as the model blood vessel in this simulation. This model takes into account the enhancing effects of RBCs on platelet diffusivity in whole blood as well as the non-Newtonian behavior of whole blood, and uses, as an initial condition, a non-uniform platelet concentration profile across the model blood vessel. The predicted transient platelet wall fluxes were compared with experimentally-measured platelet wall fluxes obtained under identical flow conditions.

6.2 Effects of red blood cells on Platelet Transport - A Review -

Accurate mathematical description of platelet transport and reaction at the vessel wall is complicated by the presence of RBCs in blood. The RBCs affect the thrombosis
process in two ways. First, the presence of RBCs leads to greatly increased rates of transport of platelets and large molecular weight species in blood vessels (Baumgartner, 1973; Turitto and Weiss, 1980; Eckstein, 1982). Second, interactions between platelets and RBCs lead to a non-uniform radial distribution of platelets across the flow field (Beck and Eckstein, 1980; Tangelder et al., 1985). This section is a review of the latest experimental as well as theoretical findings concerning these two hemodynamical phenomena, and introduces many important concepts used in developing the model's theory.

6.2.1 Augmented Diffusion

When RBCs are present in a non-uniform velocity field, such as exists in tube or parallel plate flow, the cells rotate. This situation is illustrated in Figure 6.1, where the non-uniform velocity field is depicted in undisturbed form by the broken line (Report of the National Heart, Lung, and Blood Institute Working Group, 1985b). When the RBCs rotate, small fluid layers surrounding the cells also rotate. Because the RBCs are large (8-10 μm) compared to platelets (1-2 μm), the platelets can become entrained in the rotating fluid layer surrounding the RBCs. These local secondary flows lead to greatly augmented effective diffusion coefficients for platelets and large molecular weight species when compared to Brownian diffusion alone. Keller (1971) has developed a theoretical model which gives the effective diffusivity, $D_e$, of small solutes as:

$$D_e = D_b + D_i$$  \hspace{1cm} (6.1)
Figure 6.1: Red blood cell rotating in a uniform shear flow with a frequency $\omega$, leading to the augmented transport of small solutes present in the fluid layer $g$ adjacent to the cell surface since there is no slip there. The local non-uniform velocity profile is represented in undisturbed form by the broken line, the local shear rate being the slope of that line. (From Report of the National Heart, Lung, and Blood Institute Working Group, 1985b).
where $D_b$ is the Brownian molecular diffusion coefficient and $D_\gamma$ is the rotation-induced diffusion coefficient. In this model, he came up with the following expression for $D_\gamma$:

$$D_\gamma(y) = 0.18 \delta^2 \dot{\gamma}(y)$$  \hspace{1cm} (6.2)

where $\delta$ is the equivalent RBC radius; $\dot{\gamma}$ is the shear rate; and $y$ represents the direction perpendicular to the flow direction.

The Brownian diffusion coefficient is due primarily to random thermal motion which generates intermolecular collisions between solute and solvent molecules. The magnitude of this diffusion coefficient for large molecules or particles in an aqueous solution can be predicted from the Stokes-Einstein equation (Karino and Goldsmith, 1985):

$$D_b = \frac{BT}{6\pi \mu r}$$  \hspace{1cm} (6.3)

where $B$ is the Boltzmann's constant; $T$ is the absolute temperature; $\mu$ is the solvent viscosity; and $r$ is the solute radius. The average distance, $\Delta y$, a cell travels toward the tube wall due to Brownian motion alone in a time interval, $\Delta t$, can also be computed according to the following equation (Karino and Goldsmith, 1985):

$$\Delta y = (2\Delta t D_b)^{0.5}$$  \hspace{1cm} (6.4)

For platelets in plasma at 37°C, with an equivalent sphere diameter of 1.3 $\mu$m, a plasma
viscosity of 1.2 mPa-s, $D_b = 1.5 \times 10^{-13} \text{ m}^2 \text{ s}^{-1}$, and $\Delta t = 1 \text{ sec}$. $\Delta y$ was calculated to be 0.5 μm which is almost negligible compared to the distance traveled axially in the flow by the platelets.

In presence of RBCs, however, the radial distance traveled by platelets increases considerably as reported by Goldsmith and Marlow (1979). Using transparent ghost cell suspensions at various concentrations, they measured the fluctuations in radial position of RBCs and 2 μm latex sphere (platelet analogues) due to the presence of the ghost cells. From such plots they were able to calculate a radial dispersion coefficient, $D_r$, from the measured and calculated mean square radial distance, $\Delta r^2$, traveled by the particles in the time interval $\Delta t$:

$$D_r = \frac{\Delta r^2}{2 \Delta t}$$  \hspace{1cm} (6.5)

Data for the radial dispersion coefficients of platelets ranged from $10^{-12}$ to $1.5 \times 10^{-11}$ m$^2$ s$^{-1}$ and were from 7 to 100 times greater than the Brownian translational diffusion coefficient of a platelet in plasma (Karino and Goldsmith, 1985; Goldsmith and Marlow, 1979).

The magnitude of the platelet diffusivity has been directly measured in flowing blood, and was inferred from measurements of rates of platelet adhesion on various substrates, including the subendothelium (Turitto, 1984; Feuerstein et al., 1975). Those measurements indicated that the platelet diffusivity depends strongly on both shear rate and hematocrit. Typical values of platelet diffusivity in whole blood have been estimated to range from 3 to $86 \times 10^{-7}$ cm$^2$ s$^{-1}$ as shear rate is increased from 50 to 10,000
sec⁻¹. An even more striking increase in platelet adhesion on subendothelium is noted at high shear rates, as hematocrit is increased from 0 to 70 percent. Such increases in adhesion correspond to increases of greater than two orders of magnitude in platelet diffusivity as determined from equation (6.3).

Because equation (6.2) does not take into consideration all the parameters influencing diffusivity of platelets in flowing whole blood, including hematocrit, particle size, RBC deformability, and collision between RBCs and platelets, the augmentation effect was further investigated by several investigators, including Wang and Keller (1979 and 1985). In their studies, a convenient electrochemical method was developed to measure the augmented transport of extracellular solutes due to erythrocytes in a couette flow. They found that augmentation for suspensions of normal bovine and human erythrocytes at 10 and 40 percent hematocrit increased with shear rate and hematocrit, although the smaller bovine cells (60% of the volume of a human cell) exhibited a lower augmentation at a given shear rate and hematocrit. Augmentation was defined as following:

$$A = \left( \frac{D_e - D_s}{D_s} \right) \times 100$$  \hspace{1cm} (6.5)

where $D_e$ and $D_s$ are, respectively, the effective diffusivity in the flowing and stationary suspensions. To examine how deformability affects augmentation, erythrocytes were modified in two ways. They were either treated with gluteraldehyde to form hardened cells or mildly lysed in hypotonic phosphate buffer and resealed to form highly deformable ghost cells. Augmentation was found to increase with particle rigidity at all
shear rates. Furthermore, the augmentation in hardened cell suspensions increased at a much greater rate with increasing hematocrit than it did for normal cells and ghost cells. For normal cells at 30% hematocrit, they found that augmentation could be well-predicted on the basis of the following empirical equation:

$$A = 8.7 \text{Pe}^{0.82}$$ (6.7)

Pe is the Peclet number and is defined as $\text{Pe} = \frac{\delta^2 \gamma}{D_{sf}}$, where $\delta$ is the equivalent RBC radius, defined as the radius of a sphere having the same volume as a RBC, and $D_{sf}$ is the molecular effective diffusivity of a solute in a stationary suspension, which can be predicted from Fricke's model as follows:

$$D_{sf} = D_b \left[ \frac{(1 - \phi)}{1 + \left( \frac{\phi}{1.05} \right)} \right]$$ (6.8)

where $D_b$ is the Brownian diffusivity and $\phi$ is the volume fraction of erythrocytes in the suspending medium.

Several other studies were undertaken to find out the exact mechanism of augmentation for different flow conditions. The results obtained by Wang and Keller (1985) seem to agree well with those reported by Goldsmith and Marlow (1972 and 1979). Indeed, Wang and Keller showed that augmentation decreased with increasing $\phi$ for suspensions of normal and ghost RBCs, but increased with $\phi$ for suspensions of hardened RBCs. Those results agreed pretty well with the visual observations of tracer
erythrocyte motion in ghost suspension made by Goldsmith and Marlow. Indeed, it was reported that in dilute suspension ($\phi < 0.02$) normal RBCs rotated with periodically varying angular velocities. As $\phi$ increased above 0.2, the tracers rotated erratically because of interactions with neighbors. As $\phi$ increased above 0.3, normal erythrocytes no longer rotated as a whole but deformed into various irregular shapes with the membrane rotating about the cell interior. In addition, the paths of tracer erythrocytes exhibited erratic displacements normal to the direction of flow and increasing in the range of $\phi$ from 0.1 to 0.4. The decrease in the normalized augmentation for normal erythrocytes at $\phi > 0.2$ was probably due to the impeded particle rotation and the increasing alignment of the erythrocytes. Although the collision frequency and the erratic displacements actually increased in this concentration range, their contribution couldn't compensate for the impeded particle rotation. At $\phi = 0.1$ the specific augmentation for hardened erythrocyte suspensions was similar to those of normal and ghost RBCs, indicating that particle deformability is apparently not important at $\phi \leq 0.1$. At $\phi > 0.1$ however, the augmentation increased with increasing $\phi$, suggesting that the multiplet collisions of hardened cells contribute significantly to the augmentation and that the effects are large enough to compensate for the impeded particle rotation and increasing alignment in such highly concentrated suspensions (Wang and Keller, 1985).

From the theoretical and experimental results obtained by those two groups, important predictions have been made concerning the mechanisms of augmentation. In dilute RBC suspensions ($\phi < 0.1$), which may occur for instance in the region near the wall surface, the main mechanism of augmentation is particle rotation for suspensions of both deformable and rigid cells. However, in more concentrated solution ($\phi > 0.1$), as
may occur in the bulk of the circulation, the mechanisms of augmentation for suspensions of deformable cells are membrane rotation and cell deformation, whereas the mechanisms of augmentation for hardened cell suspensions are multiplet collisions and particle rotation.

From those conclusions one may predict that, to model transport of small solutes, such as the clotting factors released by activated platelets from a growing thrombus attached to the vessel wall, equation (6.2), which only accounts for augmentation due to RBC rotation, may be sufficient, because the concentration of RBCs near the vessel wall is small (see Chapter 5). On the other hand, in order to model platelet transport which occurs from the bulk of the fluid, where the RBC concentration is large, to the reactive wall, equation (6.7), which takes into account not only particle rotation but also membrane rotation, cell deformability, and intercellular collisions, might be more appropriate to describe enhanced platelet diffusion accurately under normal physiological conditions.

When compared to effective platelet diffusivities measured by the Taylor dispersion technique (Turitto et al., 1972) or estimated from the platelet fluxes to a tube wall (Grabowski et al., 1972), the values obtained from equation (6.7) at 100 and 200 sec\(^{-1}\) agree to better than a factor of two. Although Keller's turbulent mixing model [equation (6.2)] predicts that rotation of a rigid sphere in a shear field would lead to a linear dependence of augmentation on Pe, equation (6.7) shows that for both normal and hardened RBC suspensions, augmentation is proportional to Pe\(^{0.82}\), supporting the view that interaction of flow fields around particles and multiplet interactions in the concentrated suspensions result in a Peclet number which is less than for dilute
suspensions.

6.2.2 Non-Uniform Platelet Distribution

RBCs not only affect platelet diffusivity in flowing blood but also platelet distribution normal to the flow direction. Indeed, when blood flows through a tubular or a parallel plate flow chamber, platelets tend to concentrate very rapidly in the region near the vessel wall. Eckstein's group (Tilles and Eckstein, 1987; Eckstein et al., 1987) and Sixma's group (Aarts et al., 1989) have been the primary investigators in this particular area.

Eckstein used methods involving epifluorescence video microscopy to obtain concentration profiles of platelet-sized beads during flow through glass channels 50 or 100 μm wide. In one study (Tilles and Eckstein, 1987), his group showed that a near-wall excess of beads occurred when the suspension contained a significant fraction of RBCs (>7%). For hematocrit of 15 to 45%, the excess was about 5 times the concentration in the core region at 1630 sec\(^{-1}\). Furthermore, the region of excess beads was 5 to 8 μm thick. At 15% hematocrit, they also observed that near-wall excesses were large only for wall shear rates of 430 sec\(^{-1}\) and above. Clearly then, the near-wall excess of platelet-sized particles is a function of both hematocrit and wall shear rate.

In another study (Eckstein et al., 1987), they used a freeze-capture method together with the same video microscopy equipment to analyze platelet distribution in a 200 μm-wide capillary tube over a wide range of hematocrit (0 to 60%) at 500 sec\(^{-1}\). Their freeze-capture method allowed them to stop blood flow instantaneously by
submerging the tube in liquid N$_2$ and then to determine the particle distribution from the digitized images of the video micrographs. They found that, for hematocrit from 7 to 60%, the region of each distribution ranging from 5 to 35 $\mu$m from the wall, could be fit with a decaying linear exponential whose constant varies between 0.02 and 0.04 $\mu$m$^{-1}$. The exponential distribution is consistent with a rheological model in which platelet motions are the result of two distinct radial fluxes. One flux has the nature of a convective velocity ($v_y$) and is directed toward the wall; the other is a conventional Fickian flux that describes the movement of particles down a concentration gradient and uses an effective diffusion coefficient, $D_e$. At steady-state, when platelet distribution does not change with time across the tube, the radial convection of particle is balanced by diffusion along a concentration gradient such as:

$$v_y C_{ph} = -D_e \left( \frac{dC_{ph}}{dy} \right)$$  \hspace{1cm} (6.9)$$

where $v_y$ is an average lateral velocity; $C_{ph}$ is the local platelet concentration; $D_e$ is an effective diffusion coefficient; and $y$ is the distance from the wall. Solution of this equation provides a concentration profile of the form $C_0 \exp{-v_y y/D_e}$, where $C_0$ is a constant of integration.

Eckstein also noticed that there were two other regions of particular interest: the central region of the flow, where the slope of the distribution is small or zero, and the peak of the distribution, located near the wall. There is no lateral convective motion in the center of the tube, because symmetry implies the lack of a preferred direction. The
net lateral convective motion near the wall is also zero since there is a wall-induced force acting on each particle which counter-balances the convective force which moves platelets toward the wall. The decreased concentration of platelets near the wall (between 0 and 5 μm) is an illustration of this balance.

Eckstein's group (Eckstein and Tilles, 1989) also studied the occurrence of the near-wall excess of platelet analogues for additional parameters, namely: bead size, channel height, suspending fluid viscosity, and RBC deformability. Their experiments revealed that beads with diameter of 1 μm or less have little or no near-wall excess, while all other sizes (2.0 to 5.2 μm) exhibited a significant near-wall excess at 1630 sec\(^{-1}\) and 15% hematocrit in a 50 μm-wide channel. Their data also showed that, using a suspending fluid with 3.4 times the viscosity of the normal fluid, the near-wall excess was large at the wall shear rate of 210 sec\(^{-1}\), a value of wall shear rate at which only small near-wall excess was seen with the usual suspending fluid. Experiments with RBCs hardened with gluteraldehyde showed only small near-wall excess compared to those where normal RBCs were used, indicating that large near-wall excess is strongly dependent upon RBC deformability. Finally, using channels of either 30, 50, or 100 μm in diameter, and beads of 2.5 μm in diameter, large near-wall excesses were observed in all three cases.

Sixma's group (Aarts et al., 1989) investigated platelet and RBC distributions in flowing blood through a 3 mm-wide tube for various hematocrits and shear rates using the laser-Doppler technique. Qualitatively their results were very similar to those found by Eckstein's group. Indeed, their data showed that RBC ghosts are crowded near the axis of the tube, with a local hematocrit elevated above the bulk hematocrit, and
decreasing steadily toward the wall. In the absence of ghost, platelets exhibited the "tubular pinch effect", typical of rigid particles (maximum at 0.6x tube radius and zero at the wall). In the presence of ghosts however, the platelets were expelled toward the wall region. This high concentration at the wall increased with increasing bulk hematocrit and wall shear rate. On the other hand, they also showed that the actual wall concentration of platelets did not demonstrate a linear increase with bulk concentration. Increasing the platelet bulk concentration 10 times only caused the wall concentration to increase 3 times.

6.2.3 Random Walk Model

The wall-directed convective flux [left hand side of equation (6.9)], is primarily caused by collisions between platelets and RBCs. In fact, a mathematical model showing that biased collisions of a random walk result in a directed flux is available (Chandrasekhar, 1954). Eckstein and coworkers have tried to explain most of their experimental results in terms of this biased random walk as a model for platelet transport in flowing whole blood. The concepts behind this random walk theory and the reasons for which it can be applied to platelet transport are the subjects of the following discussion.

Goldsmith (Karino and Goldsmith, 1985) was the first one to apply random walk theory to platelet transport. He showed experimentally that lateral motion of individual particles depends upon the particle Reynolds number, the deformability of the particle, and the volume fraction of particles in the suspension. More specifically, rigid particles interacting in dilute suspensions show little or no net motion across streamline, while
deformable particles in dilute suspensions are associated with directed lateral motion. In contrast, both normal RBCs and latex bead platelet analogues show significant lateral diffusive motion in concentrated ghost cell suspensions. Since a single equation governs both simple random walk and the diffusion process [see equation (6.5)], they were able to calculate the effective lateral diffusion coefficient.

This model is reasonable for collisions among particles of equal sizes and assumes that one step is independent of the previous one and that there is no preferred outcome of the collisions. Although RBCs and platelets differ in size and other properties, this model seems to adequately describe platelet transport in blood when the collisions between RBCs and platelets occur in flows in low shear rates. Since in most physiological situations the shear rates are large, Eckstein's group has considered a more complex theory of random walks (Eckstein et al., 1987). They considered walks for which the probability of outcome is not equal in all directions. Indeed, it seems reasonable to expect that the probabilities describing the lateral displacement of a rigid body, such as a platelet, after collision with a body of different rheological character, such as a deformable RBC, may be unequal. The larger and more flexible RBCs would have a greater tendency to be displaced toward the center, while the smaller, more rigid platelets would tend to be displaced toward the wall. In addition, the non-uniform distribution of RBCs in the near-wall region, causing an environmental anisotropy there, may be another source of bias on platelet transport. The theory for random walk with persistence and external bias was developed by Patlack (1953). The major effect of using random walks biased to account for non-uniform interactions among platelets and RBCs, is that lateral platelet motion is no longer purely diffusive, but includes a
convective term as shown in the following equation for the diffusion constant, D:

\[ D = \left( \frac{1}{2} \right) \frac{\lambda_0}{(1/c)} \tag{6.10} \]

where \( \lambda_0 \) is the "average" distance the particle will travel independently of direction and \( c \) is the average speed of the particle between collisions, both variables being functions of position and time.

Although this equation, which is only a simplification of the original, more complex expression derived by Patlack (1953), is not applicable as such in platelet transport models because of the lack of quantitative information on \( \lambda_0 \) and \( c \), the random walk concepts are useful tools to predict platelet distribution during steady-state blood flow in tubes. Indeed, in the central part of the tube, where collisions between RBCs and platelets are unbiased, the local distribution of platelets will be uniform, which is what is observed experimentally. On the other hand, in the near-wall region, where collisions are biased, the random walk theory predicts a convective-type of platelet motion which tries to build an excess concentration near the wall. That excess is limited, however, by conventional Fickian diffusion which always acts to reduce concentration gradients.

6.3 Theory

Consider whole blood with an hematocrit of 30% entering a parallel-plate flow chamber, 200 \( \mu \)m-thick and 5 mm-long, as a non-Newtonian fluid at a wall shear rate of 500 sec\(^{-1}\). The bottom plate of the flow chamber is a thrombogenic surface on which
platelets react immediately upon contact, while the top plate is an inert surface. The geometry of this model blood vessel is shown in Figure 6.2.

The non-Newtonian behavior of blood is described accurately by the Casson equation (Cokelet, 1987; Self et al., 1976):

\[ \frac{\tau_{yx}}{\tau_y} = \tau_{y}^{1/2} + (\eta \gamma)^{1/2} \quad \text{for} \quad \tau_{yx} > \tau_y \]  

and

\[ \dot{\gamma} = -\frac{dv_x}{dy} = 0 \quad \text{for} \quad \tau_{yx} < \tau_y \]  

where \( \tau_{yx} \) is the shear stress; \( \tau_y \) is the yield stress; \( \eta \) is the fluid viscosity; \( \dot{\gamma} \) is the shear rate; and \( y \) is now the distance from the centerline.

Equation (11) can be solved analytically for the velocity profile across the flow chamber using the following boundary conditions:

at \( y = 0 \) \quad \tau_{yx} = 0 \n
at \( y = \pm B_y \) \quad \tau_{yx} = \tau_y \n
at \( y = \pm B \) \quad v_x = 0 \n
The resulting velocity profile, also shown graphically in Figure 6.3, is given by:
Figure 6.2: Geometry of the model blood vessel used in the computer simulation. Blood flows from left to right with platelets reacting on the bottom plate. The fluid enters as a non-Newtonian fluid with a slightly blunt velocity profile.
Figure 6.3: Velocity profile across the model blood vessel.
\[ v_x(y) = \left[ \frac{\Delta P}{L} \right] \left[ \frac{B^2}{2 \eta} \right] \left[ 1 - \left( \frac{\gamma}{B} \right)^2 \right] - \left( \frac{4 \eta B}{3 \gamma} \right) \left( \frac{\Delta P \tau_y B}{L} \right)^{0.5} \left[ 1 - \left( \frac{\gamma}{B} \right)^{1.5} \right] + \left( \frac{\tau_y B}{\eta} \right) \left[ 1 - \left( \frac{\gamma}{B} \right) \right] \]

for \( |y| > B_y \)

and

\[ v_{\text{MAX}} = v_x(B_y) \quad \text{for} \quad |y| \leq B_y \]  \hspace{1cm} (6.12)

where \( \Delta P/L \) is the pressure drop per unit length and \( B \) is half the width of the flow chamber. The shear rate can be computed from equation (6.12) and is given by:

\[ \dot{\gamma}(y) = - \frac{dv_x}{dy} = \left[ \frac{1}{\eta} \right] \left[ \frac{\Delta P y}{L} - 2 \left( \frac{\Delta P y \tau_y}{L} \right)^{0.5} \right] \]  \hspace{1cm} (6.13)

Given the steady-state platelet concentration profile across the channel, the following model predicts the platelet fluxes at the reactive wall in the situation where platelets are induced to react on the bottom plate of the flow chamber. As will be shown, the platelet wall flux varies with the position along the reactive wall. The two dimensional transient advection-diffusion equation describing that physical situation is given by:

\[ \frac{\partial C_{\text{pt}}}{\partial t} + v_x(y) \frac{\partial C_{\text{pt}}}{\partial x} + v_y(y) \frac{\partial C_{\text{pt}}}{\partial y} = \frac{\partial}{\partial y} \left( D_{\text{pt}}(y) \frac{\partial C_{\text{pt}}}{\partial y} \right) + D_{\text{pt}}(y) \frac{\partial^2 C_{\text{pt}}}{\partial y^2} \]  \hspace{1cm} (6.14)
where $C_{plt}(x,y,t)$ is the platelet concentration; $D_{plt}$ is the platelet effective diffusion coefficient; $v_x(y)$ is the axial velocity given by equation (6.12); and $v_y(y)$ is an effective lateral velocity, which developed due to the RBCs present in the medium, as was explained earlier. It should be pointed out that the flow disturbances introduced at the reactive wall by the growing thrombi are not taken into account in this model. This is a valid assumption since the size of those thrombi are relatively small compared to the width, $2B$, of the flow chamber. That equation was solved numerically using a finite element method described in detail in Appendix D, with the following boundary and initial conditions:

at $t = 0$ and $x = x_0$ for $0 \leq |y(\mu m)| \leq 65$  
$C_{plt} = C_{BLK}$

for $65 < |y(\mu m)| \leq 95$  
$C_{plt} = K \left[ \exp \left( 0.04 \times 10^4 |y(y)| \right) \right]$ 

for $95 < |y(\mu m)| \leq 100$  
$C_{plt} = M |y| + H$

at $x = x_e$  
$\frac{\partial C_{plt}}{\partial x} = 0$

at $y = +B$  
$C_{plt} = C_{WALL}$ (non-reactive wall)

at $y = -B$  
$C_{plt} = 0$ (reactive wall)

The initial or steady-state platelet concentration profile was obtained from recent experimental measurements performed by Eckstein's group (Eckstein et al., 1987). As
mentioned earlier, they used a freeze-capture method together with a video microscopy equipment to analyze platelet distribution in a 200 μm capillary tube over a wide range of hematocrit at a wall shear rate of 500 sec\(^{-1}\). For an hematocrit of 30%, they found that the steady-state platelet distribution across their model blood vessel could be fit by (1), a straight line in the region within 5 μm from the wall, (2), a decaying linear exponential in the region from 5 to 35 μm from the wall, and (3), a constant in the center of the tube (35 to 100 μm from the wall). The same initial platelet distribution was used in this model, as shown in Figure 6.4. The constants of integration in those initial conditions, namely K, M, and H, were estimated from a platelet mass balance on a cross-section of the flow chamber, which is given as:

\[
C_{\text{AVG}} = \left( \frac{130}{200} \right) C_{\text{BLK}} + \left( \frac{2}{200} \right) \int_{65}^{95} K \exp(0.04y) \, dy + \left( \frac{2}{200} \right) \int_{95}^{100} (My + H) \, dy \quad (6.15)
\]

where \(C_{\text{AVG}}\), the average platelet concentration in stationary whole blood (30% Ht), was measured in this study with an electronic particle counter for a random healthy male donor A. The constants of integration K, M, and H were determined by solving equation (6.15) with the following two relationships:

at \(y = 95 \mu m\) \hspace{1cm} K \exp(0.04y) = My + H

and

at \(y = 65 \mu m\) \hspace{1cm} K \exp(0.04y) = C_{\text{BLK}}
Figure 6.4: Initial platelet concentration profile across the model blood vessel.
The effective lateral velocity in equation (6.14), \( v_y \), was estimated from Eckstein's steady-state model given by equation (6.9). Furthermore, it was assumed here that a convective motion not only exists in the region extending 5 to 35 \( \mu \)m from the wall as originally proposed by Eckstein, but also in the region within 5 \( \mu \)m from the reactive wall. Although, at steady-state, the vessel wall is repulsive towards platelets, in this model, the thrombogenic wall has an attractive effect on platelets that may react there. Therefore, it is safe to postulate that there might be a net convective flux of platelets in that region. In other words, the thrombogenic surface introduces an additional external bias on platelet motion. The solution of equation (6.9) is a decaying linear exponential whose constant equal \( v_y/D_{\text{plt}} \). For an hematocrit of 30\% and a wall shear rate of 500 sec\(^{-1}\), that constant was reported to be 0.04 \( \mu \)m\(^{-1}\) (Eckstein et al., 1987). Knowing \( D_{\text{plt}} \), one could then calculate \( v_y \). Five different approaches were used to estimate \( D_{\text{plt}} \).

First, a constant average effective platelet diffusion coefficient of 10\(^{-7}\) cm\(^2\)/sec (model #1) (see Eckstein et al., 1987) or 10\(^{-8}\) cm\(^2\)/sec (model #5) was used, and \( v_y \) was calculated to be a constant equal to 0.4 \( \mu \)m/sec and 0.04 \( \mu \)m/sec, respectively. Second, \( D_{\text{plt}} \) was estimated from Keller's mixing model [see equations (6.1) and (6.2)], using equation (6.13) to estimate the shear rate. In this case, \( v_y \) varied with the distance from the wall, ranging from 27.0 \( \mu \)m/sec near the wall to 17.0 \( \mu \)m/sec 35 \( \mu \)m away from the wall (model #2). Third, using Keller and Wang's semi-empirical model given by equations (6.6) and (6.7), and equation (6.8) as an approximation for \( D_s \), \( D_{\text{plt}}(y) \) was estimated from:
Again, \( v_y \) varied with the distance from the wall, ranging here from 192.0 \( \mu \text{m/sec} \) near the wall to 133.0 \( \mu \text{m/sec} \) 35 \( \mu \text{m} \) away from the wall (model #3). Last, a combination of the last two models were used to estimate \( D_{\text{plt}} \) (model #4). Near the wall (0 to 5 \( \mu \text{m} \)), where the RBC concentration is low, Keller's mixing model was used to estimate \( v_y \), while closer to the bulk (from 5 to 35 \( \mu \text{m} \)), Keller and Wang's semi-empirical model was used.

Two different approaches were used to estimate the effective platelet diffusion coefficient, \( D_{\text{plt}} \), in equation (6.14). The first approach consisted in estimating a constant average effective diffusion coefficient, \( \langle D_{\text{plt}} \rangle \), using the diffusivity models given by equations (6.1) and (6.2), or (6.16), and calculated on the basis of an average shear rate across the model blood vessel, \( \langle \dot{\gamma} \rangle \), given by:

\[
\langle \dot{\gamma} \rangle = \frac{1}{B} \int_{0}^{B} \dot{\gamma}(y) \, dy
\]

\[
= \left( \frac{\Delta P}{L} \right) \left( \frac{B}{2\eta} \right) - \left( \frac{\Delta P B \tau_{y}}{L} \right) \left( \frac{2}{1.5\eta} \right) + \left( \frac{\tau_{y}}{\eta} \right)
\]

(6.17)

where \( \dot{\gamma}(y) \) is given by equation (6.13). In the second approach, the medium was considered anisotropic with \( D_{\text{plt}} \) varying with the local shear rate, and therefore with the \( y \)-direction according to equations (6.2) or (6.16).
**Table 6.1: Physical Properties and Model Parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield Stress $^*$, $\tau_y$</td>
<td>0.045 dynes/cm$^2$</td>
</tr>
<tr>
<td>Average Hematocrit</td>
<td>30 %</td>
</tr>
<tr>
<td>Wall Shear Rate, $\dot{\gamma}_w$</td>
<td>500 sec$^{-1}$</td>
</tr>
<tr>
<td>Average Wall Shear Rate, $&lt;\dot{\gamma}&gt;$</td>
<td>242.2 sec$^{-1}$</td>
</tr>
<tr>
<td>Average Viscosity, $\eta$</td>
<td>0.038 poise</td>
</tr>
<tr>
<td>Brownian Diffusivity $^\dagger$, $D_B$</td>
<td>$1.6 \times 10^{-9}$ cm$^2$/sec</td>
</tr>
<tr>
<td>$C_{\text{BULK}}$</td>
<td>$153.85 \times 10^6$ platelets/cm$^3$</td>
</tr>
<tr>
<td>$C_{\text{AVERAGE}}$</td>
<td>$198.23 \times 10^6$ platelets/cm$^3$</td>
</tr>
<tr>
<td>$C_{\text{WALL}}$</td>
<td>$151.02 \times 10^6$ platelets/cm$^3$</td>
</tr>
<tr>
<td>Integration Constants:</td>
<td></td>
</tr>
<tr>
<td>$K$</td>
<td>$11.43 \times 10^6$</td>
</tr>
<tr>
<td>$M$</td>
<td>$-719.5 \times 10^9$ platelets/cm$^4$</td>
</tr>
<tr>
<td>$H$</td>
<td>$7.35 \times 10^6$ platelets/cm$^3$</td>
</tr>
<tr>
<td>$\Delta P / L$</td>
<td>$2.09 \times 10^3$ dynes/cm$^3$</td>
</tr>
<tr>
<td>$B_y$</td>
<td>0.2154 $\mu$m</td>
</tr>
</tbody>
</table>

$^*$ obtained from Self et al. (1976).
$^\dagger$ calculated from equation (6.3).
Figure 6.5: Average platelet surface density as a function of axial position along a glass cover slip coated with type I human collagen fibrils.
All physical properties and model parameters are summarized in Table 6.1.

6.4 Experimentally-Measured Platelet Wall Fluxes

The platelet wall fluxes were also measured experimentally in this study. For that purpose, whole blood from the same donor A, reconstituted to an hematocrit of 30%, was perfused in a parallel plate flow chamber, 200 μm-thick, for 2 minutes at a wall shear rate of 500 sec⁻¹. Prior to perfusion, the bottom plate of the flow chamber was coated with type I human collagen fibrils, a predominant component of the subendothelium of injured blood vessels and a very thrombogenic protein. The coating technique was detailed in Chapters 4 and 5. The accumulation of fluorescent-labelled platelets on the coated plate was monitored, real-time, using an EPI-fluorescent video microscopy equipment, and the end-point accumulation after 2 minutes of flow recorded continuously as a function of axial position along the coated plate by a computerized and motorized microphotometric measurement system. This equipment was described in depth in Chapter 2. The integrated average platelet density, fitted with a power function, is shown in Figure 6.5 as a function of axial position along the coated surface. The platelet flux at the wall was estimated from those data by assuming a linear rate of platelet accumulation.

6.5 Results and Discussion

The platelet wall fluxes resulting from the different simulations are shown in
Figures 6.6 to 6.10 as a function of axial position along the reactive surface. All the simulated wall fluxes shown were calculated after 120 seconds of flow. Figure 6.6 illustrates the differences between the platelet wall fluxes resulting from the different estimations of the effective lateral velocity, $v_y$, in the case where $D_{pt}$ in equation (6.14) is equal to the constant platelet Brownian diffusion coefficient, $D_B$. Also shown in that figure is the curve for the power function fit of the measured fluxes. Clearly, model #1, which assumes a constant average lateral velocity of 0.4 $\mu$m/sec, leads to a significant underestimation of the platelet wall flux at any axial position, when compared to the measured fluxes. In addition, whereas the measured flux decreases with axial position, the curve for model #1 shows an increase of platelet wall flux with axial position. The experimental data suggest that the location of maximum platelet aggregation is at and near the inlet and that the process of mural thrombogenesis becomes slowly diffusion-limited downstream, as noticed before (Folie and McIntire, 1988; Hubbell, 1986). Clearly, the effective lateral velocity in model #1 is not large enough to totally account for the enhanced platelet transport in whole blood due to the presence of RBCs. In this particular case, the thrombogenesis process does not become diffusion-limited because of the relatively slow diffusion and convection of platelets towards the reactive wall. The curve for model #2 shown in Figure 6.6, which uses Keller's mixing model to account for the enhanced platelet transport, is closer to the experimental data. Furthermore, this model predicts a decrease in platelet wall flux with increasing axial position, in agreement with the experimental data. The platelet wall fluxes are still underestimated at practically all axial positions, however. Although, in this case, the effective lateral velocity averages around 22 $\mu$m/sec, which is considerably larger than
Figure 6.6: Platelet wall fluxes resulting from the different estimations of the lateral velocity term, $v_y$, vs. axial position along the reactive surface. In all the models, $D_{plt}$ was equal to the Brownian diffusivity, $D_B$. The curve-fitted experimental data are also shown for comparison.
the 0.4 µm/sec proposed by Eckstein and coworkers (1987), the results of this simulation suggest that it is still not sufficient to accurately model platelet transport to the reactive surface. Model #3 utilizes Keller and Wang's semi-empirical model to calculate $D_{pt}$ and estimate $v_y$. The platelet wall flux predicted by this model, in platelets/cm²-sec, drops from about 35,000 at the inlet to a minimum of about 7,000 half way downstream, and then, increases to a maximum of around 60,000 at the outlet. Although a platelet surface density curve with a similar type of shape was observed experimentally at higher hematocrits and with a different collagen type (Folie and McIntire, 1989b), this result demonstrates that even a significant increase in the lateral convective term - to an average value of 163 µm/sec - will not lead to platelet wall fluxes similar to the experimentally-measured ones. The following physical argument is a possible explanation for the shape of that curve. After all the platelets contained in the fluid layer adjacent to the reactive surface have quickly reacted on the surface at the inlet, this layer becomes depleted of platelets and is only slowly replenished with platelets due to slow platelet diffusion and decreasing lateral convection as one moves away from the wall. By the time the platelets contained in the next fluid layer, adjacent to the first one, have reached the surface, those platelets have been carried further downstream by the large axial convective forces. The peak at the inlet is due to the fact that there is a continuous arrival of fresh non-reacted platelets in the layer adjacent to the surface. Similarly, the platelet wall flux curve generated from model #4, which uses Keller’s mixing model in the region within 5 µm from the wall and Wang and Keller’s semi-empirical model in the region extending from 5 to 35 µm from the wall to predict $v_y$, does not match at all the experimental data, as shown again
in Figure 6.6. In fact, near the inlet, that curve is very similar to the curve generated from model #3, increasing at a much slower rate near the outlet, however. The results presented in Figure 6.6 clearly show that modeling the enhanced platelet transport in flowing whole blood using an effective lateral velocity only is not sufficient and/or appropriate to explain the platelet wall fluxes observed experimentally in a parallel plate flow chamber. As noted by Eckstein and coworkers (1987), it is likely that a lateral convective term is needed to initially form and maintain the non-uniform platelet concentration profile shown in Figure 6.4 in the case of non-reactive walls. In this context, the average effective lateral velocity of 0.4 μm/sec proposed by Eckstein might be sufficient to maintain the non-uniform concentration profile at steady-state, but probably underestimates the initial forces needed to establish that profile. Although enhanced transport due to interparticle collisions is probably the main mechanism generating that non-uniform platelet concentration profile across blood vessels, it is unlikely to be the prevailing mechanism leading to enhanced platelet transport to a reactive wall, chiefly because the hematocrit near the wall is considerably reduced compared to its bulk value. In this context, the physical forces, diffusive and convective, involved in the augmented cellular transport in flowing whole blood could be arbitrarily divided into three categories: those needed to establish the non-uniform platelet concentration profile, leading to an elevated platelet concentration near the wall compared to the bulk concentration; those needed to maintain that profile at steady-state, that is in the case of non-reactive walls; and those carrying platelets to the surface in the case of reactive walls.

Figure 6.7 shows the platelet wall fluxes predicted from the different models in the
Figure 6.7: Platelet wall fluxes resulting from the different estimations of the lateral velocity term, $v_y$, and the average effective platelet diffusivity, $\langle D_{plt} \rangle$, vs. axial position along the reactive surface. The curve-fitted experimental data are also shown for comparison.
case where $D_{plt}$ in equation (6.14) is set equal to a constant average effective platelet diffusivity, $<D_{plt}>$. As mentioned earlier, that average coefficient was estimated using an average shear rate across the model vessel ($<\gamma> = 242.2 \text{ sec}^{-1}$), and the same diffusivity model as the one used to estimate $v_y$. The platelet wall fluxes predicted from model #1, for which $<D_{plt}>$ is $1.0\times10^{-7} \text{ cm}^2/\text{sec}$, agree reasonably well with the measured fluxes. Indeed, the platelet wall flux curve for that model decreases with increasing axial position and slightly overestimates the measured fluxes at all axial positions. This is what should be expected since the model assumes that all platelets contacting the surface immediately react there. Therefore, the results predicted by this model should represent upper-bounds on those observed experimentally, simply because some platelets may detach from the surface under the effect of drag forces or bounce back from the surface if not sufficiently activated to bind to the collagen fibrils. As shown in Figure 6.7, model #2 (Keller’s mixing model) and model #3 (Keller and Wang’s semi-empirical model) largely overestimate the platelet wall fluxes compared to the measured fluxes. $<D_{plt}>$ was calculated to be $3.3\times10^{-6}$ and $26.6\times10^{-6} \text{ cm}^2/\text{sec}$, respectively, for those two models. Clearly then, the platelet wall flux predicted by the model is highly dependent upon the magnitude of the effective platelet diffusion coefficient. A possible explanation for that is that the initial platelet concentration increases as one moves away from the wall, up to 5 $\mu$m from the wall, where it reaches a maximum. According to Fick’s law, in the situation where platelets are induced to react at the wall, there will be a diffusive platelet flux trying to counterbalance this platelet
concentration gradient and, in this case, it will be directed towards the wall. This wall-directed platelet diffusive flux will increase, of course, with increasing average platelet diffusivity, \( <D_{\text{plt}}> \), resulting in increasing platelet wall fluxes. Model #5, which uses a constant average effective diffusion coefficient of \( 10^{-8} \) cm\(^2\)/sec, leads to an underestimation of the platelet wall flux at all axial positions.

Figure 6.8 shows the platelet wall fluxes resulting from model #1 in the presence and the absence of the effective lateral velocity, \( v_y \). By comparing those two curves, it is clear that the lateral convective term is only of minor importance compared to the diffusive term in modeling platelet transport to a reactive surface in flowing whole blood.

Figure 6.9 shows the platelet wall fluxes resulting from model #2 and #3 in the case where the medium is considered anisotropic, meaning that \( D_{\text{plt}} \) in equation (6.14) varies with the distance from the wall, \( y \). Again, those two models overestimate by one or two orders of magnitude the measured fluxes. Since it is unlikely that that many platelets detach from the surface during such a short perfusion time (2 minutes), the diffusion coefficients must be overestimated. Indeed, Wang and Keller's semi-empirical model given by equation (6.7) was derived for a bulk hematocrit of 30%, which is certainly much larger than the local hematocrit near the wall. At such high hematocrit, they mention that the mechanisms of augmentation for suspensions of deformable cells are membrane rotation and cell deformation, which are very unlikely to occur near the wall where the local RBC concentration is so low. Similarly, Keller's theoretical model given by equations (6.1) and (6.2) assume that particle rotation is the main mechanism
Figure 6.8: Platelet wall fluxes resulting from model #1, using a constant average effective platelet diffusivity of $10^{-7}$ cm$^2$/sec and, with or without the lateral velocity term, $v_y$. 
Figure 6.9: Platelet wall fluxes resulting from different estimations of the lateral velocity term, $v_y$, and the effective platelet diffusivity, $D_{plt}(y)$, vs. axial position along the reactive surface. The medium was considered to be anisotropic in this case, with $D_{plt}$ varying with the distance from the wall, $y$. The curve-fitted experimental data are also shown for comparison.
of augmentation. However, that model assumes that the cells are free to rotate and that enough RBCs are present. Those two conditions may only be partially met in the region near the reactive wall, where RBC rotation may be impeded due to contacts with the wall surface and where the local hematocrit may be too low for that mechanism to be significant. In fact, it should be noted that the effective platelet diffusion coefficient, $D_{plt}$, is not only a function of the local shear rate but also a function of the local hematocrit inside the blood vessels. This latter dependence was not taken into account in the model because of the lack of information concerning the hematocrit profile across the model blood vessel, the relationship between hematocrit and $D_{plt}$, and the relationship between hematocrit and initial platelet concentration profile.

Similarly to Figure 6.8 for model #1, Figure 6.10 illustrates the fact that, including the lateral convective term, $v_y$, in models #2 and #3, does not significantly increase platelet transport to the surface, compared to the role played by the effective diffusion coefficient.

In conclusion, it can be said that it is model #1, which uses a constant average platelet diffusivity of $1.0 \times 10^{-7}$ cm$^2$/sec, about two orders of magnitude larger than the Brownian diffusion coefficient as predicted by the Stokes-Einstein equation ($1.6 \times 10^{-9}$ cm$^2$/sec), that fits the most accurately the experimental data. In addition, it is likely that enhanced platelet transport to the surface occurs through an "impeded" particle rotation mechanism, depending upon the local hematocrit near the wall and the distance separating the reactive surface and the location of maximum platelet concentration at steady-state.
Figure 6.10: Effect of the lateral velocity term, $v_y$, on platelet transport to a reactive surface. The same comments as those of Figure 6.9 apply.
CHAPTER 7

SUGGESTIONS FOR FUTURE WORK

The study of Chapter 3 with the antithrombotic agents could be extended to investigate their efficacy in blocking platelet deposition on various biomaterials and human collagens. In addition, the effect of wall shear rate on their inhibitory capacity could be studied as well. Regarding ATA, further studies could be pursue to determine with more certitude its mechanism of action. In particular, experiments confirming that ATA does not bind to collagen fibrils or to other plasma proteins involved in the adhesion and aggregation processes, such as fibrinogen or fibronectin, should be done.

More work could be done with von Willebrand Factor. Specifically, the role played by platelet-released vWF in the thrombogenesis process could be further investigated at different wall shear rates by replacing von Willebrand's disease (vWD) platelets with normal platelets in physiologic concentrations. Also, the difference in the ability of the unusually large vWF multimers secreted by endothelial cells (ECs) and the largest plasma forms to augment platelet adhesion and aggregation on collagen could be examined more thoroughly by using vWD whole blood and collagen-coated surfaces preincubated with the purified vWF forms of interest. In this context, the role played by those unusually large vWF multimers secreted by ECs in the extracellular matrix could also be examined in a more realistic model of the vascular system. An endothelial cell
monolayer could be grown on a collagen-coated glass substrate and the cells lysed, exposing the extracellular matrix containing those unusually large vWF multimers.

The roles of other proteins such as fibrinogen and fibronectin could also be investigated by using fibrinogen or fibronectin-free plasma and/or platelets lacking those substances. With respect to the study on the relative thrombogenicity of different human collagen subtypes presented in Chapter 4, one might want to investigate in more detail the structure-function relationship of those various collagen types using electron microscopy techniques, their rate of fibrillogenesis, and the optimal experimental conditions that would favor potent fibril formation in each case.

The model of Chapter 5 could be extended to a transient model with moving boundary conditions since the size and rate of growth of a thrombus can be measured experimentally with the image processor at discrete times. Also, more realistic model thrombus shapes or contours could be used. Knowing the forces acting on the thrombus surface, the stresses and strains developing inside the thrombus could also be estimated numerically, in order to determine the locations of eventual fractures, possibly leading to embolization of part of the thrombus. Also, the platelet-activating agents' wall fluxes used in this model could be estimated experimentally with other surfaces, such as biomaterials, a subendothelial matrix, or yet other types of human collagen.

In order to develop a more sophisticated model than the one presented in Chapter 6 one would need additional data on platelet concentration across the model blood vessel vs. wall shear rate, bulk hematocrit, and vessel diameter. In this context, one might want to consider using the laser-Doppler technique with circular tubes of various dimensions. In addition, as mentioned in Chapter 6, one might
also want to develop three separate transport models: one that would simulate the forces needed to establish the non-uniform platelet concentration profile, leading to an elevated platelet concentration near the wall compared to the bulk concentration, a second model that would simulate the forces necessary to maintain that profile at steady-state, and a last model that would account for the forces carrying platelets to the surface in the case of reactive walls.
REFERENCES


Hyman, M.A. 1972. Shear flow over a protrusion from a plane wall. J. Biomechanics. 5: 45.


Moake, J.L., N.A. Turner, N.A. Stathopoulos, L.H. Nolasco, and J.D. Hellums. 1988. Shear-induced platelet aggregation can be mediated by vWF released from platelets, as well as by exogenous large or unusually large vWF multimers, requires adenosine diphosphate, and is resistant to aspirin. Blood. 71:1366.


inhibitory target sites assumed to involve membrane phospholipids. Biophys. J. 37:130.


White, J.G. 1984. The ultrastructure and regulatory mechanisms of blood platelets. *In*


APPENDIX A

MACROSCOPIC DATA ANALYSIS: BIOCHEMICAL ASSAYS AND COMPUTER PROGRAMS

A lactate dehydrogenase (LDH) assay and an hemoglobin assay were used to determine the total number of platelets accumulated on the reactive surface during the perfusion period, as mentioned in Chapter 2. Those two assays are detailed in some depth below.

**Cell Count.** The concentrations of platelets and RBCs in the control samples were determined using a high speed electronic particle counter (Coulter Electronics, Inc., Hialeah, FL; Coulter Counter Model Z) coupled with a multi-channel particle size analyzer (Coulter Electronics, Inc.; Coulter Channelyser II). First, PRP was obtained from whole blood by centrifugation at 400 g for 15 minutes. PRP was then separated from the RBCs and a fraction of it placed in a microcentrifuge for 3 minutes in order to obtain PPP for later use. 3.33 μl of PRP were pipetted in 10 ml of ISOTON II and the cell suspension was gently mixed by inversion. The platelet count was then determined with the 50 μ aperture size. Similarly, 20 μl of RBCs were added to 10 ml of ISOTON II, and 100 μl of that suspension further dissolved in 10 more ml of ISOTON II. The RBC count was done using the 100 μ aperture size. In order to account for the presence of white blood cells (WBCs) in that count, a WBC suspension was prepared by first mixing
20 µl of RBCs in 10 ml of ISOTON II, and then adding 3 drops of ZAP-OGLOBIN II, an erythrocytic agent. The WBC count was subtracted from the RBC count in order to obtain the true RBC count. All counts were corrected using coincidence correction charts provided with the equipment.

*LDH Assay.* After lysing the cells by sonication, all experimental and control samples were assayed for the cytoplasmic enzyme lactate dehydrogenase spectrophotometrically (Wroblewski and LaDue, 1955). LDH catalyzes the reduction of pyruvate to L-lactate with concomitant oxidation of nicotinamide adenine dinucleotide from NADH to NAD⁺:

\[
\text{LDH} \\
\text{Pyruvate} + \text{NADH} \rightarrow \text{L-lactate} + \text{NAD}^+ 
\]

One of the reactant NADH has a strong absorbance peak at 340 nm, while its product NAD⁺ does not. Therefore, since the oxidation of NADH is directly proportional to the reduction of pyruvate by LDH in equimolar amounts, the LDH activity in any sample can be estimated by measuring the rate of decrease in absorbance at 340 nm.

A UV-VIS microprocessor-controlled spectrophotometer System 2600 (Gilford Diagnostics, Oberlin, OH) was programmed to read absorbance (A) continuously for 3 minutes at 20 seconds intervals and to calculate the slope, in units of ΔA/min, between each two successive absorbance readings. A LDH kit (Gilford Diagnostics, Oberlin, OH) provided the necessary reagents for that assay. 50 µl of the control samples (PRP, PPP, and RBC) or 100 µl of the experimental samples were rapidly added to a test tube containing 1.0 ml of the reconstituted reagent (0.75 mM pyruvate, 0.25 mM NADH, pH
= 7.2 ± 0.1). Next, the test tube was immediately inverted 3 or 4 times, its content fully aspirated using the Gilford Model 2443-A rapid sampler, and the absorbance readings started. The control samples for this assay were prepared by mixing respectively 20 μl of RBCs with 1 ml of 1% triton X-100 cell lysing buffer, 480 μl of PRP with 20 μl of 25% triton X-100, and 480 μl of PPP with 20 μl of 25% triton X-100. The experimental samples were prepared as indicated in Chapter 2. For a 50 μl sample volume, the LDH activity, in units of IU/l, is given by the following formula (Gilford Diagnostics, Oberlin, OH):

\[
\text{activity (IU/l)} = \frac{(\Delta A/\text{min} \times 10^3 \times 1.05)}{(6.3 \times 1 \times 0.05)} \tag{A1}
\]

where IU stands for international unit and is defined as the amount of enzyme that catalyses the transformation of 1 μmole of substrate per minute; l stands for liter; 6.3 is the millimolar absorptivity of NADH; 10^3 is the conversion factor from milliliter to liter; 1 is the light path in cm; 1.05 is the total reaction volume in ml; and 0.05 is the sample volume in ml.

**Hemoglobin Assay.** Because RBCs contain a large amount of LDH activity, all control samples were also assayed for the conjugated protein hemoglobin spectrophotometrically (Eilers, 1967). The test principle is as follows. Erythrocytes are first lysed by a detergent, after what they release their hemoglobin. The ferrous iron of hemoglobin is oxidized to the ferric state by potassium ferricyanide to form methemoglobin. Next, methemoglobin reacts with cyanide ions, from potassium cyanide,
to form cyanmethemoglobin, which has a strong absorbance peak at 540 nm. The absorbance is directly proportional to the hemoglobin concentration, which itself is proportional to the number of RBCs. An hemoglobin kit (Sigma Chemical Co; Kit No. 525) provided the necessary reagents for the assay as well as for the calibration.

Typically, 20 µl of RBCs were mixed with 5 ml of the reconstituted reagents in a quartz cuvette, while 0.5 ml of PRP or PPP were mixed with 2.5 ml of the same reagents in a similar cuvette. Each cuvette was then placed in the cuvette holder of the Gilford System 2600 spectrophotometer and the optical density recorded. The hemoglobin concentration in any sample was determined from a calibration curve obtained previously with hemoglobin solutions of known concentrations. A typical calibration curve for that assay is shown in Figure A.1, where the data were curve-fitted using a least square method.

Calculation of the Total Platelet Number. The total number of platelets accumulated on the reactive surface was calculated as follows. First, the amount of LDH activity per platelet was computed according to:

\[
\text{IU/platelet} = \frac{[\text{IU/l(PRP)} - \text{IU/l(PFP)} - \text{IU/l(RBCs in PRP)}]}{[\text{platelets/l(PRP)}]}
\]  
(A2)

where IU/l(RBCs in PRP) was estimated in several steps. First, the LDH activity per RBC was computed as:
Figure A.1: Calibration curve for the hemoglobin assay
where 5.1x10^{-5} accounts for dilutions and the conversion between liter and microliter.

Second, the amount of hemoglobin per RBC was estimated from:

\[ \text{gr. hemoglobin/RBC} = \frac{\text{OD(RBC)} \times \text{slope}}{10^{-3} \times \text{(RBC/μL)}} \]  \hspace{1cm} (A4)

where OD is the measured optical density; slope is the slope of the calibration curve; and 10^{-3} is a dilution factor. Last, IU/l(RBCs in PRP) was computed from:

\[
\text{IU/l(RBCs in PRP)} = \frac{\text{OD(PPP)} - \text{OD(PPP)}}{\text{slope} \times \left(\frac{\text{IU/RBC} \times 10^3}{\text{gr. hemoglobin/RBC}}\right)}
\]  \hspace{1cm} (A5)

where 10^3 accounts for dilutions and conversion from microliter to liter. Finally, the total number of platelets accumulated on the thrombogenic surface was computed according to:

\[
P_T = \frac{\text{IU/l(EXP) \times vol(DEC)}}{\text{IU/platelet}}
\]  \hspace{1cm} (A6)

where EXP stands for values in experimental samples and vol for the sample volume.

**Computer Programs.** Referring to equation (7) of Chapter 2, the integral in that equation was estimated numerically using the following approach. First, the fluorescence
intensity data points, \( A(z) \), were curve-fitted using a least square technique. Next, the surface under the generated curve was calculated numerically using the Romberg algorithm. Knowing the value of that integral, \( P_T \), and \( w \), the constant \( c \) could then be easily calculated. Two programs, written in TURBO PASCAL as shown in the next few pages, were used for that purpose.
A) PROGRAM FOR NUMERICAL CURVE-FITTING

program Least_Squares;

{-------------------------------------------------}
{ Purpose: This program averages data files from different experiments
  and curves fit the resulting macroscopic data points
  on platelet fluorescence intensity using a least square
  method. (B. Folie)
{-------------------------------------------------}
{ Units used: IOSelection

---
var OutFile : text;
    OutName : string;
    InFile : text;
    InName : string;
    IOErr : boolean;

procedure DisplayWarning
procedure DisplayError
procedure IOCheck
procedure GetInputFile
procedure GetOutputFile

LeastSquares

---
function ModuleName
procedure LeastSquares

---

{$I-} { Disable I/O error trapping }
{$R+} { Enable range checking }

{$R IOSelection.rsrc} { Resource file for IOSelection unit }

{SU IOSelection}
{SU LeastSquares}

uses
  MemTypes, QuickDraw, OSIntf, ToolIntf, PackIntf, PasPrinter,
  LeastSquares, IOSelection;

var
XData, YData : TNCOLUMNVECTOR; { Data points (X,Y) }
NumPoints : integer; { # of points }
NumTerms : integer; { # of terms in least squares }
Solution : TNCOLUMNVECTOR; { Coefficients of the l.s. fit }
YFit : TNCOLUMNVECTOR; { Least squares solution }
    at XData points }
Residual : TNCOLUMNVECTOR; { YFit - YData }
StandardDeviation : Extended; { Square root of variance; }
Variance : Extended; { Indicates goodness of fit }
Error : byte; { Variance in the solution }
Fit : FitType; { Flags if something went wrong }
    { The type of fit to be performed }

procedure Initialize(var XData : TNCOLUMNVECTOR;
var YData : TNCOLUMNVECTOR;
var YFit : TColumnVector;
var Residual : TColumnVector;
var Solution : TRowVector;
var Error : byte;

begin
  FillChar(XData, SizeOf(XData), 0);
  FillChar(YData, SizeOf(YData), 0);
  FillChar(Solution, SizeOf(Solution), 0);
  FillChar(YFit, SizeOf(XData), 0);
  FillChar(Residual, SizeOf(XData), 0);
  Error := 0;
end; // procedure Initialize

procedure GetData(var NumPoints : integer;
  var NumTerms : integer;
  var XData : TColumnVector;
  var YData : TColumnVector);

var
  Index : integer;
  NumberFiles : integer;

begin
  FillChar(XData, SizeOf(XData), 0);
end; // procedure GetData

procedure GetTwoVectorsFromFile(var NumPoints : integer;
  var XData : TColumnVector;
  var YData : TColumnVector);

begin
  FillChar(Y, SizeOf(Y), 0);
  Writeln;
  Write('enter background intensity');
Readln(Background);
IOCheck;
WriteLn;
Write('enter calibration constant k');
Readln(k);
IOCheck;
WriteLn;
NumPoints := 0;
while not (EOF(InFile)) do
begin
  NumPoints := Succ(NumPoints);
  Readln(InFile, Y[NumPoints]);
  Y[NumPoints] := (Y[NumPoints] - Background) * k;
  if Y[NumPoints] < 0.0 then
    Y[NumPoints] := 0.000001;
  YData[NumPoints] := YData[NumPoints] + Y[NumPoints];
  IOCheck;
end;
XData[1] := 0.1;
for Index := 2 to NumPoints do
  XData[Index] := XData[Index - 1] + (22.86 / Float(NumPoints - 1));
Close(InFile);
end; { procedure GetTwoVectorsFromFile }

procedure GetTwoVectorsFromKeyboard(var NumPoints : integer;
var XData : TColumnVector;
var YData : TColumnVector);
{------------------------------------------------------------------------}
{ - Output: NumPoints, XData, YData                                  - }
{ -                                                            - }
{ - This procedure reads in the data points from the keyboard. -    }
{------------------------------------------------------------------------}

var
  Term : integer;

begin
  NumPoints := 0;
  WriteLn;
  repeat
    Write('Number of points (1-?, TColumnSize, ?)? ');
    Readln(NumPoints);
    IOCheck;
  until ((NumPoints >= 1) and (NumPoints <= TColumnSize) and not IOerr);
  Write('Type in the X ');
  WriteLn('and Y values, separated by a space (not a comma):');
  for Term := 1 to NumPoints do
  repeat
    Write('X[', Term, '], Y[', Term, ']:');
    Read(XData[Term], YData[Term]);
    WriteLn;
    { Read in the XData and YData }
    IOCheck;
  until not IOerr;
end; { procedure GetTwoVectorsFromKeyboard }

begin { procedure GetData }
  Write('enter number of files to be averaged');
  Readln(NumberFiles);
IOCheck;
Writeln;
for Index := 1 to NumberFiles do
begin
  GetInputFile('Input data from:', InFile, InName);
  if InName = 'KEY' then
    GetTwoVectorsFromKeyboard(NumPoints, XData, YData)
  else
    GetTwoVectorsFromFile(NumPoints, XData, YData);
end;
for Index := 1 to NumPoints do
  YData[Index] := YData[Index] / Float(NumberFiles);
Writeln;
NumTerms := 0;
repeat
  Write('Number of terms in the least squares fit (<= ', NumPoints, ')? ');
  Readln(NumTerms);
  IOCheck;
  if NumTerms <= 0 then
    IOerr := true;
  if NumTerms > NumPoints then
  begin
    IOerr := true;
    Writeln;
    Writeln('The number of terms in the fit must');
    Writeln('be less than the number of points.');
    Writeln;
  end;
until not IOerr;
end; "procedure GetData"

procedure Results(NumPoints : integer;
  var XData : TNCOLUMNVECTOR;
  var YData : TNCOLUMNVECTOR;
  var NumTerms : integer;
  var Solution : TNCOLUMNVECTOR;
  var Error : extended;
  var Residual : TNCOLUMNVECTOR;
  var StandardDeviation : extended;
begin

var
  Index : integer;
const
  : byte);

output files. The first one gives the results of the
-- curve fitting, and the second one creates x-y data points--
"to be used in CRICKET-GRAPH.
"-------------------------------------------------------------------

Index : integer;
begin
  GetOutputFile(OutFile);
  Write('enter 1 if k=1.0 or 0 otherwise');
  Readln(constant);
  IOCheck;
  Writeln;
  Writeln;
  Writeln(OutFile, 'The Modified Data Points:');
  if constant = 1 then
    Writeln(OutFile, 'axial position  fluorescence intensity');
if constant <> 1 then
  Writeln(OutFile,' axial position platelet density(plt/1000 sq.\mu m)');
for Index := 1 to NumPoints do
  Writeln(OutFile,XData[Index] : 8 : 3, ' ' : 10, YData[Index] : 12 : 7);
Writeln(OutFile);
if Error >= 1 then
  DisplayError;
case Error of
  0 : begin
    Writeln(OutFile, '************************************************************************');
    Writeln(OutFile, ModuleName(Fit));
    Writeln(OutFile, '************************************************************************');
    Writeln(OutFile);
    Writeln(OutFile, 'Coefficients in least squares approximation:');
    for Index := 1 to NumTerms do
      Writeln(OutFile, '   Coefficient', Index-1, ': ',
      Solution[Index]:19);
    Writeln(OutFile);
    Writeln(OutFile, 'Standard Deviation: ', StandardDeviation : 15);
  end;
  1 : Writeln(OutFile,'There must be more than one data point.');
  2 : Writeln(OutFile,
      'The number of terms in the fit must be greater than zero.');
  3 : begin
    Writeln(OutFile, 'The number of terms in the solution must be');
    Writeln(OutFile, 'less than the number of data points.');
  end;
  4 : Writeln(OutFile,
    'There is no least squares solution to this set of data.');
end; { case }
Close(OutFile);
GetOutputFile(OutFile);
for Index := 1 to NumPoints do
  Writeln(OutFile,XData[Index] : 8 :4, chr(9), YFit[Index] : 19, chr(9),
  YData[Index] : 12 : 7);
Close(OutFile);
end; { procedure Results }

begin { call program LeastSquares from TURBO PASCAL NUMERICAL METHODS TOOLBOX
FOR MACINTOSH }
  Initialize(XData, YData, YFit, Residual, Solution, Error);
  GetData(NumPoints, NumTerms, XData, YData);

  {------- Change "Fit" to the type of Least Squares fit you need -------}
  Fit := Poly;
  {--------------------------------------------------------------------------}

  LeastSquares(NumPoints, XData, YData, NumTerms, Solution,
  YFit, Residual, StandardDeviation, Variance, Error, Fit);
  Results(NumPoints, XData, YData, NumTerms, Solution, YFit,
  Residual, StandardDeviation, Error);
  WaitForReturnOrClick;
end. { program LeastSquares }
B) PROGRAM FOR NUMERICAL INTEGRATION

program Romberg;

{-----------------------------------------------}
{-
{- Purpose: THIS PROGRAM USES THE ROMBERG INTEGRATION PROCEDURE
{- TO CALCULATE THE AREA UNDER THE CURVE FITTING THE
{- EXPERIMENTAL DATA ON FLUORESCENCE INTENSITY.
{- IT ALSO CALCULATES THE CALIBRATION CONSTANT C AND
{- THE GLOBAL AVERAGE PLATELET DENSITY (APD).
{- (B. FOLIE)
{-
{- Units used: IOSelection
{- var OutFile : text;
{- OutName : string;
{- InFile : text;
{- InName : string;
{- IOerr : boolean;
{- procedure DisplayWarning
{- procedure DisplayError
{- procedure IOCheck
{- procedure GetInputFile
{- procedure GetOutputFile
{-
{- Integration
{- procedure Romberg
{-

{-[SI-]  { Disable I/O error trapping }
{-[SR+]  { Enable range checking }

{SR IOSelection.rs}  { Resource file for IOSelection unit }

{$U IOSelection}
{$U Integration}

uses
  MemTypes, QuickDraw, OStrf, ToolIntf, PackIntf, PasPrinter,
  Integration, IOSelection;

var
  LowerLimit, UpperLimit: Extended;  { Limits of integration }
  Tolerance : Extended;              { Tolerance in the answer }
  MaxIter : integer;                { Maximum number of iterations }
  Integral : Extended;              { Value of the integral }
  Iter : integer;                   { Number of iterations to find answer }
  Error : byte;                     { Flags if something went wrong }

function TIntTargetF(X: Extended): Extended;

{-----------------------------------------------}
{- This is the function to integrate
---
}
begin
{ TNTargetF := EXP(LN(1.60713e-1) - 5.759815e-1 * LN(X));
 TNTargetF := 9.6412e-2 - (1.9736e-2 * X) + (1.551e-3*X*X) -
 (3.8702e-5*X*X*X); 
 end; { function TNTargetF }

procedure Initialize(var LowerLimit : Extended;
vart x
LowerLimit := 0;
UpperLimit := 0;
Integral := 0;
Tolerance := 0;
MaxIter := 0;
Iter := 0;
error := 0;
end; { procedure Initialize }

procedure GetData(var LowerLimit : Extended;
vart x
LowerLimit := 0;
UpperLimit := 0;
Integral := 0;
Tolerance := 0;
MaxIter := 0;
Iter := 0;
error := 0;
end; { procedure GetLimits }

{ - Output: LowerLimit, UpperLimit, Tolerance, MaxIter
  -
  - This procedure assigns values to the above variables
  - from keyboard input
  -
procedure GetLimits(var LowerLimit : Extended;
vart x
LowerLimit := 0.0;
UpperLimit := 26.3;
end; { procedure GetLimits }

procedure GetTolerance(var Tolerance : Extended);
{ - Output: Tolerance }
begin
    Writeln;
    repeat
        Tolerance := 1E-8;
        Write('Tolerance (> 0): ');
        Readln(Tolerance);
        IOCheck;
        if Tolerance <= 0 then begin
            IOerr := true;
            Tolerance := 1E-8;
        end;
        until not IOerr;
    end;  { procedure GetTolerance }

procedure GetMaxIter(var MaxIter : integer);
{
- Output: MaxIter
- }
{
- This procedure reads in the accepted MaxIter
- from the keyboard.
- }
begin
    Writeln;
    repeat
        MaxIter := 100;
        Write('Maximum number of iterations: (> 0): ');
        Readln(MaxIter);
        IOCheck;
        if MaxIter <= 0 then begin
            IOerr := true;
            MaxIter := 1000;
        end;
        until not IOerr;
    end;  { procedure GetMaxIter }

begin  { procedure GetData }
    GetLimits(LowerLimit, UpperLimit);
    GetTolerance(Tolerance);
    GetMaxIter(MaxIter);
    GetOutputFile(OutFile);
end;  { procedure GetData }

procedure Results(LowerLimit : Extended;
    UpperLimit : Extended;
    Tolerance : Extended;
    MaxIter : integer;
    Integral : Extended;
    Iter  : integer;
    Error : byte);

var
    k : Extended;
    NumberPlatelets : Extended;
AveragePlateletDensity : Extended;

(-- This procedure outputs the results to an output file --)
(-- the width of the flow chamber is 12.7 mm. --)

begin
Write('enter number of platelets calculated from LDH assay');
Readln(NumberPlatelets);
IOCheck;
Writeln;
Writeln(OutFile);
Writeln(OutFile, 'Lower Limit:' : 30, LowerLimit : 23);
Writeln(OutFile, 'Upper Limit:' : 30, UpperLimit : 23);
Writeln(OutFile, 'Tolerance:' : 30, Tolerance : 23);
Writeln(OutFile, 'Maximum number of iterations:' : 30, MaxIter:4);
Writeln(OutFile, 'Number of iterations:' : 30, Iter:4);
Writeln(OutFile);
if Error = 3 then
  DisplayWarning;
if Error in [1, 2] then
  DisplayError;
case Error of
  0 : Writeln(OutFile, 'Integral:' : 25, Integral:23);
  1 : Writeln(OutFile, 'The tolerance must be greater than zero.');
  2 : Writeln(OutFile,
     'The maximum number of iterations must be greater than zero.');
  3 : begin
     Writeln(OutFile, 'Tolerance not reached in ', Iter, ' iterations.');
     Writeln(OutFile, 'The last iterate of the integral is:',
     Integral:23);
     end;
end; { case }
Writeln(OutFile);
c := (NumberPlatelets / (Integral * 12.7)) * 1e-3;
Writeln(OutFile, 'The calibration constant c is:', c:6);
Writeln(OutFile);
AveragePlateletDensity := (NumberPlatelets / (26.3 * 12.7)) * 1e-3;
Writeln(OutFile, 'The average platelet density(plt/1000 sq.μm) is:',
AveragePlateletDensity:6);
end; { procedure Results }

begin { call program Romberg from THE TURBO PASCAL NUMERICAL METHODS TOOLBOX
FOR MACINTOSH }
  Initialize(LowerLimit, UpperLimit, Integral, Tolerance,
     MaxIter, Iter, Error);
  GetData(LowerLimit, UpperLimit, Tolerance, MaxIter);
  Romberg(LowerLimit, UpperLimit, Tolerance, MaxIter, Integral, Iter, Error,
     @TNTargetT);
  Results(LowerLimit, UpperLimit, Tolerance, MaxIter, Integral, Iter, Error);
  Close(OutFile);
  WaitReturnOrClick;
end. { program Romberg }
APPENDIX B

TOTAL PROTEIN ASSAY

A total protein assay based on Peterson's modification of the micro-Lowry (Peterson, 1977) method was used in the studies of Chapter 3 and 4 in order to estimate bovine and human collagen concentrations in various suspensions. In principle, an alkaline cupric tartrate reagent complexes with the peptide bonds of the proteins and forms a purple-blue color when a folin and ciocalteu's phenol reagent is added. The absorbance was read at 750 nm. Sodium dodecylsulfate was also used to facilitate the dissolution of the proteins. A protein kit (Protein Assay Kit No. P 5656, Sigma Chemical Co) provided the necessary reagents for the assay as well as for the calibration.

For this assay, 1.0 ml of the collagen suspension of interest was first mixed with 1.0 ml of the reconstituted reagents and that mixture allowed to stand at room temperature for 20 minutes. Next, 0.5 ml of the phenol reagent was added and the color allowed to develop for 30 minutes. The quartz cuvette containing that solution was then placed in the cuvette holder of a Gilford System 2600 spectrophotometer and the optical density recorded. The protein concentration in the sample was read from a calibration curve obtained previously with bovine serum albumin solutions of known concentrations. A typical calibration curve for that assay is shown in Figure B.1.
Figure B.1: Calibration curve for the total protein assay
APPENDIX C

NUMERICAL TECHNIQUE FOR THE COMPUTATIONS OF

CHAPTER 5

The system of weakly-coupled PDEs and their boundary conditions, given by equations (10) to (18) in Chapter 5, was solved by a finite element routine for fluid flow, FIDAP (Fluid Dynamics International, Inc., Evanston, IL). A preprocessor, with automatic mesh refinement capability, was utilized to discretize the flow domain into a large number of elements. For a typical rectangular model thrombus 20 \( \mu \)m-long, with the dimensions of the flow domain shown in Figures 5.1 and 5.2, 361 isoparametric quadrilateral elements, each containing 9 nodes, were used, yielding a total of 1549 nodal points. A typical element mesh plot of the flow domain is shown in Figure C.1. The Galerkin form of the Method of Weighted Residuals was the minimization technique chosen in this simulation. The velocity and concentration were approximated by a series of identical biquadratic interpolation functions, while discontinuous linear approximation functions were used for the pressure. The finite element method (FEM) was applied to a perturbed system of equations in which the continuity requirement, equation (10) in Chapter 5, was replaced by:

\[ \nabla \cdot \bar{V} = -\varepsilon P \]

(C1)
Figure C.1: Element mesh plot. The two-dimensional flow domain was discretized into quadrilateral elements, each containing 9 nodes. The diameter of the semi-circular model thrombus is 20 μm in this case. The flow domain extends 200 μm in the y-direction and 220 μm in the x-direction.
where $\varepsilon$, the penalty parameter, was chosen as $10^{-6}$. This approach has the advantage of eliminating the dependent variable $P$ from the momentum equation without losing any significant accuracy, provided $\varepsilon$ is small enough (Bercovier, 1976).

The application of the FEM to the momentum and convection-diffusion equations resulted in a set of nonlinear algebraic equations, represented in matrix (bold face) form as:

$$
L(U)U = F \quad (C2)
$$

where $L$ is the global system matrix; $U$ is the global vector of unknowns (velocities and concentrations), and $F$ is a vector which includes the effect of body forces and boundary conditions.

A combination of two different iterative algorithms were used to solve this system. First, the successive substitution (SS), or Picard iteration, scheme, given below, was applied:

$$
L(U_t)U_{t+1} = F \quad (C3)
$$

The Stokes solution for the velocity field and a null concentration vector served as the initial iterate $U_0$. The advantages of this method are its large radius of convergence and its insensitivity with respect to $U_0$. Because of its slow convergence rate, however, this technique was replaced, after 3 iterations, by the faster quasi-Newton (Broyden, 1965)
method of iteration, written as:

\[ U_{i+1} = U_i - 0.8D_i \quad ; \quad D_i = S_iR_i \quad (C4) \]

\[ S_{i+1} = S_i + \Delta S_i \quad (C5) \]

\[ \Delta S_i = \frac{\left(I_{i+1} - S_i\Gamma_{i+1}\right)\lambda_{i+1}^T}{\lambda_{i+1}^T S_i\Gamma_{i+1}} \quad (C6) \]

where \( \lambda_i = U_i - U_{i-1} \); \( \Gamma_i = R_i - R_{i-1} \); \( R_i \) is the residual force vector \( R(U_i) = L(U_i)U_i - F \); and \( S_i \) is the inverse of the Jacobian matrix of the system of equations \( J^{-1}(U_i) = (\partial R_i/\partial U_i)^{-1} \). The initial \( S_0 \) was calculated from \( J^{-1}(U_0) = (\partial R_0/\partial U_0)^{-1} \), where \( U_0 \) is the solution vector of the last iteration by the SS method. This technique is more economical than the Newton-Raphson method, for instance, because it only updates \( S_i \) at every iteration instead of recomputing it.

Iteration for the steady-state solution was terminated when the following two convergence criteria were satisfied simultaneously:

\[ \frac{||U_i - U_{i-1}||}{||U_i||} \leq 0.01 \quad (C7) \]

\[ \frac{||R_i - R_{i-1}||}{||R_0||} \leq 0.01 \quad (C8) \]
where the norm $\| \cdot \|$ is a root mean square norm summed over all nodes in the mesh.

The method adopted for solving the assembled matrix equation at each iteration step was the direct Gauss elimination with a skyline out-of-core storage mode initially developed by Hasbani and Engelman (1979). Finally, a postprocessor was used to output the various solution variables in graphical form.

A typical preprocessor input data file is shown on the next pages.
FIPREP input file for calculation of thrombin concentration profile over two rectangular model thrombi separated by a distance of 200 microns.

*TITLE
BLOOD FLOW OVER TWO CUBES (THROMBIN, WALL SHEAR RATE = 1500/sec)
*FIMESH (2-D, IMAX=13, JMAX=5)

[Mesh generation]
EXP1
1 0 7 0 19 0 33 0 47 0 59 0 69

[Mesh refinement]
EXPJ
1 0 9 0 19

[Topology data]
POINT
/ # I J K X Y
1 1 1 1 0.0
2 2 3 1 1 0.2 0.0
3 3 3 1 0.20 0.10
4 5 3 1 0.4 0.1
5 5 1 1 0.4 0.0
6 7 1 1 1.4 0.0
7 7 3 1 1.4 0.1
8 8 1 1 2.4 0.0
9 9 3 1 2.4 0.1
10 11 3 1 2.6 0.1
11 11 1 1 2.6 0.0
12 13 1 1 3.0 0.0
13 13 3 1 3.0 0.1
14 14 3 1 3.0 2.0
15 11 5 1 2.6 2.0
16 16 9 5 1 2.4 2.0
17 17 7 5 1 1.4 2.0
18 18 5 5 1 1.4 2.0
19 19 3 5 1 0.2 2.0
20 20 1 5 1 0.0 2.0
21 21 1 3 1 0.0 0.1

LINE
/P1 P2 RATIO IRAT
1 2 4. 4
2 3 2. 4
3 4
5 4 2. 4
6 6 15. 3
8 6 15. 3
9 2. 4
6 7 2. 4
4 7 15. 3
9 7 15. 3
9 10
10 11 2. 3
11 12 5. 3
10 13 5. 3
12 13 2. 4
13 14 15. 3
10 15 15. 3
9 16 15. 3
7 17 15. 3
4 18 15. 3
3 19 15. 3
21 20 15. 3
14 15 5. 4
15 16
16 17 15. 3
SURFACE
1 19
3 18
5 16
9 15
11 14

BCNODE (UX)
/ P1 P2 UX
1 2 0.
2 3 0.
3 4 0.
4 5 0.
5 8 0.
8 9 0.
9 10 0.
10 11 0.
11 12 0.
14 20 0.

BCNODE (UY)
/ P1 P2 UY
1 20 0.
1 2 0.
2 3 0.
3 4 0.
4 5 0.
5 8 0.
8 9 0.
9 10 0.
10 11 0.
11 12 0.
14 20 0.

BCNODE (UX, PARABOLIC=0)
1 20 1.0

[velocity boundary conditions]

BCNODE (CONCENTRATION)
/ P1 P2 CONCENTRATION
1 20 0.
14 20 0.

[concentration boundary conditions]

BCFLUX (CONC, NODES=3)
/ P1 P2 FLUX
2 3 4.58059E-3
3 4 4.58059E-3
4 5 4.58059E-3
5 9 3.63981E-3
9 10 3.63981E-3
10 11 3.63981E-3

[flux boundary conditions]

ELEMENTS (QUADRILATERAL, NODES=9)
1 14

END

*PROBLEM (NONLINEAR, WEAKLY-CPLD)
*EXECUTION (NEWJOB)
*PRESSURE (PENALTY=1.E-6, DISCONTINUOUS)
*DENSITY (CONSTANT=1.)
*VISCOSITY (CONSTANT=.2963)
*SPECIFICHEAT (CONSTANT=1.)
*CONDUCTIVITY (CURVE=2)
-2000.0, 2000.0, 2.773E-4, 2.773E-4
*IC-VELOCITY (STOKES)

[physical parameters]
*SOLUTION (Q.N.=10)
*STRATEGY (S.S.=3)
*DATA PRINT (NORMAL, PAGE, NODES=1)
*POST PROCESS
*NODES (FIMESH)
*ELEMENTS (QUADRILATERAL, NODES=9, LINEAR, GLOBAL, FIMESH)
*BODYFORCE (CONSTANT)
0  -1.744
*HTSOURCE (SUBROUTINE) [user-supplied FORTRAN subroutine for reaction term]
*RENUMBER (PROFILE)
*END
APPENDIX D

NUMERICAL TECHNIQUE FOR THE COMPUTATIONS OF

CHAPTER 6

The transient advection-diffusion equation given by equation (4) in Chapter 6 was solved again by the finite element routine for fluid flow, FIDAP. A preprocessor, with automatic mesh refinement capability, was utilized to discretize the flow domain into a large number of elements. For a two dimensional parallel plate geometry, 200 μm-thick and 5 mm-long, 1078 isoparametric quadrilateral elements, each containing 9 nodes, were used to discretize the domain, yielding a total of 4455 nodal points. The Galerkin form of the method of Weighted Residuals was the minimization technique chosen in this simulation. The concentration variable was approximated by a series of biquadratic interpolation functions.

Application of the FEM to the advection-diffusion equation resulted in a set of first order linear ordinary differential equations (ODEs), represented in matrix form as:

\[ [M]\{U\} + [K]\{U\} = \{F\} \]

where \([K]\) is the global system matrix; \([U]\) is the global vector of unknowns (concentrations); \([M]\) is the global capacitance matrix; and \([F]\) is a vector which
includes the effect of boundary conditions. Because the velocity field is constant in time for this type of problem, the matrix \( K \) is independent of the unknown concentrations in vector \( U \) and the problem becomes linear. A second-order accurate time integration scheme, the trapezoid rule, with a variable time increment determined by control of the local time truncation error, was used to solve the equation above. Typically, it took 57 time steps, \( \Delta t \), to solve that problem, starting with an initial \( \Delta t \) of 0.01 sec and ending with a \( \Delta t \) equals to 3.15 sec. The system of linear algebraic equations resulting at each time step was solved by direct Gauss elimination with a skyline out-of-core storage mode, as mentioned in Appendix C.

A typical preprocessor input data file used for this simulation is shown on the next few pages.
FPREP input data file for calculations of bulk platelet concentration profile and platelet wall fluxes using Keller and Wang’s model of effective platelet diffusivity in flowing whole blood.

*TITLE
2-D ADVECTION-DIFFUSION MODEL (Vy=400*De,Db)
*FIMESH(2-D,IMAX=99,JMAX=45)  [mesh generation]

POINT
//  
I   J   K   X   Y   [topology data]
 1, 1, 1, 0.0, -100.0E-4
 2, 45, 1, 1, 2500.0E-4, -100.0E-4
 3, 39, 1, 1, 5000.0E-4, -100.0E-4
 4, 99, 7, 1, 5000.0E-4, -95.0E-4
 5, 99, 15, 1, 5000.0E-4, -65.0E-4
 6, 99, 23, 1, 5000.0E-4, 0.0
 7, 99, 31, 1, 5000.0E-4, 65.0E-4
 8, 99, 39, 1, 5000.0E-4, 95.0E-4
 9, 99, 45, 1, 5000.0E-4, 100.0E-4
10, 45, 45, 1, 2500.0E-4, 100.0E-4
11, 45, 39, 1, 2500.0E-4, 95.0E-4
12, 45, 31, 1, 2500.0E-4, 65.0E-4
13, 45, 23, 1, 2500.0E-4, 0.0
14, 45, 15, 1, 2500.0E-4, -65.0E-4
15, 45, 7, 1, 2500.0E-4, -95.0E-4
16, 1, 45, 1, 0.0, 100.0E-4
17, 1, 39, 1, 0.0, 95.0E-4
18, 1, 31, 1, 0.0, 65.0E-4
19, 1, 23, 1, 0.0, 0.0
20, 1, 15, 1, 0.0, -65.0E-4
21, 1, 7, 1, 0.0, -95.0E-4

LINE
/ P1   P2   RATIO   IRAT
 1, 2
 2, 3
 21, 15
 15, 4
 20, 14
 14, 5
 19, 13
 13, 6
 18, 12
 12, 7
 17, 11
 11, 8
 16, 10
 10, 9
 1, 21
 3, 4
 2, 15
 21, 20
 15, 14
 4, 5
 20, 19, 4, 3
 14, 13, 4, 3
 5, 6, 4, 3
 13, 18, 4, 4
 13, 12, 4, 4
 6, 7, 4, 4
 18, 17
 12, 11
 7, 8
SURFACE
1,9
ELEMENTS (QUAD, NODES=9)
1,9
BCNODE (CONCENTRATION, CONSTANT)
1 2 0.0D0
2 3 0.0D0
16 10 151.0164E6
16 9 151.0164E6
20,19,153.8472E6
19,18,153.8472E6
BCNODE (CONCENTRATION, SUBROUTINE=2)
17,16,510.7727E6,151.0164E6
BCNODE (CONCENTRATION, SUBROUTINE=3)
21,1,510.7727E6,151.0164E6
18,17,153.8472E6,510.7727E6
20,21,153.8472E6,510.7727E6
END
*PROBLEM (TRANSIENT, NONLINEAR, ADV-DIFFUSION)
*EXECUTION (NEWJOB)
*DENSITY (CONSTANT=1.0)
*SPECIFICHEAT (CONSTANT=1.0)
*DIFFUSIVITY (SUBROUTINE=0)
*DIFFUSIVITY (CONSTANT=1.0E-9)
*IC-VELOCITY
/NODE Vx Vy
1 1 0.0 0.0
99 0 0.0 0.0
100 1 0.0414838 -.01911
108 0 0.0414838 -.01911
199 1 0.08260365 -.01897
297 0 0.08260365 -.01897
298 1 0.12335925 -.01883
396 0 0.12335925 -.01883
397 1 0.16375434 -.01869
495 0 0.16375434 -.01869
496 1 0.20377935 -.01855
594 0 0.20377935 -.01855
595 1 0.24344352 -.01842
693 0 0.24344352 -.01842
694 1 0.41743399 -.01779
792 0 0.41743399 -.01779
793 1 0.58406802 -.01716
891 0 0.58406802 -.01716
892 1 0.74335358 -.01652
990 0 0.74335358 -.01652
991 1 0.89529917 -.01588
1089 0 0.89529917 -.01588
1089 1 1.03991388 -.01523
1188 0 1.03991388 -.01523
1189 1 1.17720747 -.01458
1287 0 1.17720747 -.01458
1288 1 1.30719043 -.01392
1386 0 1.30719043 -.01392
1387 1 1.42987413 -.01326
1485 0 1.42987413 -.01326
1486 1 1.55492833 -.01253
1584 0 1.55492833 -.01253
1585 1 1.67139551 -.01180
1683 0 1.67139551 -.01180
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</tr>
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</tr>
<tr>
<td>99</td>
<td>151.01638E6</td>
</tr>
<tr>
<td>100</td>
<td>210.97566E6</td>
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<tr>
<td>198</td>
<td>210.97566E6</td>
</tr>
<tr>
<td>199</td>
<td>270.935E6</td>
</tr>
</tbody>
</table>
297 0  270.935E6
298 1  330.89415E6
299 0  330.89415E6
300 1  390.86832E6
301 0  390.86832E6
302 1  450.81315E6
303 0  450.81315E6
304 1  510.77274E6
305 0  510.77274E6
306 1  439.64154E6
307 0  439.64154E6
308 1  378.40298E6
309 0  378.40298E6
310 1  325.69446E6
311 0  325.69446E6
312 1  280.32782E6
313 0  280.32782E6
314 1  241.28039E6
315 0  241.28039E6
316 1  207.67196E6
317 0  207.67196E6
318 1  178.74491E6
319 0  178.74491E6
320 1  153.8472E6
321 0  153.8472E6
322 1  178.74491E6
323 0  178.74491E6
324 1  207.67196E6
325 0  207.67196E6
326 1  241.28039E6
327 0  241.28039E6
328 1  280.32782E6
329 0  280.32782E6
330 1  325.69446E6
331 0  325.69446E6
332 1  378.40298E6
333 0  378.40298E6
334 1  439.64154E6
335 0  439.64154E6
336 1  510.77274E6
337 0  510.77274E6
338 1  450.81315E6
339 0  450.81315E6
340 1  390.86832E6
341 0  390.86832E6
342 1  330.89415E6
343 0  330.89415E6
344 1  270.935E6
345 0  270.935E6
346 1  210.97568E6
347 0  210.97568E6
348 1  151.01638E6
349 0  151.01638E6

*TIME (TRAPEZOID, NSTEPS=200, TSTART=0.0, TEND=120.0, DT=0.01, VARIABLE=0.1, TCHAR=0.6, UCHAR=2.5, VCHAR=0.02, KCHAR=1.6E-9, LUMPED) [time integrator]

*POSTPROCESS (NBLOCKS=2)
  0 8 1
  8 24 4

*PRINTOUT (NBLOCKS=2)
  0 8 1
  8 24 4

*DATAPRINT (NORMAL, PAGE, NODES=1, INITIAL=2)
  *NODES (FIMESH)
*ELEMENTS (QUAD, NODES=9, LINEAR, GLOBAL, FIMESH)
*RENUMBER (PROFILE)
*END