The Relation Among Vessel Injury, Thrombus Formation and Platelet Survival

by

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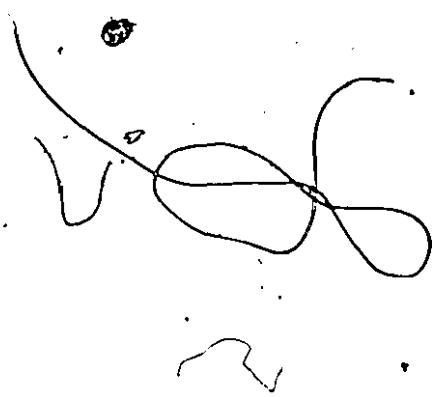
The Relation Among Vessel Injury, Thrombus Formation and Platelet Survival



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Abstract

Shortened platelet survival observed in individuals with thromboembolic vascular disease could be due to platelet consumption or turnover in thrombi or platelet turnover on the damaged vessel wall. The object of this study was to examine the relationship between:

a) platelet consumption and exchange in thrombi and platelet survival

b) platelet accumulation and turnover on the injured vessel wall and platelet survival.

Indwelling catheters were inserted into rabbit aortae to induce repeated vessel injury and macroscopic thrombus formation. Radiolabelled platelets monitored platelet participation in thrombosis and platelet accumulation on the injured vessel wall. Morphological studies assessed the thrombus characteristics and the cellular events on the vessel wall.

During the first 24 hours following the insertion of indwelling aortic catheters, thrombi formed, attained their maximum size and maintained a constant weight thereafter. ^{51}Cr-labelled platelet incorporation into thrombi following the initial growth, decreased by about 50% by 3 days. Loss of radioactivity from the thrombus during the time when thrombus weight remained constant indicated platelet lysis, phagocytosis or loss of whole platelets from the thrombus.

Both morphological studies and studies using platelets doubly labelled with ^{125}I and ^{51}Cr demonstrated that some of the platelets initially incorporated into thrombi lysed. Studies of changes in platelet density as well as studies showing thrombi retained some

capacity to accumulate circulating platelets indicated that platelets exchanged in thrombi and some of the platelets that had participated in thrombus formation returned to the circulation as less dense platelets.

The consumption and exchange of platelets in thrombi during the acute phase of growth correlated with maximal shortening of platelet survival time. When platelet survival studies were carried out 1 week after insertion of the catheter (at a time when thrombus growth had ceased and platelet accumulation into thrombi was decreased) platelet survival continued to be shortened. At this time, the extent of platelet accumulation on the injured vessel wall was similar to that observed at the time of insertion of the catheter suggesting platelet interaction with the injured vessel wall influenced platelet survival.

Studies using long and short catheters demonstrated that short catheters induced comparatively less vessel injury than long catheters but thrombus formation was significantly increased. Platelet survival studies carried out at 3 days after insertion of long or short catheters (when thrombus growth had ceased) indicated that the short catheters had no significant effect on platelet survival whereas long catheters continued to shorten platelet survival time.

In conclusion, extensive thrombus formation with evidence of platelet exchange in thrombi is associated with significant shortening of platelet survival time. However, this effect is detectable only during the acute phase of thrombus formation. In contrast, repeated vessel injury in association with continuing platelet turnover on the vessel wall can shorten platelet survival time.

Therefore, shortened platelet survival time is a manifestation of acute thrombus formation, repeated vessel injury or both.

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CHAPTER 1

INTRODUCTION

1. Introduction

Platelets play a major role in the thromboembolic complications of vascular disease. Active consumption of platelets is suggested by their presence in atherosclerotic lesions, thrombi and emboli (Mustard and Packham, 1979). Since platelet survival time is shortened in a large proportion of individuals with thromboembolic vascular disease, and since platelet survival has been proposed as a method of detecting platelet activation in these patients, the relationship among vessel injury, thrombus formation and platelet survival is of particular interest.

Normally, the vessel endothelium constitutes a thromboresistant surface which is non-reactive to circulating platelets, leucocytes, red blood cells or coagulation proteins (Stemerman, 1974). However, when a vessel wall is injured and sub-endothelial tissues are exposed to the blood, platelets adhere and aggregate on the surface (Baumgartner, 1973).

In veins, although thrombi may be initiated by activated platelets, low shear forces promote contact activation of the coagulation system resulting in thrombi composed primarily of red blood cells in a fibrin network (Patterson, 1969).

In arteries, if the endothelium is removed from the surface by a single balloon catheter injury, a monolayer of platelets adheres to the subendothelium as high shear forces promote platelet diffusion and adhesion while minimizing accumulation of coagulation factors. Thrombi composed primarily of platelets form on the surface but are rapidly embolized to the microcirculation (Baumgartner, 1973).

The vessel quickly becomes non-reactive to further platelet adhesion and those platelets which had initially interacted with the damaged vessel are gradually lost from the surface. Additional thrombus formation does not occur and platelet survival is unaffected as too few platelets are utilized at the site of injury (Groves et al., 1979).

If the endothelial cell injury is repeated or continuous, or if blood flow is disturbed, thrombin generation is promoted through activated and accumulated coagulation factors and platelets are continuously stimulated to release aggregating agents. This results in thrombus formation at these sites. Since platelets play a primary role in continued thrombus formation and are activated and consumed during the process, it is not surprising that repeated vessel injury and thrombus formation result in shortened platelet survival (Kinlough-Rathbone et al., 1983).

The mechanisms that are responsible for changes in platelet survival in conjunction with repeated vessel injury and thrombus formation have remained largely unknown.

Therefore, in the present study, the possible causes of shortened platelet survival were examined in rabbits under experimental conditions in which repeated vessel injury and thrombus formation occurred.

Before the mechanisms which influence platelet survival under pathological conditions can be effectively examined, it is essential to have a clear understanding of the factors which contribute to platelet survival in normal physiological conditions. These factors include:

a) platelet production

b) platelet distribution within the vascular system

c) platelet utilization during normal functions in vivo

d) platelet destruction (removal from the circulation).

This review will begin with a description of the origin of platelets and the factors which normally regulate their production and influence their survival in the circulation.

1.1 The Origin of Platelets

The earliest descriptions of circulating blood platelets appeared in the literature when Donné (1842) described the origin and fate of 'les globules du sang'. Donné maintained that platelets were small fat particles resulting from the ingestion of milk. His conclusions were based on the evidence that similar globules could be experimentally induced by giving animals an intravenous infusion of milk. These original experiments initiated the development of an extensive literature concerning the origin of platelets and almost every blood cell and blood forming tissue was suggested as the precursor to the circulating platelet (Bizzozero, 1882; Wright, 1906).

In 1906, on the basis of morphological and histochemical evidence, Wright suggested that platelets originated from cytoplasmic budding of bone marrow megakaryocytes. However, Wright's proposal remained a matter of controversy for the next half century.

In 1952 Zajicek et al. (1957) discovered that the enzyme acetylcholine esterase (AchE) was distributed differently between circulating erythrocytes (RBC) and platelets of different species. This

observation provided the first biochemical evidence as to the origin of platelets. Zajicek noted that circulating RBC's in humans contained high concentrations of AchE while their circulating platelets lacked this enzyme. When human bone marrow was examined, RBC precursors were also found to have high AchE activity whereas the activity of marrow megakaryocytes was very low. Conversely, in other species such as rabbits and cats, the AchE activity of circulating platelets was found to be high while that of the RBC was low. Bone marrow studies of these animals revealed that the RBC precursor AchE activity was minimal while megakaryocyte activity was high. It was apparent to Zajicek that the only cell system in the bone marrow which always corresponded with the AchE activity of the circulating platelets was that of the megakaryocyte. He therefore concluded that platelets must arise from megakaryocytes.

In 1955, Humphrey provided immunological data to support Zajicek's conclusions. Humphrey produced antisera against rabbit platelets and labelled the antibodies with fluorescent dyes. When rabbit bone marrow preparations were stained with the antibody, fluorescence was seen only in the cytoplasm of megakaryocytes.

Since then, other techniques including direct visualization of platelet formation through bone windows (Kinosita and Ohno, 1961), have clearly demonstrated that Wright's proposal in 1906 had been correct and that platelets do originate from megakaryocytes.

Within the bone marrow, the first recognizable cell in the megakarycyte series is the basophilic (Stage I) megakaryoblast (Ebbe, 1970; Zucker-Franklin, 1970). These cells are smaller (25-30um) than the

more mature forms of megakaryocytes and have a high nuclear/cytoplasmic ratio. They comprise approximately 19% of the megakaryocytes in the marrow and are the only cells in the series which actively synthesize DNA. Polyploidy, a distinguishing feature of all stages of megakaryocytes is a result of nuclear replication in the blast stage without accompanying cytokinesis. The ploidy state of the megakaryocyte is important as it has been demonstrated that megakaryocytes with a ploidy of less that 8N do not produce platelets. In humans, the majority of marrow megakaryocytes (66%) belong to the 16N class while in other species such as the rabbit, most belong to the 32N class.

Megakaryoblasts mature into promegakaryocytes (Stage II; Ebbe, 1970; Zucker-Franklin, 1970) which make up about 25% of the marrow megakaryocyte population. These cells are much less basophilic than their precursor blast forms and have a small nuclear/cytoplasmic ratio resulting from extensive cytoplasmic growth.

The mature megakaryocyte (Stage III; Ebbe, 1970; Zucker-Franklin, 1970) makes up about 56% of the marrow megakaryocyte population and is the largest (100 μm) polymorphonuclear cell normally present in the bone marrow. A low nuclear/cytoplasmic ratio, lobulated nucleus with numerous indentations and loss of basophilia during the phase of platelet production characterize these cells.

The cytoplasm of the mature megakaryocyte is divided into three zones:

a) perinuclear zone
b) intermediate zone
c) marginal zone.

The perinuclear zone surrounds the nucleus and contains numerous ribosomes, mitochondria and rough endoplasmic reticulum. Thus the major synthetic and metabolic activity of the cell occurs in this area.

The intermediate zone comprises the major cytoplasmic portion and contains the granules characteristically found in circulating platelets. This area also contains the distinguishing feature of the megakaryocyte, the demarcation membrane system (DMS) composed of tubular invaginations of the cell membrane which approach the centre of the cell thereby greatly increasing the area of the surface membrane.

The marginal zone contains the fibrils resembling those found in circulating platelets. This zone is where thrombopoiesis takes place.

1.2 Platelet Formation and Release from Megakaryocytes

The process of platelet formation and release from the megakaryocyte was also first described by Wright (1906). He observed that megakaryocytes in the marrow maintained a close relationship with the blood vessels. As pseudopodia emerged from the megakaryocyte membranes, small portions were pinched off into platelet sized fragments which subsequently entered the circulation. Larger segments of pseudopodia were also observed in blood vessels of the marrow and Wright speculated that they would probably undergo further fragmentation into platelets.

More recently, Kinosita and Ohno (1961), described similar findings after extensive observations through windows inserted into the marrow. As the marrow and blood vessels grew into the thin space between the windows, the process of platelet formation could be visualized directly.

Electron microscopy studies by Radley et al. (1980) clearly demonstrated how platelets were formed and released into the circulation from the mouse bone marrow. Megakaryocytes were found adjacent to the bone marrow sinusoids. Cytoplasmic processes extended from these megakaryocytes into the abluminal side of the endothelial cells lining the sinusoid to form migration pores through the cells. The cytoplasmic processes contained longitudinally oriented microtubules and all the other organelles such as secretory granules and dense tubular systems characteristically found in circulating platelets. The cytoplasmic processes were shown to constrict at the ends of platelet sized segments releasing the segment into the sinus. Large multi-platelet portions of megakaryocyte cytoplasm which undergo further fragmentation into platelets have also been found in the marrow sinusoids (Fedorko and Lichtman, 1982).

Most megakaryocytes shed only their cytoplasm into the bone marrow sinusoid leaving a bare nucleus surrounded by a thin rim of residual cytoplasm. The infrequent presence of bare megakaryocyte nuclei in normal marrow has led to suggestions that they are degraded by marrow phagocytes (Fedorko and Lichtman, 1982).

In dogs, it has been shown that a proportion of mature megakaryocytes pass through the endothelial cell migration pores and enter the peripheral circulation (Kaufman et al., 1965). These cells are trapped in the microcirculation of the lungs from where they continue to produce platelets.

In man, the bone marrow megakaryocyte matures in three days and a single cell produces an average of 4000 to 8000 disc-shaped anucleate

platelets. There are approximateley 250,000 to 450,000 platelets per microlitre of whole blood.

Circulating platelets are heterogeneous with respect to size, density, metabolism and function. Two hypotheses have been proposed to explain the origin of this heterogeneity.

Based on the experimental evidence that megakaryocytes of the higher ploidy classes (32N, 64N) produce larger platelets, Penington et al. (1976a, 1976b) and Harker and Finch (1969) suggest that platelet heterogeneity is a result of differences in characteristics of precursor megakaryocytes.

Alternatively, numerous other investigators (Karpatkin (1969a, 1969b; Ginsburg and Aster, 1972; Corash et al. 1978, 1982; George et al., 1976a, b; Rand et al., 1981) have provided evidence that the biological effects of aging in the circulation contribute significantly to the heterogeneity of circulating platelets.

Ginsberg and Aster (1972) prepared young and old rat platelets using platelet membrane antibody infusions and whole body irradiation techniques. They demonstrated that young platelets survived longer, were larger, and were functionally more responsive to ADP-induced aggregation in vitro. Biochemical analysis of young and old rat platelet populations demonstrated that younger platelets contained greater proportions of phospholipids, cholesterol and tyrosine. These workers were unable to demonstrate a density difference between young and old platelets, however, Ginsberg was able to calculate that 22% of the platelet mass was lost as platelets aged in vivo.

Metabolic and kinetic investigations of human platelets by Karpatkin

(1969a, b) have provided results similar to those observed in the animal studies.

Karpatkin (1969a, b) separated human platelets on the basis of their density on discontinuous fixed density oil gradients. He demonstrated that the young platelet population was enriched in large, heavy platelets while the older platelet population was enriched in smaller, lighter platelets. Metabolic measurement showed that the young platelets had increased rates of glycogenolysis, glycolysis and protein synthesis compared to older platelets. In addition, younger platelets contained increased concentrations of total adenine nucleotides and orthophosphate. Karpatkin concluded that in humans, young heavy platelets change with age *in vivo* to light, small platelets with diminished metabolic potential.

Animal experiments in which rabbit megakaryocytes were labelled *in vivo* with ^{75}Se-selenomethionine (Amorosi *et al.*, 1971) or ^{35}S, (Charmatz and Karpatkin, 1974; Rand *et al.*, 1981; Corash and Shafer, 1982) have shown that the labelled platelet subpopulation which first appears in the circulation is enriched in large, dense platelets. Subsequently, there was an apparent migration of the label intensity from the most dense platelet subpopulation to the least dense platelet subpopulation. Rand *et al.* (1981) also observed that the most dense platelet subpopulation had a significantly longer mean platelet survival time upon reinfusion into normal animals.

Based on the results of these density and metabolic studies, it was concluded that as platelets age in the circulation, they become less dense and that this was a major basis of platelet heterogeneity.

The observations that circulating platelets become less dense with time *in vivo* may also reflect repeated platelet participation in hemostasis or thromboembolic events since it has been demonstrated that when platelets are exposed *in vitro* to agents such ADP (Packham *et al.*, 1979; Gear, 1981) or thrombin (Cieslar *et al.*, 1979) they become less dense. In addition, several investigators have noted that increased proportions of least dense platelets were present in the circulation of animals (Packham *et al.*, 1979; Gear, 1981) or humans (Van Oost *et al.*, 1982) which had active thromboembolic disease.

These experimental findings were compatible with results from George *et al.* (1976a, 1976b, 1978a, 1978b) who simultaneously labelled platelet surface membrane glycoproteins with the non-penetrating compound ^{125}I-diazodiiodosulphanilic acid (DD^{125}ISA) and platelet cytosol with ^{51}Cr. They showed that as platelets aged in the circulation, they continually lost portions of the plasma membranes and became smaller in size.

Thus, the available evidence suggests that the newly formed platelet population released from megakaryocytes are enriched in large, dense platelets. As these platelets circulate, they continually interact with their environment. This results in depletion of their metabolic capacity as well as changes in their size and density.

1.3 Regulation of Thrombopoiesis

The number of circulating platelets is so precisely regulated that any significant change in peripheral platelet count is followed by rapid

adjustment in the rate of platelet production (Cooper, 1970).

In 1958, Keleman et al. suggested the term thrombopoietin for the hormone which regulates thrombopoiesis. The exact nature of the hormone has not been established although inactivation by trypsin suggests the molecule is primarily protein (Keleman et al., 1958). In addition, it has been established that thrombopoietin is distinct from erythropoietin, the physiological regulator of erythropoiesis produced in the kidneys. (Keleman et al., 1958, 1963)

Serum (Odell et al., 1961), plasma and urine (de Gabriele and Penington, 1967a, b) have all been investigated for the presence of thrombopoietin and the results have been as varied as the methods used. All investigators however, agree that thrombopoietic activity is detectable in plasma or serum of animals following thrombocytopenia.

Thrombopoietin initiates many changes in the megakaryocyte population in the bone marrow including increase in numbers, size and rate of maturation (Ebbe, 1970; Harker, 1968).

The spleen, kidney, pituitary and adrenal glands as well as platelets have all been considered as the source of thrombopoietic activity, however, the site has not been established.

Following studies in nephrectomized rats, de Gabriele and Penington (1967a, 1967b) concluded that kidneys were not essential for the production of thrombopoietin. However, recent work by McDonald (1975, 1980) clearly demonstrated that the medium from cultured human embryonic kidney cells contains a thrombopoietic factor which specifically stimulates platelet production in mice.

The influence of the spleen (Abilgaard and Simone, 1967; de

Gabriele and Penington, 1967a, b; Bessler et al., 1981) on thrombopoietic regulation has also been studied. Spleen homogenates, spleen extracts and numerous other splenic derivatives have been administered to animals to determine thrombopoietic effects. Conflicting data and lack of reproducibility between and within laboratories have not resolved the role of the spleen as a source of thrombopoietin.

de Gabriele and Penington (1967a, b), postulated that thrombopoietin may be bound or adsorbed to the platelet membrane. In this manner, the concentration of the hormone would be dependent on the number of platelets present in the circulation or sequestered in the spleen.

Podolsak et al. (1980) have also shown that a thrombopoietic stimulating factor may be contained in platelets. These investigators injected platelet homogenates into the circulation of rabbits and produced a significant increase in circulating platelet number within 5 days. The absence of hematocrit changes during this experiment supported their conclusion that the changes seen in platelet numbers were absolute rather than relative.

Despite the uncertainty in the literature as to the nature of thrombopoietin and its site of production, all the studies agree in one respect; there is an inducible hormone present in plasma which regulates the rate of platelet production.

1.4 Morphology of Platelets

Platelets are the smallest formed elements of the blood measuring about 2-4 μm in diameter. They are anuclear; they do not contain DNA;

nor do they synthesize significant amounts of protein.

Anatomically platelets can be divided into four major sections (White and Gerrard, 1982):

a) peripheral zone

b) sol-gel zone

c) organelle zone

d) dense tubular zone

1.4.1 Peripheral Zone

The outermost covering of the platelet is a glycocalyx rich in glycoproteins and glycolipids in which the receptors for various stimuli are located. Beneath the glycocalyx is the plasma membrane. This is a typical unit membrane derived from the demarcation membrane of the megakaryocyte. The plasma membrane is rich in phospholipids and is closely associated with a submembranous region rich in microfilaments. The peripheral zone of the platelet also contains the surface connected canalicular system which consists of deep invaginations of the surface membrane forming a system of channels through the cytoplasm.

1.4.2 Sol-Gel Zone

The sol-gel zone consists of numerous contractile actin-myosin elements in various states of polymerization. A circumferential band of microtubules which circles the platelet in the equatorial plane exists immediately beneath the plasma membrane. Together, the contractile elements and microtubules comprise the cytoskeleton that maintains the normal discoid shape of unstimulated platelets. Glycogen particles are also apparent in this zone.

1.4.3 Organelle Zone

Collectively, the numerous formed elements within the platelet cytoplasm constitute the organelle zone. These structures include a few small mitochondria, some peroxisomes and the organelles (α-granules, dense bodies and lysosomes) involved in the platelet release reaction.

The electron opaque dense bodies primarily store 5-hydroxytryptamine (5-HT), adenine nucleotides and the bivalent cations Ca^{++} and Mg^{++}.

The α-granules are storage sites of many types of high molecular weight protein molecules including platelet specific proteins such as β-thromboglobulin (βTG), platelet factor 4 (PF4) in addition to the platelet derived growth factor (PDGF).

The contents of the lysosomal granules are released from platelets by strong stimulating agents and include proteases, glycosidases phosphatases and sulphatases.

Table 1.1 lists the organelles found within platelets and the various constituents contained in them.

1.4.4 Dense Tubular Region

The dense tubular system of platelets is thought to be derived from the smooth endoplasmic reticulum of megakaryocytes (Daimon and Gotoh, 1982). The tubules form a randomly dispersed system of channels throughout the platelet cytoplasm. They maintain a close relationship with the surface connected canalicular system at the cell periphery. The dense tubular system is the primary site of intracellular Ca^{++} sequestration and contains the enzymes of prostaglandin synthesis.

The ultrastructural components in the different zones of normal,

Table 1.1

Platelet Organelles and Their Constituents

Organelle	Constituent	
Dense bodies	5-HT, catecholamines, histamine	
	ATP, ADP, GTP, UTP	
	Calcium, magnesium	
	Pyrophosphate	
	Antiplasmin	
α-granules	Platelet factor 4 (PF4)	
	β-thromboglobulin (βTG)	
	Thrombin sensitive protein (TSP) (or glycoprotein-G)	
	Platelet Derived Growth Factor (PDGF)	
	Fibrinogen	
	Chondroitin-4-sulphate (PF4 carrier)	
	Fibronectin (cold insoluble globulin)	
	Factor V	
	Factor VIII-von Willebrand Factor	
	Permeability Factor	
	Bactericidal Factor (β lysin)	
	High molecular weight kininogen	
Lysosomes	Proteases	collagenase
		elastase
		cathepsin A, D, E
		neutral protease
	Glycosidases	β-glucuronidases
		β-galactosidase
		β-N-acetyl glucosaminidases
		α-mannosidase
		endoglucosidases
	Phosphatases	acid phosphatase
	Sulphatases	

from da Prada et al., 1982; Niewiarowski and Kodungallore, 1982; Holmsen, 1982.

unactivated platelets are shown in Figure 1.1.

1.5 Platelet Function

1.5.1 Maintenance of Endothelial Integrity and Hemostasis

Experimental evidence and clinical evidence showing morphological endothelial cell changes in thrombocytopenic animals and humans have led to suggestions that platelets play a major role in the maintenance of endothelial cell integrity and that there is a constant demand for normal numbers of platelets in the circulation (Danielli, 1940; Woods et al., 1953).

In 1969, Gimbrone et al. demonstrated that the extent and frequency of endothelial cell damage in perfused organs was significantly reduced when the perfusate contained intact platelets. More recently, endothelial cell culture techniques have shown that cell numbers, cell shape, DNA synthesis and the ability to migrate into a culture scratch wound were all enhanced when intact platelets were present in the culture medium (Maca et al., 1977).

It is not known what the platelet contributes to the endothelial cell or whether this interelationship contributes in some way to the eventual removal of platelets from the circulation. However, the requirement for intact platelets indicates that the active agent is not a secreted protein, rather, a trophic factor continuously supplied by the platelets themselves. The observations by Sweetman et al. (1981) that exogenously administered serotonin normalizes the vascular fragility of thrombocytopenic guinea pigs have led to suggestions that platelets may

Figure 1.1

Ultrastructural Features of Disc-shaped Platelets

The platelets were:

A) sectioned in equatorial plane

B) cross sectioned

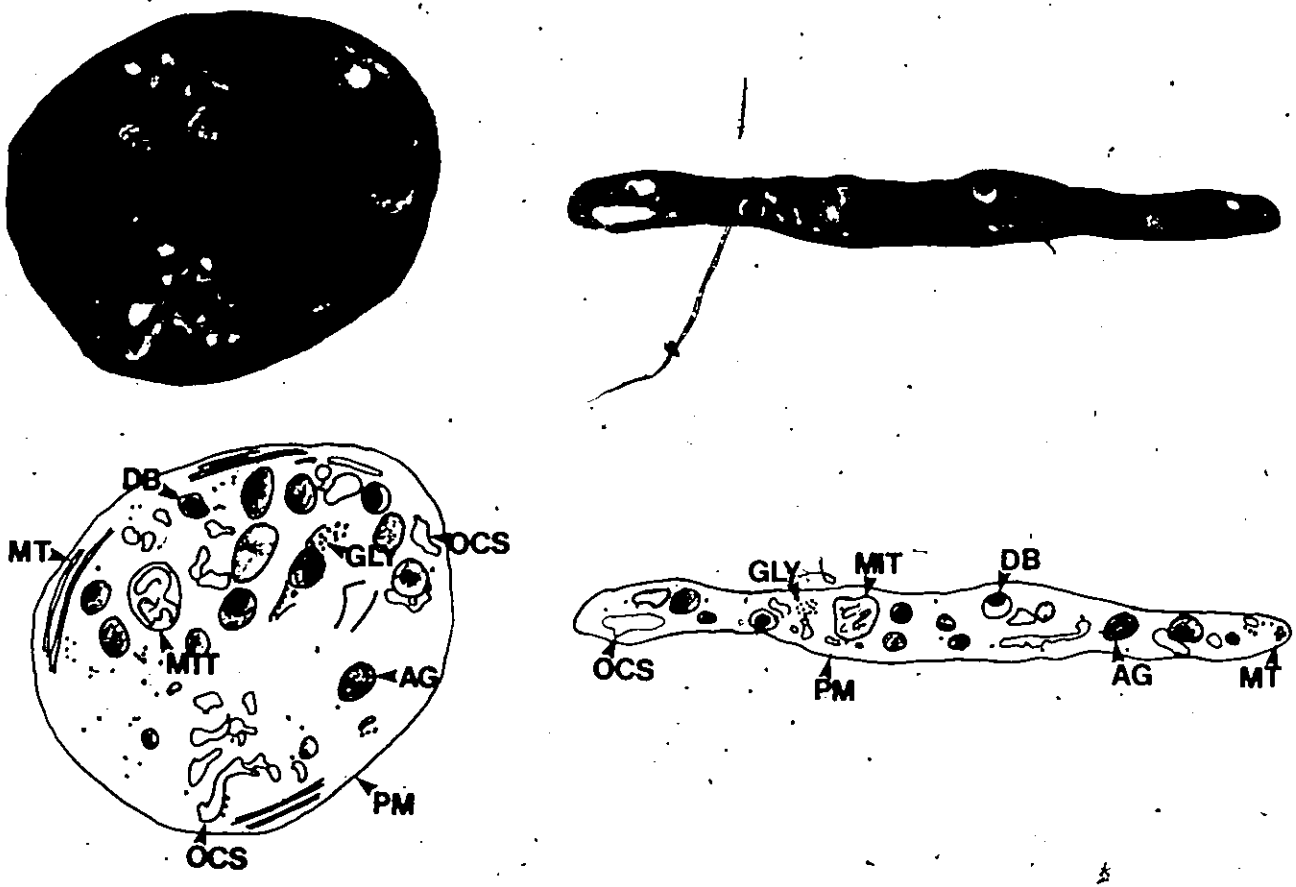
The diagrams C, D summarize the components of the different zones.

The peripheral zone includes the exterior coat, cell membrane (PM) surface connected canalicular system and submembranous filaments.

The components of the sol-gel zone comprise platelet matrix and contain actin-myosin elements, structural filaments and the microtubule band (MT) as well as some glycogen granules (GLY)

The organelle zone is composed of formed elements such as α-granules (AG), dense bodies (DB), lysosomes (LYS) and mitochondria (MIT) embeded in the sol-gel zone.

The dense tubular system maintains a close relationship with the open surface connected canalicular system (OCS).


A
B
DB
MT
GLY
OCS
MTT
AG
PM
OCS
C
GLY
MIT
DB
OCS
PM
AG
MT
D

transport serotonin to the microvasculature where it is taken up by endothelial cells.

In addition to a nurturing function, platelets also contribute to the maintenance of the vascular endothelium by forming hemostatic plugs at sites of vessel injury thereby inhibiting extravasation of blood (Bizzozero, 1882).

Normally, circulating platelets do not adhere to other blood cells, to each other, or to normal vascular endothelium.

When vessel injury occurs, the endotheliun is disrupted and subendothelial tissues such as collagen, microfilaments and basement membrane are exposed to the blood.

Circulating platelets adhere to these components and undergo shape change by extending pseudopods from their surface membranes. Shape change probably involves mobilization of membrane Ca^{++} and alteration of the microfilament contractile system beneath the plasma membrane (Carroll et al., 1982; Gerrard et al., 1981).

The adherence of platelets to collagen under conditions of high shear is mediated by von Willebrand factor (FVIII-VWF) produced primarily by endothelial cells (Jaffe et al., 1973). The importance of this factor in the adhesion process is exemplified in patients with von Willebrand disease. The characteristic bleeding disorder in these individuals is due to decreased concentrations of plasma FVIII-VWF factor resulting in defects in platelet adhesion and subsequent platelet plug formation. It is thought that platelet adhesion to subendothelium under conditions of high shear is due to an interaction between platelet membrane glycoproteins and VWF adsorbed to exposed subendothelial tissue (Sixma et al., 1982; Baumgartner et al. 1981).

Although various types of collagen differ in their ability to stimulate platelets (Chiang et al., 1980; Barnes, 1981), some of the collagen types (type I, type III) found in the vessel wall can cause platelets to undergo the release reaction. The release reaction probably involves mobilization of intracellular Ca^{++} from the dense tubular system (Gerrard et al., 1981; Carroll et al., 1982). During the release reaction, the subcellular organelles (dense bodies, α-granules, lysozomes) undergo internal reorganization and become centralized by a network of contractile filaments and the band of circumferential microtubules. The organelle membranes fuse with the surface connected canalicular system and the contents of the granules are expelled to the platelet exterior (White and Gerrard, 1976, 1982: Carroll et al., 1982).

The release of the contents of platelet granules follow different kinetics (Holmsen and Day, 1970; Witte et al., 1978; Kaplan et al., 1979; Niewiarowski, 1982). Primarily, the constituents of the dense bodies and α-granules are released. With higher concentrations of stimulating agents, the proteolytic contents of lysosomal granules are also released (Holmsen and Day, 1970).

Adherence of platelets to collagen also stimulates platelet phospholipases which cleave arachidonic acid from membrane phospholipids. Arachidonic acid is metabolized by enzymes of the prostaglandin system to the pro-aggregatory endoperoxides PGG_2 and PGH_2 and finally to thromboxane A_2 (TXA_2; Moncada and Vane, 1982).

Released ADP and TXA_2 formed by activated platelets act synergistically and cause other platelets in the vicinity to aggregate around adherent platelets and to undergo the release reaction. Released

serotonin (5-HT), as well as enhancing further platelet aggregation also acts as a vasoconstrictor thereby reducing blood loss (D'Amore, 1978).

In addition to the ultrastructural and metabolic changes in platelets at sites of vessel injury, the blood coagulation system also is activated. The release of tissue factor from injured endothelial cells initiates the extrinsic pathway of coagulation by acting as a cofactor for the activation of factor VII. The intrinsic system of coagulation is initiated by the exposure of the negatively charged structures in the sub-endothelium resulting in contact activation of several plasma proteins including kallikrein, high molecular weight kininogen and Factor XII.

The simultaneous activation of both the extrinsic and the intrinsic coagulation pathways may result in localized thrombin generation at sites of injury.

Thrombin has numerous functions in the process of hemostasis. Thrombin causes additional platelets flowing past the injury site to change shape and aggregate around the adherent mass. Thrombin also stimulates the release reaction and the prostaglandin pathway resulting in the liberation of additional ADP and TXA_2. As well, thrombin cleaves fibrinogen to fibrin monomers which polymerize in and around the platelet mass to provide structural stability.

Thrombi which form at injury sites may undergo transformation and become incorporated into the vessel wall (Jørgensen et al., 1967). Alternatively, thrombi may undergo dissolution by the action of proteolytic enzymes such as plasmin generated at the sites of injury or by proteolytic enzymes released from platelets and leucocytes.

Plasmin digests the fibrin in and around the platelet aggregates and disrupts the stability of the thrombus. Portions of the weakened thrombus may embolize to the distal microcirculation and some of the platelet aggregates may undergo de-aggregation. Some of the freed or de-aggregated platelets may return to the circulation.

1.5.2 Platelets as a Source of Phospholipids and Coagulation Factors

Activated human platelets release coagulation proteins such as Factor V (FV), Factor VIII von Willebrand factor (FVIII-VWF, dependent upon species), kininogen and some fibrinogen from their granules (da Prada, 1982 et al.; Walsh, 1982; Niewiarowski and Kodungallore, 1982; Schmaier et al., 1983). Small amounts of thrombin generated at sites of injury cleave and activate some of these released factors.

As well, when platelets are activated by adhesion to collagen or by thrombin, the acidic phospholipid, phosphatidyl serine, is translocated from the inner leaflet of the platelet membrane to the outer surface (Bevers et al., 1982). Together with phosphatidyl inositol and phophatidyl ethanolamine these phospholipids form platelet factor 3 (PF-3). PF-3 provides the catalytic surface which binds activated coagulation factors Va and Xa in close proximity to each other in order to optimize the interactions necessary for prothrombin conversion to thrombin (Kane and Majerus, 1982; Marcus et al., 1982; Bevers et al., 1982).

1.5.3 Platelet Phagocytic Function

Many substances that adhere to the platelet plasma membrane can

eventually be found within the platelet cytoplasm. Large latex particles, ferritin, mycoplasmas, viruses, immune globulins and chylomicrons have been shown to be endocytosed by platelets (Zucker, 1981).

Interiorization of large particles such as latex is associated with the platelet release reaction but soluble substances are internalized without simultaneous degranulation.

The uptake of particles by platelets is energy dependent and can only be arrested by inhibition of glycolysis and oxidative phosphorylation (Zucker, 1981).

Bacterial endotoxin and bacterial products are also endocytosed by platelets (Jirillo et al., 1982) and it has been suggested that platelets represent a physiological clearing mechanism whereby noxious agents are removed from the circulation as platelets are cleared.

Whether the process of platelet endocytosis is completely comparable to leucocyte phagocytosis remains unresolved although platelets have been shown to migrate in vitro toward stimulants such as collagen (Lowenhaupt et al., 1982).

1.5.4 Platelet Secretion

Blood platelets secrete three main classes of proteins of biological significance.

The first class of proteins secreted from platelets are similar or identical to plasma proteins such as coagulation factors, fibronectin, fibrinogen, α_2-macroglobulin and α_1-antitrypsin. The importance of some of these proteins during the process of hemostasis have been described previously (see Section 1.5.1).

The second class of secreted proteins includes enzymes such as collagenase (Chesney et al., 1974) elastase (Robert et al., 1971; Legrand et al., 1977) and acid hydrolases (da Prada et al., 1982; Niewiarowski and Kodungallore, 1982). Collagenase and elastase may have important functions in limiting thrombus formation by proteolytically altering exposed subendothelial tissue. Acid hydrolases have been implicated as mediators of additional endothelial cell damage at sites of vessel injury (Robert et al., 1971; Chesney et al., 1974). In addition, acid hydrolases may play a significant role in thrombus dissolution since it has been shown that the rate of clot lysis in vitro is increased if clots had previously been formed in the presence of platelets (Taylor and Muller-Eberhard, 1970).

The third class of secreted proteins is specific to platelets and function biologically in a variety of ways. This class of protein includes β-thromboglobulin (βTG), platelet factor 4 (PF4), platelet derived growth factor (PDGF) and platelet basic protein.

PF4 has an important role in mediation of the inflammatory response at sites of vessel injury by its positive chemotactic action on polymorphonuclear leucocytes, monocytes (Deuel et al., 1981) and fibroblasts (Senior et al., 1983). As well, PF4 neutralizes the anti-coagulant and growth inhibitory activity of heparin.

Platelet derived growth factor is thought to be the primary mitogenic component in whole blood which stimulates rapid reproduction of tissue cells in culture. In vivo PDGF is chemotactic for smooth muscle cells and fibroblasts and thus has an important role in tissue repair and

wound healing (Ross, 1981; Seppa et al., 1982). Moreover, PDGF has been implicated as the mediator of smooth muscle cell proliferation characteristically seen in many atherosclerotic lesions.

Very recently, Doolittle et al. (1983) demonstrated that the amino acid sequences of PDGF bear a striking homology to the sequences encoded within an open reading frame of the oncogene v-sis from the simian sarcoma virus. This observation raised the possibility that the transforming virus acquired its oncogenic gene from the cellular genes which encode PDGF. This observation is relevant to the etiology of atherosclerosis since Benditt (1977) and Pearson et al. (1979) have both demonstrated that organizing arterial thrombi and atherosclerotic lesions have monoclonal characteristics.

1.6 Investigation of Platelet Survival

Numerous procedures have been developed to investigate platelet survival time. Differences in methodology probably account for the variable results encountered in the literature. The following sections will therefore describe:

a) the various experimental techniques which have been used to measure platelet survival and some of the limitations of these techniques

b) the methods which have been used to analyze and calculate platelet survival time.

1.6.1 Depletion Studies

In the early 1900's, Duke (1911) and Firket (1922) performed some of the first studies designed to measure platelet survival time. Animals (dogs and rabbits) were rendered thrombocytopenic by repeated transfusions of platelet-deficient blood. Within 3 to 5 days after the last exchange transfusion, a normal platelet count was restored and was thought to represent the rate of platelet synthesis in the bone marrow. From these estimates it was inferred that approximately 30% of the circulating platelet number was manufactured daily, balanced by a similar rate of destruction. However, the circulating platelet deficit induced by thrombopheresis renders this method unsatisfactory for studying platelet survival as the background demand for platelets by the vessel endothelium may influence survival measurements. As well, the platelets produced in the marrow under 'stress' conditions may have different survival profiles from normal platelets.

Shouse et al. (1931) studied platelet survival time in dogs which had been subjected to whole body radiation. The time of complete platelet disappearance from the circulation (7 to 8 days after irradiation) was thought to represent platelet survival time. Mean platetet survival values obtained using this procedure are also inconclusive as whole body radiation may affect organs which influence platelet survival and destruction. In addition, the time at which the bone marrow megakaryocytes are completely suppressed following radiation is not clearly defined.

1.6.2 Transfusion Studies

Platelet lifespan also has been determined by transfusing platelet-rich whole blood from polycythemic patients into individuals with thrombocytopenia. Daily platelet counts determined the time it took for transfused platelets to disappear from the circulation and the time was considered to represent normal platelet lifespan (Stefanini and Chatterjea, 1951; Mueller, 1953). The disadvantage of using this method is that the underlying causes of thrombocytopenia such as isoimmunization, hemorrhage or disease processes may play a major role in the mechanism leading to platelet destruction. Transfused platelets would also be affected by these factors and their apparent survival altered. Furthermore, platelets from some patients with polycythemia have been reported to be abnormal (Davey and Lander, 1964). The circulating platelet deficit in thrombocytopenic recipients will also enhance the loss of transfused platelets. The additional limitations imposed on this method by the inaccuracies of visual platelet counting make this procedure unacceptable for studying platelet survival time.

1.6.3 Platelet Acetylation Studies

Recently, Stuart et al. (1975) developed a method for studying platelet survival making use of the effects of aspirin on platelets. This method has proven successful for use in children and pregnant women in whom radionuclide studies are contraindicated. Aspirin irreversibly acetylates the platelet enzyme cyclooxygenase necessary for the normal metabolism of arachidonic acid to its end products which include malondialdehyde (MDA). The degree to which MDA formation is inhibited in

aspirin treated individuals, plotted against time, gave an estimate of the proportion of circulating platelets which had been previously exposed to aspirin. The rate of disappearance of aspirin treated platelets from the circulation can be determined as well as the rate of appearance of new platelets. The human platelet life-span (8-11 days) determined by this method is in close agreement with survival studies using radionuclide labels.

The MDA regeneration time as a procedure for measuring platelet survival time has some limitations. It can be used only on individuals who are not ingesting aspirin continually. The use of aspirin treated platelets is inappropriate for determining the effects of other drugs on platelet survival. The technical difficulty of measuring small amounts of MDA and the variable time range required to normalize MDA synthesis in platelets may make interpretation of results difficult (Baldini and Myers, 1980). Furthermore, the recent reports of a 24 to 48 hour delay in recovery from aspirin treatment has raised the possibility that aspirin may inhibit cyclooxygenase activity in megakaryocytes (Catalano et al., 1979). This would result in the release of new platelets into the circulation which are deficient in MDA production thus complicating survival measurements.

1.6.4 Radionuclide Studies

The first studies in which radionuclide labelling techniques were used for investigating platelet survival time were performed by Julliard et al. (1952; human platelets) and Mueller (1953; rabbit platelets) using radioactive phosphate ($Na_2{}^{32}PO_4$) as an in vitro label. Since then, many

investigators (Odell et al., 1955; Aas and Gardner, 1958; Najean et al., 1969a, b; Thakur et al., 1976) have developed procedures for labelling platelets in vivo or in vitro with radioactive compounds in order to study platelet survival.

Basically, two methods have been used to label platelets.

In the first method, a cohort (platelets of the same age) is labelled in vivo by isotopes which are incorporated into developing megakaryocytes (Odell et al., 1955; Amorosi et al., 1971; Najean et al., 1969a, b). Subsequently, when the labelled megakaryocytes mature and liberate platelets, a peak of circulating platelet radioactivity is reached which must be determined by careful blood sampling.

^{75}Se-selenomethionine (^{75}Se; Najean et al., 1969a, b; Cohen et al., 1965; Dassin et al., 1979) and ^{35}Sulphur ($Na^{35}SO_4$; Odell, 1955 et al.; Rand, 1982) have been use with some success as cohort labels. However, due to limitations of radioactive dosage, use of these two isotopes for in vivo cohort labelling is almost exclusively limited to animal studies.

In vivo cohort labelling is also limited by the fact that little is known about the fate of labelled materials during normal platelet metabolism. Reutilization of the label from effete platelets may necessitate the application of correction factors in the calculation of survival time (Odell et al., 1955; Amorosi et al., 1971; Dassin et al., 1979). Furthermore, neither the period of time in which the label is available to megakaryocytes, nor the actual time of platelet labelling is clearly defined when using cohort labels in vivo.

In the second method commonly used to label platelets, the radioactive isotope is randomly attached to all platelets either in vivo

or in vitro, independent of platelet age. This method is known as 'random' or 'population labelling'.

^{14}C-hydroxytryptamine (^{14}C-5HT) was thought ideal for this purpose because of its specificity for platelets and ease of labelling. However, platelets rapidly exchange and reutilize this isotope rendering it unsatisfactory as a random label (Zucker et al., 1964; Najean et al., 1969a; Cohen et al., 1965).

^{32}P as $Na_2{}^{32}PO_4$ has been used as an in vivo population label to study platelet survival in humans, rabbits and dogs (Julliard et al., 1952; Mueller, 1953; Adelson et al., 1961; Baldini and Myers, 1980). Elution and reutilization of this isotope limits its usefulness in platelet survival studies.

Diisopropylfluorophosphate (DFP) labelled with ^{32}P, ^{3}H, or ^{14}C, labels all circulating platelets by binding to serine residues of esterases (Leeksma and Cohen, 1956; Geissinger et al., 1963; Bithell et al., 1967). Of the three isotopes, DF^{32}P has been the most commonly used.

DFP has a significant advantage as a population label since it is an in vivo label and this reduces possible platelet damage associated with in vitro manipulations. Unfortunately, labelled DFP is taken up by circulating erythrocytes and leucocytes as well as plasma proteins. Therefore, the labelled platelets must be separated from other blood elements before platelet-associated radioactivity is determined. This introduces possible technical difficulties since the platelet isolation procedure may provide platelets that are not representative of the total population.

DFP may also label megakaryocytes resulting in labelled young platelets continually entering the ciculation and lengthening the apparent platelet survival time.

Furthermore, it has been demonstrated that ^{32}P, a metabolic product from $DF^{32}P$, is reutilized and thus presents limitations to survival studies (Geissinger et al., 1963; Gardner and Cohen, 1966; Cooney et al., 1968; Baldini and Myers, 1980).

In vitro labelling techniques require that platelets are first isolated from the circulation.

Early methods of platelet collection, using ethylene diamine-tetraacetic acid (EDTA) as anticoagulant resulted in sequestration of large numbers of labelled platelets when they were reinfused (Davey and Lander, 1964; Gardner and Cohen, 1966; Kinlough et al., 1966). After this initial sequestration, only 30% of the labelled platelets returned to the circulation.

Improved handling techniques and the use of acid-citrate-dextrose (ACD) anticoagulant at pH 6.5 (which prevents shape change and aggregation; Aster, 1972; Baldini, 1968) have overcome largely the sequestration problems. The net result of these improvements is that between 70-90% of platelets labelled in vitro can be recovered in the circulation within two hours after injection.

In 1958, Aas and Gardner introduced the use of ^{51}Chromium (^{51}Cr) as an in vitro label for platelets. Subsequently, ^{51}Cr has become one of the most widely used isotopes for random labelling of platelets for survival investigations.

^{51}Cr in the hexavalent state as $Na_2{}^{51}CrO_4$ is actively transported

into platelets primarily in an energy consuming process (Tsukada et al., 1971). Within the platelet, ^{51}Cr is reduced to a stable trivalent state (Davey and Lander, 1964; Ebbe et al., 1965).

Seventy percent of the ^{51}Cr taken up by platelets binds to cytoplasmic components, particularly cytoplasmic nucleotides which bind 50-60% (Steiner and Baldini, 1970; Baker et al., 1977; Baldini and Myers, 1980). Platelet granule nucleotides do not become labelled so that platelet aggregation and release in response to agonists such as thrombin or collagen fails to free significant amounts of ^{51}Cr from pre-labelled platelets. ^{51}Cr is lost from platelets when lysis occurs but in its reduced state it cannot be reutilized (Steiner and Baldini, 1970).

Whether or not labelled platelets elute loosely bound ^{51}Cr has remained a matter of controversy. Davey and Lander (1964) and Tessier et al. (1974) reported that as much as 10-20% of the label elutes immediately after autologous ^{51}Cr labelled platelets were infused into humans or rabbits. After this time, further elution did not occur. This may be a result of poor labelling technique as Steiner and Baldini (1970) have shown that the elution of loosely bound ^{51}Cr is reduced to less than 3% per day (in vitro) when platelets are adequately washed after the labelling procedure.

Platelets in balanced salt solution, labelled with ^{51}Cr, retain normal aggregation responses (Ljungqvist and Bergentz, 1971; Bjornson, 1974) and their in vivo hemostatic capabilities are comparable to non-labelled platelets (Bjornson and Aursnes, 1977). Kattlove and Spaet (1970) have shown that ^{51}Cr inhibits collagen induced aggregation. However, Kattlove pointed out that the concentration of ^{51}Cr necessary

for this inhibitory effect were 10 to 100 times greater that the concentration of ^{51}Cr bound under usual labelling conditions.

One of the few limitations noted with the use of ^{51}Cr is the poor labelling efficiency. Labelling rarely exceeds 20%, thus necessitating large volumes of blood to achieve sufficient platelet-associated radioactivity for survival studies. As well, the low gamma emission (9% at 320 keV) limits the energy available for scintillation camera imaging. Furthermore, the long half of ^{51}Cr (27.7 days) subjects recipients of labelled platelets to extended periods of unecessary radiation (Heaton et al., 1979).

More recently, procedures using ^{111}Indium-oxine (^{111}In) have been developed for use as a random in vitro label for platelets.

Human (Scheffel et al., 1979; Heyns et al., 1979, 1980) and animal platelets (Thakur et al., 1976, 1983; Scheffel et al., 1977; Joist et al., 1978; Lotter et al., 1980) have been successfully labelled with this compound at an efficiency approximating 90%. This high labelling efficiency reduces the blood volumes required for platelet survival studies.

^{111}In-oxine, is a lipid soluble complex and is absorbed by platelets in a non-energy requiring mechanism. Within the platelet cytosol an exchange reaction takes place between the oxine carrier and platelet subcellular components which chelate indium more strongly than the oxine (Hudson et al., 1981). ^{111}In uptake is independent of the status of the platelet granules since thrombin degranulated platelets (which have lost greater than 80% of their labelled ^{14}C-5HT granule content) are labelled with ^{111}In in similar fashion to intact platelets

(Joist et al., 1978). Moreover, ^{111}In-labelled platelets respond normally to aggregating and release inducing agents such as ADP, thrombin and collagen (Scheffel et al., 1979; Heyns et al., 1980).

Elution of ^{111}In from labelled platelets is negligible and the high gamma photon yield (89% at 173 keV, 94% at 247 keV; Thakur, 1983) facilitates scintigraphic visualization of platelet distribution in vivo as well as platelet deposition at sites of vascular lesions (Thakur et al., 1976, 1983; Davis et al., 1978; Heaton et al., 1979; Scheffel et al., 1979; Callow et al., 1980).

The survival time of ^{111}In-labelled platelets is similar to that observed with ^{51}Cr-labelled platelets and some investigators have reported higher platelet recovery values when using ^{111}In (Davis et al., 1978; Heaton et al., 1979; Callow et al., 1980). As well, the short half life of ^{111}In (2.8 days) may be preferable in human studies where it is desirable to minimize radiation dosage (Thakur, 1983).

One of the few limitations noted with ^{111}In is the fact that plasma transferrin competes for the isotope thereby reducing the labelling efficiency (Scheffel et al., 1979). This may present some labelling efficiency problems in human studies in which platelets routinely are labelled in small volumes of autologous platelet poor plasma and where the number of platelets available for labelling is often reduced.

Very recently, Baker et al. (1982) and Joist et al. (1983) have suggested that platelets from different species bind ^{111}In differently and that binding is dependent on the suspending media in which the platelets are labelled. Rabbit and human platelets labelled in platelet poor plasma primarily bound ^{111}In in cytoplasmic components in a

non-releasable form. Rabbit platelets labelled in modified Tyrode's solution bound ^{111}In into dense granules such that 50% could be released upon thrombin stimulation under conditions in which platelet lysis did not occur (Baker et al., 1982).

Similar studies by Rand (1982), could not confirm Baker's findings. Rand demonstrated that rabbit platelets which had been doubly-labelled with ^{51}Cr and ^{111}In in a balanced salt solution lost less than 5% of either isotope after stimulation with thrombin at a concentration (0.5 U/mL) which caused greater than 90% secretion of the contents of the platelet dense granules. The reasons for these conflicting results have not been resolved.

1,7 Recommended Methods for Platelet Survival Studies

In the past, the numerous methods which have been developed for platelet survival studies have resulted in significant differences between results from various laboratories. For this reason, the International Committee for Standardization in Haematology (1979) has recommended technical and analytical procedures for radionuclide platelet survival studies. The methods were described for use in humans and stress the importance of:

a) sterile techniques throughout

b) ACD (Aster and Jandl, 1964) as the anticoagulant for collection of whole blood from which platelets are to be isolated

c) using autologous platelets wherever possible or, ensuring ABO and Rh compatability if homologous platelets are used

d) platelet isolation techniques which minimize contamination by erythrocytes

e) ^{51}Cr as the radiolabel, (note that these recommendations were made before the ^{111}In methodology became available and all other recommendations are applicable to studies in which ^{111}In is used)

f) maintenance of frequent sampling times, particularly in the first 24 hours after infusion of labelled platelets

g) proper radioactivity counting techniques

h) analysis of data by unbiased methods such as the Gamma Function developed by Murphy and his colleagues (1969a; 1971a, b; 1973).

These basic procedures (with some modifications) were used in the present study to investigate mean platelet survival time in rabbits.

1.8 Analysis of Platelet Survival Data

The improved techniques developed for isolating platelets in balanced salt solutions (Ardlie et al., 1970) and use of labelling procedures with isotopes which do not elute or exchange in vivo have improved recovery and survival of labelled platelets in the circulation. However, the mathematical approaches used to analyse the resultant data vary considerably and it has been suggested that differences in data analysis contributes to variation in results (Hill-Zobel et al., 1982).

The graphic plot of circulating platelet radioactivity against time does not always result in a straight line. Some survival curves are exponential while others approach linearity. This diversity arises

from the variable physiological factors which contribute to the removal of platelets from the circulation by the reticulo-endothelial system (RES).

Early studies of platelet survival attributed the disappearance of platelets from the circulation to senescence (Ginsberg and Aster, 1972; Dassin et al., 1976). The fact that most survival curves had a linear pattern was thought to support this concept.

Davey and Lander (1964) and Ginsberg and Aster (1972) postulated that the discrepancy seen in survival curve shape of isotopically labelled platelets was related to the isotope used. If platelets were labelled with ^{51}Cr (a stable cytosol label which remains in platelets until removal from the circulation due to senescence), the resulting survival curve was linear. The exponential survival curves often observed with DFP labelling was attributed to the fact that DFP labels membrane esterases and portions of the platelet membrane may be lost as platelets age in the circulation.

Murphy and Francis (1969a) have pointed out that the only means by which platelet survival curves could have been linear was if all platelets live to exactly the same age. Any variation in the finite lifespan of the platelet would produce a non-linear function.

Several investigators (Davey and Lander; 1964; Murphy and Francis, 1969a; Mustard et al., 1963a) maintain that senescence of platelets is much less important in determining the fate of the platelet in the circulation. They suggest that platelet survival is primarily governed by a random destruction and consumption of platelets which occurs as a continuous process within the circulation. Their hypothesis states that

platelets die after a given number of cumulative injuries ("hits") such that both random destruction and senescence contribute to platelet removal from the circulation.

1.9 Calculation of Platelet Survival Time

When studying platelet survival, the disappearance rate of a total population of labelled platelets will give an estimate of the mean platelet survival time.

When analyzing platelet survival data, if the disappearance of radioactivity from the circulation plotted against time is linear, mean platelet survival is the point on the time axis intercepted by the line running through all the data points (Figure 1.2A).

The linear (or Gaussian) method is dependent upon the time at which the initial radioactivity sample is obtained, since the initial sample is arbitrarily considered as 100% circulating radioactivity, and all subsequent samples are expressed as a percentage of the initial sample.

The time span chosen to be considered from the initial value is also important as this time should encompass the total time in which radiolabelled platelets are present in the circulation.

With a curve or curvilinear pattern of radioactivity decline in the circulation, mean platelet survival time is estimated by drawing a tangent to the curve from the origin which is the initial circulating radioactivity sample assigned a value of 100%. The point where the tangent intercepts the time axis will give an estimate of mean platelet survival time (Figure 1.2B).

Figure 1.2

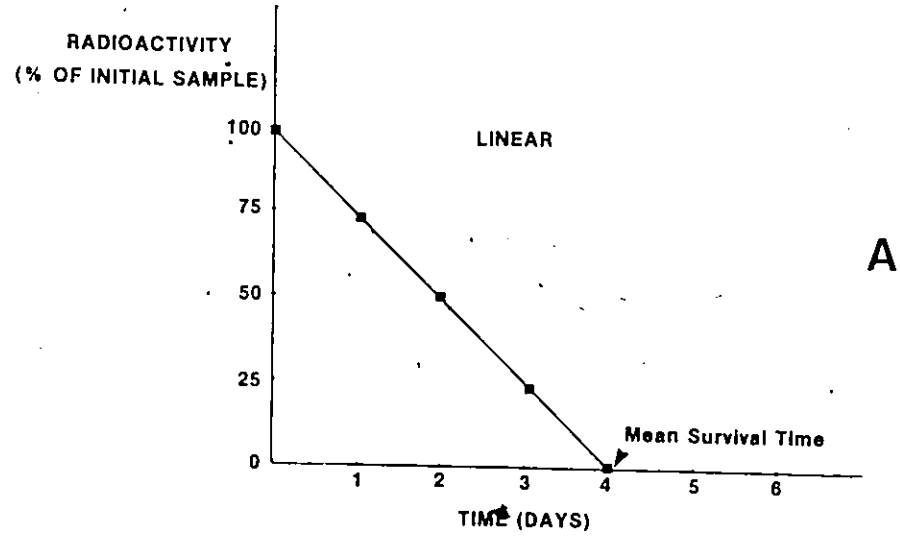
Mathematical Approaches Used to Calculate Mean Platelet Survival Time

This figure illustrates the patterns of radio-labelled platelet disappearance from the circulation and the different mathematical approaches which have been used to calculate mean platelet survival time.

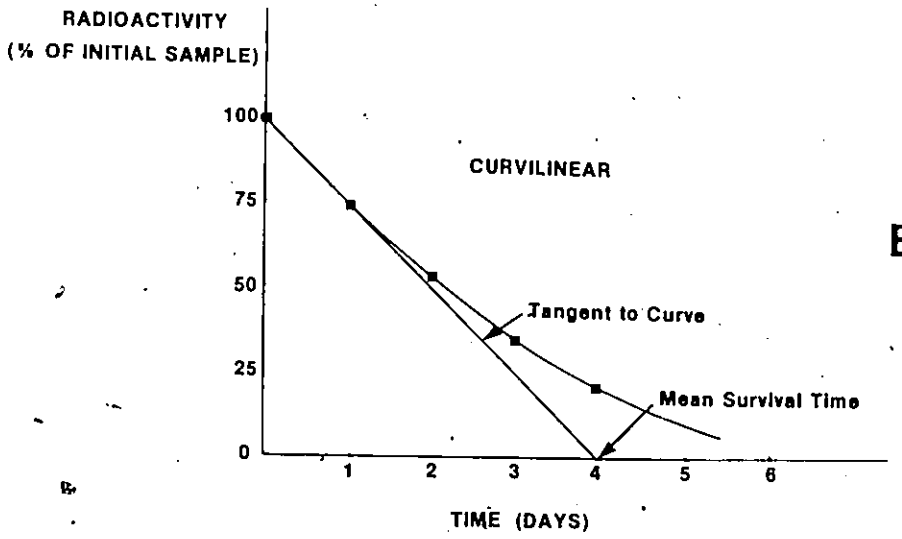
A) If the pattern is linear, mean platelet survival time is the point at which the line through all the data points intersects the base line.

B) If the pattern is curvilinear, mean platelet survival time is the point where a tangent drawn to the curve from time 0 intersects the base line.

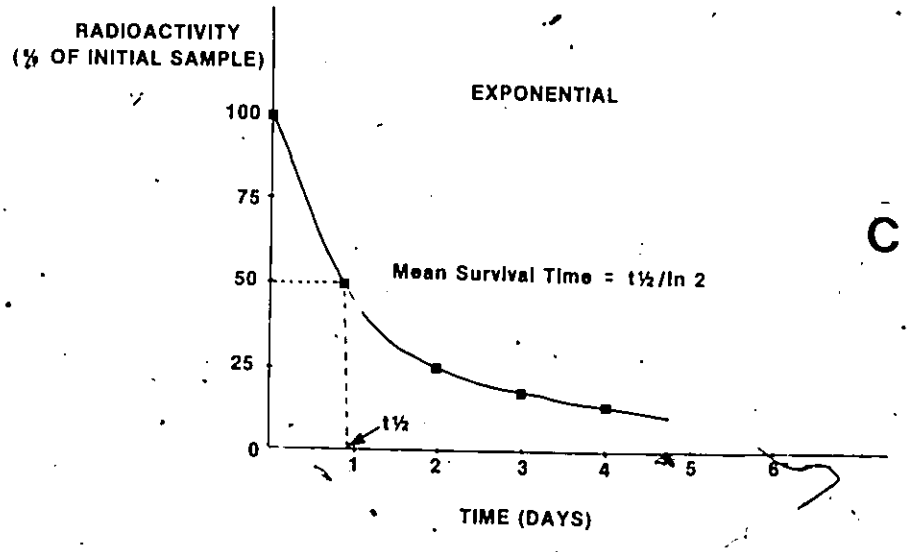
C) If the survival curve is exponential, platelet survival time is determined by calculating the platelet half-life and dividing by the natural logarithm of 2.


RADIOACTIVITY
(% OF INITIAL SAMPLE)
100
75
50
25
0
LINEAR
Mean Survival Time
1
2
3
4
5
6
TIME (DAYS)

A


RADIOACTIVITY
(% OF INITIAL SAMPLE)
100
75
50
25
0
CURVILINEAR
Tangent to Curve
Mean Survival Time
1
2
3
4
5
6
TIME (DAYS)

B


RADIOACTIVITY
(% OF INITIAL SAMPLE)
100
75
50
25
0
EXPONENTIAL
Mean Survival Time = t½/ln 2
t½
1
2
3
4
5
6
TIME (DAYS)

C

If the pattern of decline in radioactivity is exponential, platelet survival time can be estimated by calculating the half life and dividing it by the natural logarithm of two (Figure 1.2C).

The reliability of fitting lines to data by eye is subject to observer bias and may vary as much as 30% (Murphy et al., 1973). Consequently, mathematical approaches were developed which eliminated observer bias but allowed for variation in the physiological mechanisms which are likely to govern platelet survival in the circulation.

Calculation of platelet survival time using a gamma function least squares computer fitting procedure is based on the 'multiple hit model' introduced by Murphy, Francis and Bolling (1973). This model proposes that circulating platelets are randomly and repeatedly subjected to a number of insults ('hits'). This results in some damage to the platelets from which they recover sufficiently to continue to function in the circulation. Any intense 'hit' may be lethal while small 'hits' cause cumulative damage.

The gamma function includes a calculation which takes into account the intensity of a hit. This results in a continuous function which is flexible to different shapes of survival curves (linear, curvilinear or exponential) which may be encountered using total population labels.

The advantage of the gamma function is the fact that it does not require an initial assumption about the shape of the curve. Rather, with computer assistance, the best curve is fitted to the data points without observer bias.

It is now generally accepted that the objective analysis using this mathematical approach is the preferred method for calculating mean

platelet survival time.

1.10 Normal Platelet Survival Time

The original work using non-isotopic techniques to quantify platelet lifespan determined that the normal human platelet survives in the circulation for 5 to 10 days. More recently, the non-radionuclide, in vivo technique making use of the irreversible effect of aspirin on platelets (Stuart et al., 1975) has shown mean human platelet survival time to be 8 to 11 days.

Methods which employ radioisotopes that undergo minimal release, elution and re-utilization, have also demonstrated that human platelets normally survive 8 to 11 days in the circulation (Wakesaka et al., 1963; Baldini, 1968; Kutti and Weinfeld, 1971; Heyns et al., 1980). Minor variations may be noted depending on the radionuclide used and differences in methods of calculating the resultant data.

Studies performed in a variety of other species (including rabbits used in the present study) have shown that platelet lifespan is species specific.

Extensive work by Ebbe et al. (1965) in normal rabbits using four different experimental approaches including:

a) transfusion of non-labelled platelets into thrombocytopenic recipients

b) transfusion of ^{51}Cr-labelled platelet concentrates

c) transfusion of platelet concentrates previously labelled in vivo with ^{32}P-orthophosphate

d) transfusion of whole blood labelled in vivo with ^{32}P

all gave similar results of 3 to 4 days for mean platelet survival time in rabbits.

1.11 Site of Platelet Sequestration and Destruction

Radionuclide technology has made it possible to study accurately the normal sites of platelet sequestration and eventual destruction.

In the majority of platelet survival studies, particularly in human experiments, 5 to 10% of infused, labelled platelets are never recovered in the circulation despite optimum *in vitro* handling conditions. It is thought that this figure represents those platelets irreversibly damaged during the washing and labelling procedure such that they are permanently removed from the circulation.

Penny *et al.* (1966) and Najean *et al.* (1969a, b) have suggested that a dynamic equilibrium exists between platelets in the splenic pool and the platelets which circulate to maintain a constant platelet number. About 10 to 30% of the total body platelet mass is thought to reside in the spleen (Aster, 1972; Branehog *et al.*, 1973; Heyns *et al.*, 1980) and it is conceivable that platelet sequestration in this pool may account for the removal of labelled platelets from the circulation. Indeed, removal of the spleen has been observed to increase the recovery of labelled platelets from the circulation by as much as 20% (Kutti and Weinfeld, 1971; Aster and Jandl, 1964a, b).

The initial rapid elution of loosely bound radioactive ^{51}Cr has also been suggested as the mechanism for the apparent early loss of labelled platelets. However, Davey and Lander (1964) have shown that

this apparent elution occurred within a few minutes after the injection of labelled platelets; thereafter, the ^{51}Cr was platelet associated. In addition Davey and Lander noted that within 2 hours after injection of ^{51}Cr-labelled platelets a large proportion of labelled platelets returned to the circulation probably from sequestration sites.

Aster and Jandl (1964a, 1964b) using ^{51}Cr-labelled platelets and body surface scanning, demonstrated that platelets which had been mildly damaged were primarily sequestered amd destroyed in the spleen. These investigators also observed that severely damaged platelets were rapidly removed from the circulation by the liver. Body surface scanning techniques used in these procedures did not provide conclusive evidence since enlargement of the spleen or liver may have influenced the observed sequestration patterns (Aster and Jandl, 1964a).

Greenberg et al. (1975) and Kaplan and Saba, (1978) using animal models (rabbits and rats respectively) demonstrated that the removal of damaged platelets from the circulation was due to rapid clearance by the reticulo-endothelial system (RES) of the liver followed by low velocity clearance by the spleen. Heyns et al. (1980, 1982) found that the liver plays the most active role in platelet destruction and it is the liver which is the major site of accumulation of ^{111}In-labelled platelets.

The RES capacity of the liver can be impaired by infusing gelatinized lipid emulsion. The infused emulsion (90%) is preferentially sequestered by RES cells (Saba, 1970; Saba and Di Luzio, 1970) and blocks their normal clearance capacity. Pre-treatment of the liver in such a manner has been shown to reduce hepatic localization of platelets (Kaplan and Saba, 1978). Simultaneously, extra-hepatic platelet accumulation in the lungs and spleen was observed.

1.12 Factors Which Shorten Platelet Survival Time

Platelet survival time is shortened by a variety of factors. Most frequently, the causes involve intrinsic platelet abnormalities or result from platelets having interacted with an abnormal vasculature or artificial surfaces so that they become irreversibly modified or consumed.

1.12.1 Modification of Platelet Surface Membrane Glycoproteins

Modification of platelet surface membranes is associated with changes in mean platelet survival time.

The majority of proteins present at the mammalian cell surface are conjugated with carbohydrate prosthetic groups to form membrane glycoproteins. Of more than 100 different monosaccharides found in nature, only 10 occur in glycoproteins and glycolipids. Among these are glucose, fucose, mannose, galactose, glucosamine and sialic acid (N-Acetylneuraminic Acid; NANA; Bretscher and Raff, 1975). The oligosaccharide side chains of glycoproteins are usually highly branched and the monosacharides are bonded together via a variety of linkages. No serially repeating monosaccharide units are present and usually two to six different types of sugars form the carbohydrate moiety bound to the polypeptide chain. The terminal monosaccharide on the majority of glycoproteins is sialic acid which confers a net negative charge to the biological membrane (Jamieson, 1978).

Early studies of the composition of platelet membranes demonstrated that their major surface proteins are glycosylated and form a

pronounced fluffy glycocalyx around the platelet. Phillips (1972), Nachman and Ferris (1972), and Nurden et al. (1977) initially described three major glycoproteins (GP) on the surface of normal human platelets with apparent molecular weights ranging from 150,000 to 99,000. Phillips (1972) termed these glycoproteins as GPI, GPII, and GPIII and established the nomenclature whereby the GP with the highest molecular weight was termed GPI, the next lower weight GPII and so on.

Improved methods of resolution using sodium dodecyl sulfate polyacrilamide gel electrophoresis (reduced or non reduced; SDS-PAGE), lectin affinity chromatography and a variety of surface labelling techniques (George et al., 1976a, 1976b, 1978a, 1978b; Jenkins and Clemetson, 1977) have shown that platelet membrane glycoproteins are more numerous and complex than the three originally described. Now, within each major group of glycoproteins minor glycoproteins have been indentified (Phillips et al., 1975; Phillips 1980; George et al., 1976a, 1976b, 1978a, 1978b; Clemetson et al., 1979). The newly resolved species in each major molecular weight class of glycoproteins were given letters a, b and c. Confusion still exists in the current nomenclature since many investigators continue to delineate additional subgroups within each class as detection and isolation techniques vary and improve.

A surface bound GP which is functionally and immunologically related to GPI is lost from the platelet surface when the membranes are disturbed (Jamieson, 1978; Mosher et al., 1979; Phillips, 1980; Jenkins and Clemetson, 1977). This GP, termed glycocalicin or GPIs (soluble), contains approximately 10% sialic acid (Jamieson, 1978). Its loss is blocked by EDTA suggesting that a calcium activated protease may be

involved in its release.

Platelets also release GP from their granules. Upon thrombin or trypsin stimulation, thrombin-sensitive-protein (TSP), thrombospondin or GP-G (Phillips, 1972, 1980; George et al., 1978a, b) is released and binds to platelet membranes where it may be involved in platelet-platelet adhesion (Jaffe et al., 1982).

The glycoprotein composition of the cellular membrane influences cell survival in the circulation.

Initial studies by Jancik et al. (1975) and Aminoff et al. (1977) demonstrated experimentally that removal of as little as 10% of the normal sialic acid content of the erythrocyte (RBC) membrane resulted in premature clearance of these cells by phagocytic cells in the liver and spleen. Since older erythrocytes contain 10-15% less sialic acid than younger RBC, it was suggested that physiological removal of senescent RBC by the reticuloendothelial system (RES) may be a result of slow sequential desialylation as cells circulate (Jancik et al., 1975; Aminoff et al., 1977).

Similar to the observations in RBC, the glycoprotein composition - specifically, sialic acid content, on the surface of the platelet membrane plays a major role in the function and survival of these cells in the circulation.

Greenberg et al. (1977, 1979) studied the relationship between sialic acid residues on platelet glycoproteins and mean platelet survival time in rabbits. They found a negative direct correlation between the membrane concentration of sialic acid and the rate and extent of platelet clearance from the circulation. When as little as 8-10% of the total

membrane sialic acid was removed by treatment with proteolytic enzymes, platelets were cleared more rapidly from the circulation than untreated platelets. Removal of more than 15% of the membrane sialic acid resulted in complete clearance from the circulation within one hour after the platelet infusion.

Greenberg et al. (1977) also demonstrated that older platelets contain less sialic acid than younger platelets. This observation led Greenberg and his colleagues to conclude that the mechanism whereby senescent platelets are recognized and cleared from the circulation is dependent upon the sialic acid content of the surface membrane.

Additional studies by Greenberg et al. (1979) demonstrated that pretreatment of rabbit platelets with other proteolytic enzymes such as chymotrypsin, trypsin, or plasmin causes them to be cleared rapidly from the circulation by liver macrophages. Studies of membrane composition after proteolytic treatment showed that, depending upon the concentration of enzyme used, all three enzymes cleave large amounts of GPI and lesser amounts of GPII and GPIII from the platelet membrane (Phillips, 1978; Nurden and Caen, 1975; Greenberg et al., 1977, 1979;).

In contrast, platelets which were repeatedly stimulated with low concentrations of the proteolytic enzyme thrombin, or, subjected to a single treatment of high thrombin concentration (to cause them to lose almost all of their releasable serotonin) survived normally when returned to the circulation (Reimers et al., 1973). Examination of membrane GP from thrombin treated platelets have demonstrated that the concentrations of GPI and GPIII were not significantly different from untreated platelets, but the thrombin sensitive granule GP was released (Phillips,

1974) and a surface membrane GPV was modified (Phillips and Agin, 1974; Mosher et al., 1979). It was concluded by Reimers et al. (1973) that thrombin treatment has little effect on the platelet survival since thrombin does not modify any membrane GP that is important to the survival of platelets in the circulation.

Therefore, based on the available evidence, platelets which have their membranes modified experimentally by the removal of sialic acid (whether removed as sialic acid by neuraminidase or as a major glycopeptide by proteolytic enzymes) have a significantly shortened survival time in vivo.

The importance of membrane glycoproteins to platelet survival is exemplified by a number of congenital diseases in which platelet survival is shortened as a result of intrinsic defects in surface membrane glycoproteins.

Bernard-Soulier syndrome (BSS) is an inherited, autosomal recessive disease marked by thrombocytopenia, giant platelets and moderate to severe mucous membrane bleeding (Grottum and Solum, 1969; Lusher and Barnhart, 1977). The primary abnormality is a defect in platelet function characterized by decreased platelet adherence to subendothelial structures and reduced platelet agglutination in factor VIII:von Willebrand factor dependent systems (Caen et al., 1976; Nurden and Caen, 1978, 1979). BSS platelets were found to aggregate normally to ADP, collagen and thrombin (Jamieson et al., 1979; Hagen and Solum, 1978) although reduced thrombin binding has been described (Jamieson, 1978).

Platelet survival time was significantly shortened in patients with BSS (Grottum and Solum, 1969; Lusher and Barnhart, 1977; Nurden and Caen,

1979). Isologous platelets infused into BSS recipients survived normally while BSS platelets in normal recipients had a shortened survival time. These observations indicated that the defect which was responsible for reduced platelet survival was intrinsic to the platelet and was not a result of environmental effects on the platelets. Thrombocytopenia, characteristic in these patients, is thought to be due to the excessive rate of platelet removal from the circulation which exceeds the capacity of marrow production.

Analyses of platelet membrane GP from BSS platelets demonstrated specific abnormalities. Glycocalicin, the soluble portion of GPI was completely lacking and the major membrane GPI was decreased in concentration (Nurden and Caen, 1978, 1979; Caen et al., 1976; Solum et al., 1980). Since GPI contains the major portion of platelet membrane sialic acid, BSS platelets have less sialic acid. This may result in these platelets being recognized as foreign by the RES to be removed from the circulation prematurely.

Wiskott-Aldrich Syndrome (WAS) is also a congenital, sex-linked, autosomal recessive disorder affecting platelets. This disease is characterized by thrombocytopenia, eczema and disturbances in immunoglobulin synthesis (Parkman et al., 1981).

Platelets from patients with WAS are decreased in size by approximately 30%. Conflicting reports exist in regard to the organelle content and response to stimuli of WAS platelets. Lusher and Barnhart (1977) have reported reduced numbers of dense bodies, mitochondria and α-granules. White and Gerrard (1976) describe normal numbers of dense bodies, α-granules and mitochondria in patients with WAS whom they

investigated. WAS platelets have also been described to be functionally defective in energy metabolism. Aggregation to ADP, collagen and epinephrine has also been shown to be decreased (Lusher, 1977; Akkerman, 1982). White and Gerrard (1972) however, reported that WAS platelets respond normally to aggregating agents. The latter researchers suggested that the discrepancies in the results may result from lack of care during platelet concentration and isolation procedures.

Autologous, ^{51}Cr-labelled, WAS platelets have a decreased mean platelet survival time; shortened survival time is also observed when WAS platelets are infused into normal recipients. Furthermore, normal platelets maintain a normal lifespan when infused into WAS recipients (Abrahamson, 1970; Murphy et al., 1972). These observations indicated to investigators that the defect which shortened the survival of WAS platelets was intrinsic to the platelet and not due to in vivo modification of platelets by antibodies or proteolytic enzymes.

Studies of the GP profiles of WAS platelets demonstrated that GPIa and Ib were reduced while GPIIb and GPIIIa were normal (Parkman et al., 1981). In addition, bone marrow aspirates from WAS patients have shown large numbers of platelets phagocytosed by marrow macrophages and reticulum cells (White and Gerrard, 1976; Lusher and Barnhart, 1977).

It is likely that the significant reduction in membrane GP and concurrent decrease in sialic acid may make WAS platelets susceptible to removal by the RES.

Shortened platelet survival is also associated with a wide variety of other diseases such as cirrhosis of the liver (Kwaan et al., 1956; Bergstrom et al., 1960; Stein and Harker, 1982) and metastatic cancer

where circulating concentrations of proteolytic enzymes such as plasmin are often increased. These enzymes may cleave platelet membrane GP resulting in premature platelet clearance from the circulation.

It has also been suggested that the thrombocytopenia and shortened platelet survival associated with some viral diseases may be related to the ability of a a number of viruses to cleave sialic acid from platelet membrane glycoproteins (Larke et al., 1977; Scott et al., 1978).

1.12.2 Repeated Vessel Injury, Thrombosis and Thromboembolism

Platelet survival ~~is~~ shortened in a variety of conditions which cause repeated vessel injury, thrombosis and thromboembolism.

For many years it has been recognized that one of the major causes of mortality and morbidity in patients with homocysteinemia are complications resulting from thromboembolic vascular disease (Gibson, et al., 1964; Harker et al., 1974). Patients with homocysteinemia have an inborn deficiency in the enzyme cystathionine synthase which normally converts homocysteine (a metabolite of methionine) into cystathionine (Harker et al., 1974). Affected persons accumulate high concentrations of homocysteine and methionine in their plasma and they excrete homocystine in the urine.

Histological examination of vessels (particularly arteries) from these individuals demonstrate vascular changes including hyperplasia, focal and medial fibrosis and elastic degeneration (Gibson et al., 1964).

Harker and his associates (1974) and Rouland et al. (1982) observed that platelet survival was shortened in patients with homocysteinemia. Harker et al. (1974, 1976) therefore designed experimental studies to

examine the mechanism responsible for the reduction in platelet survival.

They continuously infused homocysteine into baboons over a 3 month period to maintain a range of plasma homocysteine concentrations similar to those found in humans with the disease. Examination of the aortic surface of these animals after the infusion period demonstrated that patchy endothelial cell desquamation had occurred over 10% of the vessel surface despite a 25 fold increase in the rate of endothelial cell regeneration. Platelet survival time was significantly shortened in these baboons and the degree of shortening correlated with the plasma homocysteine concentration and degree of endothelial cell loss.

Shortened platelet survival and increased platelet turnover were normalized in baboons when drugs such as dipyridamole and sudoxicam (known to inhibit platelet function) were administered in conjunction with the homocysteine infusion (Harker et al., 1976).

Harker also demonstrated that premature removal of platelets from the circulation was not a result of homocysteine induced toxic effects on platelets. In view of normal platelet function tests and increased platelet consumption, Harker proposed that shortened platelet survival in homocysteinemia was a result of increased platelet utilization in areas of chronic, chemically-induced endothelial cell damage.

More recently, two other groups (Uhlemann et al., 1976; Hill-Zobel et al., 1982) have been unable to confirm that platelet survival is significantly shortened in humans with homocyteinemia.

Methodological differences used to analyse the platelet survival curves are probably responsible for the discrepancy in results.

When the original human studies were performed by Harker the

disappearance curve of radio-labelled platelets from the circulation was fitted by eye. Based on the curve shape (exponential, linear or curvilinear) the appropriate mathematical formulas were then applied. As discussed in a previous section (Section 1.9), this method is subject to considerable observer bias. In contrast, Hill-Zobel (1982) used the gamma function to analyze platelet survival data and then analyzed the sa[illegible]ng mathematical formulas for linear or exponential dis[illegible]tterns. She demonstrated that the platelet survival data may [illegible]erably between the three methods of analysis. However, at the time w[illegible] Harker performed his studies (1974), computer assisted methods developed for analyzing survival data were not available.

Chemically-induced vessel injury has also been implicated in the effects of circulating lipids on platelet survival since platelet lifespan has been reported to be decreased in patients with hyperlipoproteinemia (Steele and Rainwater, 1978b; Harker and Hazzard, 1979b). Experimental studies by Mustard and Murphy (1962) have shown that mean platelet survival time is shortened in humans whose diets are rich in saturated fats. Conversely, platelet survival time was significantly longer in patients whose diets contained a high proportion of polyunsaturated fats. Similarly, Steele et al. (1978) showed that dietary or pharmacological reduction in circulating serum lipids in humans normalized platelet lifespan which had been shortened previous to treatment.

Ross et al. (1976) have reported that diets enriched in cholesterol result in injury to the vessel endothelium. They maintained monkeys on a

cholesterol rich diet for more than six months and demonstrated focal endothelial cell desquamation equal to 5% of the total aortic surface.

Platelet survival time was significantly shortened in monkeys on hypercholesterolemic diets. When platelets from hyperlipemic monkeys were infused into normal recipients they survived for normal time periods. Conversely, platelets from normal donors had shortened survival times in the circulation of hyperlipidemic recipients. Ross and his colleagues concluded that the shortening of platelet survival in hyperlipemia was not a direct effect of increased cholesterol on the platelets. However, in these studies, rapidly occurring lipid-mediated effects on membranes of transfused platelets were not ruled out as a mechanism responsible for shortened platelet survival. Based on the evidence that the extent of decrease in survival profile of platelets in hyperlipemic monkeys correlated directly with the degree of endothelial cell loss, Ross et al. (1976) proposed that shortened platelet survival was a result of increased platelet consumption on a continuously injured vessel wall.

Sedar et al. (1978) also provided evidence that fatty acids may injure the vessel endothelium. They observed that the infusion of a variety of fatty acids including arachidonic acid, linoleic acid and γ-linolenic acid into the circulation of rabbits resulted in endothelial cell enucleation, disruption of endothelial cell junctions and subsequent platelet adhesion to exposed subendothelial structures. Furthermore, Hessler et al. (1980) have shown that low density lipoproteins (LDL) are cytotoxic to endothelial cells and this may be why some fatty acids promote endothelial cell desquamation in vivo.

An alternative mechanism for the observed decrease in platelet survival in hyperlipidemic conditions was suggested by Armstrong et al. (1980). They observed that the livers of monkeys on hypercholesterolemic diets were considerably enlarged and that this probably resulted in an increased capacity to sequester platelets from the circulation. However, in the same studies, Armstrong reported that significant numbers of ^{51}Cr-labelled platelets accumulated on the aortic surface of monkeys on high cholesterol diets indicating that endothelial cell stimulation or damage had occurred.

In addition to the effect of dietary fats on platelet survival, other lifestyle factors such as cigarette smoking have been shown to shorten platelet survival in man (Mustard and Murphy, 1963c; Fuster et al., 1981). Although the mechanisms by which cigarette smoking shortens platelet survival have not been identified, experimental studies have shown that the vascular endothelium of rats exposed to cigarette smoke is altered (Pittilo et al., 1982). Platelets adhered to the aortic surface particularly in areas around vessel orifices. Ultrastructural changes in endothelial cells including gaps in endothelial cell junctions and subendothelial edema have been reported upon examination of coronary arteries from monkeys exposed to carbon monoxide inhalation (Thomsen, 1974). It must be pointed out however, that Thomsen could not repeat the experimental findings at a later date. Nevertheless, it is apparent from the similar evidence from a number of studies that some constituent of cigarette smoke (nicotine, carbon monoxide, tobacco antigen) probably injures the endothelium (Booyse et al., 1981; Becker et al., 1973; Thomsen, 1974).

Very recent studies by Winocour et al. (1982) performed concurrently and collaboratively in our laboratory, have provided strong evidence that repeated vessel injury contributes significantly to reduced platelet survival. Winocour inserted polyethylene catheters into rat aortae to repeatedly injure the endothelium. Under the experimental conditions used, macroscopic thrombi did not form although platelet survival was significantly reduced. The extent of reduction in platelet survival was directly proportional to the length of the indwelling catheter.

It is not known how the interaction between platelets and the repeatedly damaged vessel wall could cause shortened platelet survival. However, Winocour suggested that platelets which adhered to the injured vessel surface may subsequently be freed from the surface by the action of proteolytic enzymes such as plasmin generated at the injury site, or, by enzymes released from leucocytes or from platelets themselves. It is possible that platelets which had been cleaved from the surface to which they had adhered may thus have altered membrane glycoproteins. Based on the results of previously described studies with proteolytic enzymes (see Section 1.12.1), these platelets would be expected to be rapidly cleared from the circulation.

1.12.3 Vascular Disease

The initiating factors of arterial and venous thromboembolic disease are related to endothelial cell injury.

1.12.3.1 Arterial Thrombosis

Arterial thrombotic disease is initiated by injury to the

endothelium with subsequent platelet adhesion, aggregation and release. Thrombi composed primarily of platelets and fibrin form at sites of injury. If the injury is not repeated, the vessel wall becomes quiescent and the original lesions regress. However, if the injury stimulus is repeated or continuous, or if blood flow is disturbed, extensive atherosclerotic lesions form on the vessel wall (Moore, 1973).

Platelet survival has been shown to be shortened in association with the clinical manifestations of atherosclerosis. (Steele et al., 1975b, 1978c; Steele et al., 1978c; Kutti and Weinfeld, 1979).

It is not known whether platelet survival is shortened through continuous interactions with reactive thrombotic lesions or whether shortened survival is a result of frequent platelet-vessel wall interactions.

Platelet survival is shortened to the greatest extent in individuals with a positive family history of vascular disease and who smoke (Fuster et al., 1981). As well, platelet survival tends to be similar to normal controls in individuals who have clinical complications of atherosclerosis but who are non-smokers and have a negative family history. These observations suggest that platelet survival is shortened in association with repeated vessel injury rather than with the extentof atherosclerosis. The observations also raise the possibility that there is a genetic component to development of atherosclerosis.

Harker et al. (1979) studied the sequelae of arterial thromboembolic disease and its effects on platelet survival in baboons by implanting thrombogenic femoral arterio-venous cannulae and subsequently infusing

^{111}In-labelled platelets. In these experiments, if the cannulae were inserted as extension segments in the renal arteries, platelets actively formed thrombi on the surface. The thrombi continually embolized and progressively occluded the glomerular vessels.

Platelet survival was significantly shortened in baboons with the thrombogenic shunts although fibrinogen survival remained normal. When platelets were isolated from animals with shunts, the platelets survived normally upon infusion into normal animals demonstrating that the shunts did not cause permanent platelet modifications nor an increase in the number of young platelets in the circulation.

Based on the evidence from these studies, Harker suggested that shortened platelet survival associated with arterial thromboembolic disease was a selective process of platelet utilization, embolization and subsequent platelet destruction accompanied by little detectable fibrin formation.

It has not been established how the platelets in embolized thrombi are subsequently cleared from the circulation. It may be that thromboemboli induce anoxia and further endothelial cell injury in blocked vessels of the microcirculation. This might enhance the strong fibrinolytic activity known to be present in these vessels (Loskutoff et al., 1982; Kwaan, 1982). In combination with the reduced flow as a result of vessel blockage, high local concentrations of plasmin are likely to be achieved. In addition to digesting the fibrin and mediating dissolution of the thrombi or emboli, plasmin can modify platelet membrane glycoproteins resulting in the premature clearance of platelets from the circulation (Greenberg et al., 1977).

1.12.3.2 Venous Thrombosis

It has been suggested by some investigators that venous thrombosis is initiated by platelet activation at sites of vessel injury. These conclusions were based on histological evidence in which platelet-fibrin thrombi were regularly observed at sites of thrombus attachment to the veins (Patterson, 1969). Others (Sevitt, 1974; Thomas, 1982) maintain that recognizable intimal lesions are not pre-requisite factors for initiating deep vein thrombosis (DVT). Rather, blood stasis in venous valve pockets, local hypoxia, and subsequent accumulation and activation of coagulation factors result in high local concentrations of thrombin followed by platelet aggregation and thrombus formation.

The observation that low dose heparin has little effect in reducing the incidence of DVT in patients who have had total hip replacements suggests that a dual mechanism - local intimal damage in addition to accelerated coagulation - probably initiates DVT (Thomas, 1982).

Although the controversy regarding initiating factors in DVT has not been resolved, it has been shown that recurrent DVT is associated with shortened platelet survival (Harker and Slichter, 1972; Steele et al., 1973, 1978a). Harker et al. (1972, 1978a) also noted that platelet turnover in the circulation was increased and fibrinogen survival time was decreased in patients with DVT. This evidence led Harker and his associates to conclude that shortened platelet survival in individuals with DVT was a result of an exaggerated hemostatic response involving combined platelet and fibrinogen utilization.

Harker's data are compatible with an alternative interpretation.

Since thrombus formation activates proteolytic enzymes, it is possible that increased fibrinogen/fibrin utilization as well as shortened platelet survival time were a result of proteolysis. Reduced flow conditions in the valve pockets would enhance local concentration of proteolytic enzymes.

1.12.4 Metabolic and Other Diseases

Shortened platelet survival time is associated with a number of metabolic diseases including gout (Mustard et al., 1963b) hypertension (Steele et al., 1977; Peters et al., 1982) and diabetes (Mustard and Packham, 1977; Bern, 1978). In addition, other diseases which result in chronic hypoxemia shorten platelet survival (Steele et al., 1977; Harker and Ritchie, 1980; Peters et al., 1982). The available evidence suggests that vessel wall injury is the most likely cause of the reduction in platelet survival time.

In diabetes, the increased tendency to develop complications of occlusive vascular disease support the concept that continuous or repeated endothelial cell injury occurs in these individuals (Bern, 1978). As well, the observation that the concentration of plasma von Willebrand factor is increased in diabetic patients (Bensoussan et al., 1975) suggests stimulation or damage to vessel endothelial cells (Brinkhous et al., 1980; Humphrey, 1983).

Platelet survival has also been reported to be shortened in patients with disseminated intravascular coagulation (Harker and Finch, 1969; McKay, 1965) and in patients with Sickle cell crisis (Haut et al., 1973). The nature of of these diseases suggest that platelet consumption

in intravascular thrombi and subsequent proteolytic modification of platelets during thrombus dissolution is a mechanism of shortened platelet survival time.

1.12.5 Prosthetic Vascular Grafts and Artificial Surfaces

Since systemic emboli, occlusive thrombi and shortened platelet survival are common complications of vascular prosthetic implants, several groups have studied a variety of artificial surfaces in vivo to determine the mechanism of shortened platelet survival time (Hanson et al., 1980; Callow et al., 1980; Harker et al., 1977b).

Hanson et al. (1980) examined the thrombogenic response to eight grafted acrylic polymers and co-polymers which had been inserted as femoral arterio-venous shunts into baboons. They observed that silastic or polyethylene prostheses were almost non-thrombogenic and had no significant effect on platelet survival time. Hanson noted that the thrombogenicity of the shunts was strongly dependent on the material type and its degree of hydration.

When consumptive shunts were positioned in the renal arteries of baboons, platelet emboli caused progressive occlusion of the renal microcirculation and shortened platelet survival time.

Platelets from baboons with consumptive shunts survived normally in normal recipients showing that the shunts did not induce cumulative platelet injury.

In view of normal ^{125}I-fibrinogen kinetics in baboons with thrombogenic shunts, Hanson proposed that selective platelet consumption in the formation of microemboli on the prosthetic material is probably

responsible for the shortened platelet survival. The normalization of platelet survival time in animals (Wilkinson et al., 1979; Pumphrey et al., 1983) and humans (Norcott et al., 1982) with thrombogenic vascular prostheses by drugs (i.e., aspirin plus dipyridamole; known to inhibit platelet function) support Hanson's conclusion.

Porous dacron, the most widely used arterial replacement material is thrombogenic during the first few weeks after implantation (Harker et al., 1977b; Callow et al., 1980; Clagett et al., 1981; Pumphrey et al., 1982). However, numerous animal studies have demonstrated that increased platelet turnover and shortened platelet survival progressively normalize as the graft becomes covered with endothelium (Harker et al., 1977a; Sharefkin et al., 1982).

Harker and associates (1977a) have reported similar results in humans who received aorto-femoral fabric grafts.

Thus, it is apparent that platelet survival is shortened when the artificial surface with which blood comes in contact remains reactive toward circulating platelets.

It is not known how platelets which had adhered to prosthetic surfaces are freed to return to the circulation to be cleared rapidly. It is possible that proteolytic enzymes, activated by contact of plasma with the prosthetic surface or released from stimulated platelets and leucocytes, may cleave the protein bonds through which platelets adhere to the surface. As well, arterial shear forces may contribute significantly to the embolization process leading to dissolution of emboli in the microcirculation by proteolytic enzymes.

1.12.5.1 Cardiac Valves

Replacement of defective cardiac valves by directly sewn homografts or by porcine heterografts (Thompson et al., 1983) does not affect platelet survival time in most patients since these valves are associated with low incidences of thromboembolism.

In contrast to tissue grafts, prosthetic heart valves (particularly the older Starr-Edward's ball valves; Kinlough et al., 1968) are associated with a high incidence of thromboembolism and shortened platelet survival time.

In some patients a direct correlation exists between the area of exposed prosthetic surface, the frequency of clinical embolic episodes and the extent to which platelet survival is shortened (Steele et al., 1979).

Harker and Slichter (1970) investigated fibrinogen and platelet kinetics in patients with prosthetic heart valves to determine the mechanism responsible for shortened platelet survival. They demonstrated that fibrinogen turnover and fibrinogen survival were normal, indicating that the process leading to premature platelet clearance did not involve significant fibrin formation. As well, the normal platelet survival observed in patients with homograft valves negated any intrinsic effect on platelets by the operative procedure.

Harker and Slichter concluded that the prosthetic surface selectively interacted with platelets in a thromboembolic process resulting in shortened platelet survival time.

It is also possible that shortened platelet survival time results from platelet interaction at sites of endothelial injury where graft and

cardiac tissue are sutured. Differences in mechanical compliance between tissue and graft may induce excessive turbulence resulting in repeated endothelial injury and platelet-vessel wall interaction.

Furthermore, since hemolysis has been observed in some patients with prosthetic heart valves (thus indicating mechanical damage to erythrocytes) it has been suggested that platelets could be damaged or modified to be prematurely cleared from the circulation.

1.12.6 Immunological Mechanisms

In man and experimental animals (Friedman et al., 1975, 1977) immunological mechanisms have been associated with a high incidence of platelet destruction and premature platelet clearance from the circulation. The origin of the immunological mechanism resulting in shortened platelet survival may be induced by drugs, may be autoimmune in nature or may be secondary to other disorders such as myeloproliferative disease, bacterial or viral disease.

Idiopathic or autoimmune thrombocytopenia (ITP), characterized clinically by decreased platelet counts and markedly reduced platelet survival time, is mediated by autologous antiplatelet antibodies directed against components of the platelet membrane (Handin et al., 1973; Branehog et al., 1974; Kerneff et al., 1980). The presence of antiplatelet antibodies in plasma of patients with ITP was first demonstrated by the induction of transient thrombocytopenia in recipients receiving infusions of ITP plasma (Harrington et al., 1951; Karpatkin et al., 1971). The antibody was originally characterized by Karpatkin (1973) as belonging to the 7s, IgG3 class of immunoglobulins. More

recently all the subclasses of IgG as well as IgM have been implicated (Nel et al., 1983).

It was originally thought that binding of antiplatelet antibodies to the platelet membrane activated the C3 component of the complement system thereby initiating the activation of the full complement cascade resulting in platelet injury and removal from the circulation. Nel and Stevens (1980) have observed excess IgG binding to platelets from patients with ITP without concurrent increase in C3 binding. Others have found increased concentrations of bound C3 (Hauch and Rosse, 1977).

Platelets coated with antiplatelet antibodies are recognized and phagocytosed by the reticulo-endothelial system (RES) of the spleen and liver (Dixon and Rosse, 1975; Kernoff et al., 1980). In vitro studies have also demonstrated that granulocytes phagocytose antibody-coated platelets (Handin and Stossel, 1974).

In 1979, Cheng et al. isolated a glycoprotein from membranes of human platelets which binds the Fc portion of IgG. Others (Moore and Nachman, 1981; Karas et al., 1982) have confirmed these findings. Thus, non-specific binding of immmunoglobulins (Karas et al., 1982) or circulating immune complexes (Kutti et al., 1981) to platelet Fc receptors can also contribute to premature platelet clearance from the circulation.

This is the probable mechanism responsible for shortened platelet survival associated with myeloproliferative disorders and autoimmune diseases such as systemic lupus erythematosis and rheumatoid arthritis in which circulating concentrations of immunoglobulins or immune complexes are commonly increased.

Several investigators (Dixon and Rosse, 1975; Glockner et al., 1978; Kelton et al., 1979, 1982) have suggested that immunological mechanisms may mediate the clearance of senescent platelets from the circulation in a manner similar to that described for clearance of senescent erythrocytes (Lutz and Kay, 1981).

In 1978, Glockner and his associates demonstrated that the Thomsen-Friedenreich Antigen (T-antigen; β-O-galactosyl (1-3)-N-acetyl-D-galactosamine) exists as a cryptic antigen on the platelet membrane normally masked by a terminal sialic acid residue. Previous to this work, it had also been demonstrated that sera from normal adults contain antibodies directed against this antigen (Bird et al., 1971). Thus, Glockner proposed that the slow desialylation of platelets exposed the cryptic T-antigen on the platelet membrane. Subsequent immunoglobulin accumulation on the platelet surface would then enhance the biological elimination of platelets from the circulation.

In support of Glockners hypothesis, Kelton and Denomme (1982) have shown that older platelets (separated on the basis of density) have an increased concentration of surface associated and internalized IgG compared to younger platelets.

1.12.7 Platelet Handling and Storage

Handling and storage of platelets has a major influence on their subsequent function and viability in vivo. Several critical variables related to storage conditions have been identified.

A positive correlation exists between pH of the stored platelet concentrate and post-storage viability. Platelets stored for prolonged

periods generate and accumulate metabolic acids. Therefore, the anticoagulant used for the initial blood collection is important. Slichter and Harker (1976a, 1976b) reported that platelets prepared from blood anticoagulated with acid-citrate dextrose, pH 6.5 (Aster and Jandl, 1964a, b) and stored for longer than 24 hours had reduced survival *in vivo* because the pH in the platelet preparation dropped to less than 6.0. Platelets collected into ACD, pH 7.0 or citrate-phosphate dextrose (CPD) pH 7.2, maintained a storage pH above 6.0 for 3 days and retained their viability when returned to the circulation. Slichter and Harker also noted that reducing the number of platelets in the stored concentrates to 1.7×10^{12}/L in addition to constant gentle shaking, had a significant effect in keeping the pH above 6.0 to maintain viability.

Centrifugation speed and time during platelet preparation influences the ability of platelets to survive after they have been infused into the circulation.

Forces greater than 30,000 x g_{max} total (3,000 x g_{max} x 10 min), significantly reduces the survival time of freshly prepared platelets. When these platelets are stored, recovery and survival is further reduced.

The effect of storage temperature on mean platelet survival time has been investigated carefully (George, 1976c; Slichter and Harker, 1976a, b; Murphy and Gardner, 1969b).

Upon storage at 4°C, platelets rapidly lose their discoid configuration and become spherical. Ultra-structural studies have shown that this shape change is associated with the loss of the marginal band of microtubules (White and Krivit, 1967). Prolonged exposure to cold

results in irreversible microtubule damage as well as myosin proteolysis (Abramowitz et al., 1974).

Platelets stored at 4°C have shortened survival time but normal in vitro adhesion and aggregation responses when tested at 37°C. In contrast, platelets stored at 22°C for as long as 72 hours maintained normal lifespan despite decreased in vitro adhesion and aggregation responses. However, in vitro tests may not accurately predict in vivo function since platelets stored at 4°C were less effective hemostatically in thrombocytopenic recipients than platelets stored at 22°C.

George (1976c) and Bolin (1981), have reported that storage of platelets causes alterations to and loss of membrane glycoproteins. Dependent upon the extent of glycoprotein loss, platelet survival and platelet function may be compromized.

Furthermore, it has been demonstrated that the type of plastic bags used for platelet storage affects platelet survival (Slichter and Harker, 1976b). It has been suggested that the internal surface construction, composition of the plastic or leachable plasticizers may be responsible for the reduced survival time.

Table 1.2 summarizes the conditions associated with shortened platelet survival.

Table 1.2

Conditions Associated with Shortened Platelet Survival Time

Modification of Platelet Surface Membrane Glycoproteins

Congenital

a) Bernard-Soulier Syndrome

b) Wiskott-Aldrich Syndrome

Experimentally induced

a) Proteolytic enzymes (plasmin, trypsin, chymotrypsin)

b) Neuraminidase

c) Prolonged storage

d) Reduction or Oxidation of Membrane Sugars

Associated with Other Diseases

a) Cirrhosis of the liver

b) Infections with viruses which have neuraminidase activity

Atherosclerosis and Vessel Injury

Vascular Disease

a) Arterial thrombosis

b) Venous thrombosis

c) Atherosclerosis, coronary artery disease

d) Angina

e) Myocardial infarction

f) Rheumatic heart disease with associated thromboembolism

g) Vasculitis

h) Chronic airway obstruction, hypoxemia

Experimentally Induced

a) Chemical - continuous homocysteine infusion

b) Dietary - diets high in saturated fats

c) Indwelling aortic catheters

d) Grafts and prosthetic surfaces

Metabolic Disorders

a) Homocystinemia

b) Hyperlipoproteinemia

c) Diabetes

d) Gout

cont....

Table 1.2 (continued)

Life Style Factors

a) Diets high in saturated fats

b) Smoking

Prosthetic Surfaces and Grafts

a) Heart valves - particularly older models (ie. Starr-Edwards)

b) Vascular Grafts

c) Shunts

Immunologic

a) Isoimmune - multiple transfusion, maternal immunization

b) Autoimmune - idiopathic thrombocytopenia purpura

c) Drug induced - quinine

d) In association with other diseases - bacterial, viral, parasitic, rheumatoid arthritis, hyperthyroidism, chronic lymphocytic leukemia, systemic lupus erythematosus, lymphocytic lymphomas

e) Experimentally induced by injection of platelet antibody

In Association with other Diseases

a) Bacteremia

b) Crisis - Sickle Cell Disease

c) Hemolytic Uremic Syndrome

d) Myeloproliferative Disorders

f) Chronic Airway Obstruction

g) Disseminated Intravascular Coagulation

Surgical Trauma

Platelet Storage

1.13 Aim of the Present Study

As indicated in the introduction, many studies have reported that platelet survival is reduced in individuals with the thromboembolic complications of vascular disease. However, the mechanisms have not been clearly defined.

The aim of this study was to examine the mechanisms responsible for the shortened platelet survival observed in thromboembolic states. Theoretically, there appeared to be several possibilities. First, survival of circulating platelets could be shortened because of the incorporation of platelets into thrombi. Second, repeated injury to a vessel wall resulting in enhanced platelet interaction at injury sites could lead to platelet removal from the circulation. Third, a combination of these events may occur.

To determine which of these possibilities contributed to shortened platelet survival, it was decided to examine;

a) the rate and extent of platelet incorporation into thrombi induced in the aortae of rabbits by an indwelling catheter

b) the extent to which platelets might turn over in thrombi formed in this way

c) whether thrombi that form *in vivo* continue to attract circulating platelets or whether thrombi rapidly lose their reactivity

d) the extent to which platelets became associated with the injured vessel wall in regions where thrombi do not form

and

e) the relation between extent of vessel injury and reduced platelet survival.

These studies were conducted using radiolabelled platelets to monitor platelet participation in thrombosis and platelet accumulation at sites of injury. In addition, morphological studies assessed the nature of the thrombi and the cellular events occurring on the injured surface. Using these experimental approaches it would also be possible to examine turnover of platelets in thrombi or on the vessel wall by determining whether they were cleared as a result of phagocytosis, lysis or proteolytic modification in thrombi, or, through interaction and modification on the injured vessel wall.

Thus, it was expected that the examinations outlined above would provide useful insight into the mechanisms responsible for shortened platelet survival associated with thromboembolic diseases.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Anticoagulants

2.1.1.1 Acid-Citrate-Dextrose (ACD)

The acid-citrate-dextrose (ACD) anticoagulant of Aster and Jandl (1964) was used. The solution contained:

trisodium citrate.$2H_2O$	25.0 g
citric acid	14.0 g
(or citric acid monohydrate	15.0 g)
dextrose	20.0 g

The chemicals were dissolved in 1 L of distilled water. The pH was 4.5 without adjustment and the osmolarity was 450 milliosmoles (mosM). One part of ACD was used for six parts of whole blood; the pH of the ACD-whole blood mixture was 6.5.

2.1.1.2 3.8% (10^{-1}m) EGTA

(Ethylene glycol-bis(B-aminoethyl ether)N,N,N',N'-tetraacetic acid)

EGTA	3.8 g
distilled H_2O	80.0 ml

EGTA was dissolved in distilled water by dropwise addition of 10-20M aqueous NaOH. The pH was finally adjusted to 7.0 with 1M aqueous NaOH before bringing the total volume to 100 mL with distilled water. The osmolarity was 290 milliosmoles.

To prepare a 2% solution of isotonic EGTA, 10 mL of 3.8% EGTA (as prepared above) was added to 9.0 mL of isotonic saline.

2.1.2 Platelet Washing Solutions

All platelet washing solutions were based on Tyrode's balanced salt solution (Parker, 1962). Stock solutions were prepared and kept refrigerated at 4°C. The platelet wash solutions were freshly prepared from the stock solutions for each experiment.

2.1.2.1 Stock Solution #1

NaCl	160.0 g
KCl	4.0 g
$NaHCO_3$	20.0 g
NaH_2PO_4	1.0 g
	(or 1.16 g $NaH_2PO_4.H_2O$)

These salts were dissolved in deionized distilled water to total 1L of solution.

2.1.2.2 Stock Solution #2 (10^{-1}M $MgCl_2.6H_2O$)

$MgCl_2.6H_2O$	20.33 g

The salt was dissolved in one litre deionized distilled water.

2.1.2.3 Calcium-Free Tyrode's Albumin Solution (CF-TYR-ALB)

distilled H_2O	60.0 mL
Stock #1	5.0 mL
Stock #2	2.0 mL
D-glucose	0.1 g
Albumin	0.35 g

The ingredients were dissolved in distilled water and pH of the solution was adjusted to 6.5 with 2M aqueous HCl. The solution was made up to a final volume of 100 mL with distilled water. The osmolarity was 290 mosM.

The bovine albumin was Pentex fraction V from Miles Laboratories Inc., Elkhart, IN.

2.1.2.4 ACD-Calcium-Free Tyrode's-EGTA-Albumin (ACD-CF-TYR-EGTA-ALB; for Stractan Density Gradients)

Distilled H_2O	50.0 mL
ACD	15.0 mL
Stock #1 (no NaCl)	5.0 mL
Stock #2	2.0 mL
Albumin	0.35 g
2% EGTA	1.0 mL

The ingredients were dissolved in distilled water and the pH of the solution was adjusted to 6.5 with 2M aqueous NaOH. The total volume was made up to 90 mL. NaCl (0.6 g) or 4 mL of 30% NaCl solution was added to bring the osmolarity to 290 mosM. The final volume was adjusted to 100 mL with distilled H_2O.

The composition of the platelet washing solutions (g/100 mL) are listed on Table 2.1.

Table 2.1

Composition of Platelet Washing Solutions (g/100 mL)

Chemicals	Calcium Free Tyrode Albumin (CF-TYR-ALB)	ACD Calcium Free Tyrode-EGTA-Albumin (ACD-CF-TYR-EGTA-ALB)
NaCl	0.80	0.60
KCl	0.02	0.02
$NaHCO_3$	0.10	0.10
$NaH_2PO_4.H_2O$	0.0058	0.0058
$MgCl_2.6H_2O$	0.0406	0.0406
D-glucose	0.10	0.30
Albumin	0.35	0.35
EGTA	–	0.02
Trisodium citrate.H_2O	–	0.375
Citric acid.H_2O	–	0.225
pH	6.5	6.5

2.1.3 Radioactive Chemicals

2.1.3.1 ^{51}Chromate (^{51}Cr)

Sodium chromate ($Na_2{}^{51}CrO_4$) solution in sterile isotonic saline, specific activity 200-500 Ci/g chromium was obtained from New England Nuclear, Lachine, Quebec, (~~NEZ~~-03).

2.1.3.2 Diazo ^{125}Iodosulfanilic Acid (D^{125}ISA)

^{125}I-iodosulfanilic acid (^{125}ISA) greater than 1000 Ci/mmol was obtained as a kit (NEX-121) from New England Nuclear, Lachine, Quebec. The ^{125}I-iodosulfanilic acid was converted to the diazotized form (D^{125}ISA) immediately before use according to the instructions supplied in the labelling kit.

2.1.3.3 ^{111}Indium-oxine

^{111}Indium-oxine (^{111}In-8-hydroxyquinoline) greater than 99% radionuclide purity, carrier free, specific activity 1mCi/50 uL, was obtained from New England Nuclear, Lachine, Quebec (NEZ-154).

2.1.3.4 ^{131}I-Labelled Albumin

^{131}I-labelled albumin 1mg/mL sterile saline, labelled with 1mCi/mL ^{131}I was obtained from Charles E. Frosst and Company, Dorval Quebec.

2.1.4 Discontinuous Stractan Density Gradients

2.1.4.1 Preparation of Stractan (Arabinogalactan) Solution

Concentrated stock solutions of Stractan (Stractan 2, St. Regi's

Paper Co., Tacoma, WA) were prepared according to the method of Corash et al. (1974), with modifications (Cieslar et al., 1979). Stractan (250 g) was dissolved in 500 mL distilled water (50% w/v) and passed through 2.5 X 30 cm columns of analytical grade mixed-bed ion exchange resin (AG 501-X8, Bio-Rad Laboratories, Richmond, CA) to remove impurities and to reduce the osmolarity to less than 100 milliosmoles/L (mosmoles/L). Three to four passages of the Stractan solution over the resin bed were usually required before the desired osmolarity was reached.

Measurements of osmolarity were obtained by measuring freezing-point depression with an osmometer (Advanced Instruments Inc., Newton Highland, MD). The true osmolarity of the Stractan solution was calculated from the measured osmolarity (mosmoles/$L_{measured}$) using the formula:

$$\text{mosmoles/L} = \frac{\text{mosmoles/L}_{measured}\ (1+\text{AW/mL}) - \text{mosmoles/L}_{0.15m\ saline}}{\text{AW/mL}}$$

where AW = available water for osmotic activity and AW/mL was estimated by the formula:

$$\text{AW/mL} = 1 - 0.64cs$$

(0.64 is the volume in mL occupied by 1 g Stractan; cs is the concentration of Stractan (g/mL).

Since aqueous Stractan has the same refractive index as sucrose solution (wt/wt; Corash et al., 1974), the concentration of Stractan was obtained by refractometry and a standard sucrose table.

The effluent solution of Stractan from the final passage of the

resin bed had an osmolarity of less than 100 mosmoles/L and a concentration of greater than 20%. To each 100 mL of Stractan solution, the following ingredients were added to make the solution equivalent to CF-TYR-ALB containing 0.02% EGTA without NaCl:

0.35 g Albumin

0.02 g KCl

0.10 g $NaHCO_3$

1.0 mL 2% EGTA (0.02g)

0.0058 g $NaH_2PO_4.H_2O$

0.0406 g $MgCl_2.6H_2O$

0.10 g D-glucose

The materials were dissolved in water and the pH was adjusted to 6.5. The osmolarity of the concentrated Stractan solution was brought to 291 mosmoles/L by the stepwise addition of 30% NaCl solution (1 mL of 30% NaCl increases the osmolarity of 100 mL AW by about 100 mosmoles/L).

Solutions of 18%, 15%, and 13.5% Stractan were prepared by dilution of the concentrated solution of Stractan with CF-TYR-ALB containing 0.02% EGTA. Aliquots (50mL) of the solutions were stored at -20°C.

2.1.4.2 Preparation of Discontinous Stractan Density Gradients

Solutions of 18%, 15% and 13.5% Stractan were thawed and thoroughly mixed. The Stractan density gradients were prepared in siliconized, graduated, conical glass centrifuge tubes (12 mL) by adding 2.5 mL of the 18% Stractan solution to the tube to form the bottom layer of the gradient. A layer of 15% Stractan (2.5 mL) was gently pipetted over the 18% Stractan layer such that a distinct interface was visible between the

two volumes. Similarly, 2.5 mL of 13.5% Stractan were pipetted over the 15% volume to form the top layer of the density gradient. To avoid spontaneous mixing of interfaces between the three concentrations of Stractan solution, density gradients were prepared immediately before use.

2.1.5 Perfusion Solutions used for Rabbit Whole Body Fixation

2.1.5.1 Locke's-Ringer Solution

NaCl	9.0 g
KCl	0.42 g
$CaCl_2$	0.24 g
$NaHCO_3$	0.15 g
D-glucose	1.0 g

The materials were dissolved in water and the pH was adjusted to 7.4 with 0.1M aqueous HCl before bringing the volume to 1 L. The solution was freshly prepared for each experiment. Just before use, the solution was saturated with oxygen by bubbling with 95% oxygen for one hour and heparin was added to a final concentration of 1 unit per mL.

2.1.5.2 4% Paraformaldehyde Fixative Fluid in 0.1M Phosphate Buffer

$NaH_2PO_4.H_2O$	6.35 g
Na_2HPO_4	21.87 g
paraformaldehyde	40.0 g

Powdered paraformaldehyde was mixed with 500 mL distilled water and

warmed to 80°C stirring continuously to dissolve the powder. A few pellets of concentrated NaOH were added to aid dissolution of the paraformaldehyde. The solution was cooled and filtered through Whatman #1 filter paper. The remaining ingredients were added and dissolved. The pH was adjusted to 7.4 with 2M NaOH and the volume was adjusted to 1L.

2.1.5.3 4% Gluteraldehyde in Sodium Cacodylate Buffer

0.2M Stock Sodium Cacodylate Buffer

Na cacodylate $(CH_3)_2AsO_2Na.3H_2O$ 42.8 g

The salt was dissolved in 800 mL of water, the pH was adjusted to 7.4 with 1M HCl and the volume was increased to 1 L with distilled water.

Preparation of 4% Gluteraldehyde in Sodium Cacodylate Buffer

0.2M Stock sodium cacodylate buffer 500 mL

Gluteraldehyde (reagent grade) 40 mL

The solutions were mixed together and the total volume was increased to 1 L with distilled water.

2.1.6 Histological Dyes

2.1.6.1 Evans Blue Dye

A solution of Evans blue (CI 23860) dye was prepared just before use. The powdered dye (4.5 mg/mL) was dissolved in sterile isotonic saline. The solution was filtered through Whatman #1 filter paper to remove undissolved particles. Just before use, the solution was sterilized by passage through a millipore filter (0.22 um, Millex-GS, Millipore Corporation, Bedford, MA).

2.1.6.2 Lendrum's Martius Scarlet Blue Stain

Martius Yellow

(acid yellow 24, CI 10315)

Martius Yellow	0.5 g	
Phosphotungstic acid	2.0 g	per 100 mL
Distilled water to make up volume		

Brilliant Crystal Scarlet

(acid red 44, CI 16250)

Brilliant Crystal Scarlet	1.0 g	
Glacial acetic acid	2.0 ml	per 100 mL
Distilled water to make up volume		

Soluble Blue (Methyl Blue)

(acid blue 93, CI 42780)

Methyl Blue	0.5 g	
Glacial acetic acid	1.0 mL	per 100 mL
Distilled water to make up volume		

2.1.7 Animals

New-Zealand White rabbits were used for all experiments. The animals were housed singly in a controlled environment (20°C, 50% humidity, 12 hour light-dark cycles) in a central animal facility. All rabbits had access to food (stock rabbit chow; Ralston Purina Co., St. Louis MO) and water ad libitum. The animals weighed between 2.5-3.8 kg and animals of approximately the same age and weight were used within an experiment. Male animals were used throughout.

2.1.8 Drugs

2.1.8.1 Sodium Pentobarbital

Sodium pentobarbital was supplied by M.T.C. Pharmaceuticals, 1890 Brampton St., Hamilton, Ontario. The powder (6.5 g) was dissolved in water (99.8 mL) and a few drops of 1N NaOH were added to bring the pH to about 8.5 and maintain the drug in solution. Prior to use the solution was filtered through a sterile millipore filter (0.22 um, Millex-GS, Millipore Corporation, Bedford, MA).

2.1.8.2 Heparin (Hepalean)

Heparin was received as a sterile, isotonic, aqueous solution of

Heparin Sodium (10,000 U.S.P units/mL) from Harris Laboratories, Brantford, Ontario. The solution contained 1% benzyl alcohol as a preservative.

2.1.8.3 Ketamine (Ketaset)

Ketamine was received as a sterile aqueous solution (100mg/mL) of Ketamine.HCl from Rogar/SBT, Division of BTI Products Inc., London.

2.1.8.5 Atropine

Atropine was obtained as a sterile solution of atropine sulfate in water (0.4 mg/mL) from Squibb Canada Inc., Montreal, Quebec.

2.1.9 Catheters and Cannulas

2.1.9.1 Intra-Aortic Catheters

Intra-aortic catheters were prepared from PE-90 intra-medic polyethylene tubing (1.27mm ED, Clay Adams Division of Becton Dickinson and Co., Parsippany, NJ). Unless indicated otherwise, 20.0 cm aortic catheters were used in all experiments. The tip of the catheter, which was to be introduced into the femoral artery was sealed by aspirating several cm of warmed liquid paraffin. When the wax had cooled and solidified, the catheter was examined for leakage by attempted aspiration of saline into the sealed tube. The tip was tapered and rounded with fine scissors to facilitate insertion and minimize injury to the vascular endothelium. The distal end of the catheter was knotted and sealed by heating the end beyond the knot. The knotted end provided an anchor around which the femoral artery was ligated once the catheter was *in situ*.

2.1.9.2 Carotid and Femoral Cannulas for Whole Body Perfusion

These cannulas were used to obtain large volumes of whole blood from rabbits or to infuse perfusion fluids into the circulation of rabbits. The cannulas were prepared from 20 cm lengths of intra-medic polyethylene tubing (PE-190, 1.70mm ED, Clay Adams Division of Becton Dickinson and Company, Parsippany,NJ). One end of the cannula was fitted with an 18 gauge, 1.5" (18G-1.5) Luer-hub needle with a screw cap. The other end of the cannula was tapered and rounded for inserion into the vessels. Prior to use, the cannulas were filled with sterile saline.

2.1.10 Miscellaneous Material and Equipment

2.1.10.1 Microhematocrit Tubes

Microhematocrit tubes (Capilets) were obtained from Dade Division of the American Hospital Supply Corp., Miami, FL.

2.1.10.2 Centrifuges

The microhematocrit centrifuge (Adams MHCT II) was obtained from Clay Adams Inc., New York, NY.

The Sorvall RC-3 automatic centrifuge with a swing bucket rotor (17 cm average radii) was from Ingram and Bell Division of Maynard Scientific, Mississauga, Ontario.

The Eppendorf centrifuge was from Canlab, Toronto, Ontario.

2.1.10.3 Electronic Particle Counter

The electronic particle counter, Coulter Counter Model ZB_1 was from Coulter Electronics, Oakville, Ontario.

2.1.10.4 Isoton II Solution

The Isoton II (azide free) solution used as a diluent for platelet counting on the Coulter Counter was also obtained from Coulter Electronics, Oakville, Ontario.

2.1.10.5 Platelet Dilution Counting Cuvettes

Polystyrene platelet dilution cuvettes (Dilu-Vials) were obtained from Maynard Diagnostics, Weston, Ontario.

2.1.10.7 Hemocytometer

The improved Neubauer hemocytometer was from American Optical Corp., Buffalo, NY.

2.1.10.8 Siliconizing Solution

Surfasil was from the Pierce Chemical Co., Rockford IL. Surfasil (100 mL) was added to 900 mL carbon tetrachloride and stored in a fume hood. All glassware and pipettes were immersed in this solution and rinsed several times with tap water followed by several rinses in distilled water. The glassware was dried in an oven at 90°C.

2.2 METHODS

2.2.1 Collection of Venous Blood from Rabbits

Rabbits were placed in restrainer cages and secured comfortably. The central ear artery was swabbed with xylene which causes vascular dilatation and increases the blood flow. The marginal ear vein was dilated by washing the ear with warm water.

For platelet survival studies 1.5 mL of blood was withdrawn rapidly from the dilated ear vein through a needle (23G-1) into 0.5 mL ACD anticoagulant contained in a 3.0 mL plastic syringe. The samples were well mixed and transferred to 12 x 75 mm glass tubes.

Blood samples which were to include a platelet count contained 2.25 mL venous blood and 0.75 mL ACD. After thorough mixing, 1.0 mL of the anticoagulated blood was transferred to a polyethylene test tube for platelet counts; the remainder (2.0 mL) of the sample was transferred to a 12 x 75 mm glass test tube.

Blood samples (2.5 mL) which were to be processed for platelet density studies were drawn into 5.0 mL plastic syringes containing 0.5 mL ACD anticoagulant solution. The samples were well mixed and transferred to siliconized, graduated, conical glass centrifuge tubes.

2.2.2 Measurement of Rabbit Whole Blood Hematocrit

Anticoagulated, rabbit whole blood was aspirated into microhematocrit tubes to fill approximately 3/4 of the volume of the tube. One end of the tube was sealed with vinyl plastic putty and the tube was centrifuged for 5 minutes in a microhematocrit centrifuge. The

hematocrit (Hct.) was determined by measuring the percentage of the total blood volume occupied by the red blood cells.

2.2.3 Platelet Counts

2.2.3.1 Visual Method: Light Microscopy

Platelets in whole blood or packed red blood cells were performed visually using a light microscope. A 1:200 dilution of whole blood or packed red cells was prepared in a Thoma red blood cell diluting pipette. Whole blood or packed red cells were aspirated to the 0.5 mark on the pipette followed by 1% amonium oxalate to the 101 mark. The dilution was mixed for 5 minutes to allow the ammonium oxalate to lyse the red blood cells. The first 2-3 drops of the dilution were discarded from the pipette before both chambers of an improved Neubauer hemocytometer were filled. The hemocytometer was placed in a petri dish lined with moistened filter paper for 20 minutes to allow the platelets to settle. The platelets in the four corner squares and the central square of the red blood cell counting area of the hemocytometer were counted on a light microscope with a 10x eyepiece and 40x objective. The platelet counts from each side of the chamber were averaged and the number of platelets per μL of whole blood was calculated by the formula:

$$\text{Platelets}/\,\mu L = N \times \frac{1}{0.02} \times 200\ (\text{dilution})$$
$$= N \times 10{,}000$$

where N = the average counts of both sides of the hemocytometer

2.2.3.2 Automated Method

A Coulter Counter Model ZB_1 electronic particle counter was set as follows:

upper threshold	100
lower threshold	5
1/amplification	1/4
1/aperture current	1/2
aperture size	70 um
manometer size	100 uL

Platelet counts of rabbit platelet rich plasma (PRP) or suspensions of washed rabbit platelets were determined by diluting 5 uL of the suspension in 15 mL (1:3000 dilution) Isoton II (Coulter Electronics, Oakville, Ontario) in a dilution cuvette. The platelet dilution was well mixed and counted twice on the Coulter Counter. Duplicate counts were averaged, corrected for coincidence counts and dilution from a chart supplied by Coulter Electronics (Figure 3A; Correction Charts for Dilution and Coincidence Counts, Coulter Counter Instruction Manual p.3-6, 1979) to obtain platelet counts per uL.

Whole blood platelet counts were also measured on the Coulter Counter by a modification of the method of Bull et al. (1965). Glass tubes (6 x 50 mm) were completely filled with whole blood and placed in a sedimentation rack angled at 45° for 1-1.5 hours to allow red blood cells (RBC) to settle. When at least 2 mm of the supernatant platelet rich plasma (PRP) was visible above the layer of RBC, 5 uL of the PRP was

diluted in 15 mL Isoton II in a dilution cuvette (1:3000 dilution). The contents of the cuvette were well mixed and the platelet count (instrument count) of the supernatant PRP was measured on the Coulter Counter. The instrument count was corrected for coincident cell passage and dilution as described previously to obtain the corrected PRP platelet count. The sedimenting RBC trap some plasma which is free of platelets resulting in platelet excess in the supernatant plasma. Since the platelet excess is a funtion of the Hct. of the whole blood sample, a Hct. correction factor (Figure 3.1 B, Correction Charts for Dilution and Coincidence Counts, Coulter Counter Inctruction Manual, p. 3-6, 1979) is included in calculations of whole blood platelet counts. The whole blood platelet count was calculated from the formula:

platelet count/μL whole blood =

corrected PRP platelet count x Hct. factor

2.2.4 Preparation of Suspensions of Washed Rabbit Platelets from Large Volumes of Whole Blood

Suspensions of washed rabbit platelets were prepared according to the method of Ardlie et al. (1970), with some modifications.

Siliconized glassware or plastic test tubes and syringes were used throughout.

All centrifugations were performed in a Sorvall RC-3 centrifuge with a swing bucket rotor (17 cm average radii) at 23°C.

New Zealand White rabbits (2.5-4.0 kg) of either sex were

anesthetized with an intravenous injection of sodium pentobarbital (40-50 mg/kg). A carotid artery was isolated and a 20 cm polyethylene cannula was inserted via the artery into the ascending aorta. Blood was withdrawn into 50 mL plastic syringes containing 7.5 mL acid-citrate-dextrose (ACD; Aster and Jandl, 1964) anticoagulant (6 volumes whole blood/1 volume ACD).

The blood was transferred to 50 mL round bottom plastic tubes and the red blood cells were separated by centrifugation at 1500 x g_{max} for 3 minutes. The supernatant platelet rich plasma (PRP) was transferred by plastic syringes to 50 mL conical polycarbonate centrifuge tubes. The red blood cells (RBC) were mixed and centrifuged once more at 1500 x g_{max} for 3 minutes and the PRP was removed and pooled with the PRP from the first spin. The yield of platelets was calculated by the method of Corash et al. (1978) using the formula:

$$100\% - \left[\frac{\text{no. of platelets in packed RBC}}{\text{no. of platelets in whole blood}}\right] \times 100\%$$

The yield of platelets normally obtained using this method of separating platelets was 80.7 ± 1.6 (mean ± standard error, n=6).

Platelets were separated from the pooled PRP by centrifugation at 1500 x g_{max} for 15 minutes. The packed platelets were resuspended in calcium-free Tyrode's albumin solution (CF-TYR-ALB; first wash) containing 0.02% EGTA, pH 6.5 in a 10 mL conical glass tube. Care was taken to avoid transfer of any contaminating RBC's which had been centrifuged to the bottom of the tube. The platelets were incubated in this solution for 10 minutes before they were pelleted by centrifugation

(1500 x g_{max};10 minutes). The platelet pellet was resuspended in CF-TYR-ALB (second wash), pH 6.5 for 10 minutes before they were centrifuged at 1500 x g_{max} (10 minutes) and finally resuspended in rabbit PPP.

2.2.5 Suspensions of Washed Rabbit Platelets Labelled With:

2.2.5.1 ^{51}Chromate ($Na_2{}^{51}CrO_4$)

$Na_2{}^{51}CrO_4$ (^{51}Cr) is taken up by platelets in the hexavalent form and rapidly reduced to a trivalent state within the cytoplasm (Steiner and Baldini, 1970). ^{51}Cr primarily binds to cytoplasmic nucleotides. Elution of ^{51}Cr from labelled platelets is negligible, and it is not reutilized if platelets lyse. ^{51}Cr is not released from platelets during the release reaction.

Suspensions of washed rabbit platelets were prepared in CF-TYR-ALB solution (first wash) containing 0.02% EGTA, pH 6.5 as described. The platelets were incubated with the ^{51}Cr in this washing solution for 45-60 minutes (150 µCi of ^{51}Cr was used for the platelets obtained from the blood of one donor rabbit). Unbound ^{51}Cr was removed by centrifuging the labelled platelets at 1500 x g_{max} for 10 minutes and resuspending them in CF-TYR-ALB for 10 minutes. The labelled platelets were again separated by centrifugation (1500 x g_{max}; 10 minutes) and finally resuspended in rabbit platelet poor plasma (PPP). In some experiments, ^{51}Cr-labelled platelets were washed once with CF-TYR-ALB and once with PPP before final suspension in PPP.

The platelet count of the ^{51}Cr-labelled platelet suspension was

determined and the platelet concentration in the suspension was adjusted with rabbit PPP to a final concentration of 2×10^6 platelets/μL. Four mL of this suspension of ^{51}Cr-labelled platelets (8×10^9 platelets) were injected into the marginal ear veins of recipient rabbits.

Samples (500 μL) of the total ^{51}Cr-labelled platelet suspension were transferred to glass (12 x 75 mm) test tubes to measure total (TOT) radioactivity in the suspension. Other samples (1.0 mL) of the labelled platelet suspension were centrifuged at 12,000 x g_{max} for two minutes (Eppendorf Centrifuge) and 500 μL samples of the supernatent (SN) were transferred to 12 x 75 mm glass test tubes to measure non-platelet associated radioactivity in the suspension.

The radioactivity injected into each animal was calculated as follows:

platelet associated radioactivity injected into each animal = V x 2A

where V = volume of suspension injected (4.0 mL)

A = platelet associated radioactivity in 500 μL suspension (TOT-SN)

The fraction of free, non-platelet associated ^{51}Cr injected was always less than 2%.

2.2.5.2 ^{111}Indium-Oxine (^{111}In)

^{111}In-oxine (^{111}In-8-hydroxyquinoline) is a lipid soluble complex and diffuses passively through platelet plasma membranes. Within the cell, ^{111}In binds to cytoplasmic components and oxine elutes from the cells (Hudson et al., 1981).

Suspensions of washed rabbit platelets were prepared in CF-TYR-ALB

solution containing 0.02% EGTA, pH 6.5 as described. The platelets were labelled in this solution with ^{111}In-oxine for 40-60 minutes. The platelets from the blood of one donor rabbit were labelled with 100 µCi of ^{111}In. The platelets were then processed as described for the ^{51}Cr-labelling procedure (Section 2.2.5.1).

Samples (500 µL) of the total ^{111}In labelled platelet suspension and samples (500 µL) of the suspension supernatant were prepared to determine the platelet associated radioactivity in the suspension.

Each recipient rabbit received an injection of 4.0 mL ($8. \times 10^9$ platelets) of ^{111}In-labelled platelets suspended in rabbit PPP.

2.5.3 Simultaneous Labelling of Rabbit Platelets with $Na_2{}^{51}CrO_4$ (^{51}Cr) and Diazotized ^{125}Iodosulfanilic Acid (D^{125}ISA)

In some experiments platelets were doubly-labelled with ^{51}Cr and D^{125}ISA. As previously described, ^{51}Cr labels cytoplasmic constituents. D^{125}ISA is a non-penetrating agent which labels platelet surface membrane (glyco)proteins.

Iodosulfanilic acid obtained in solution (n-propanol:water solvent) was converted to the salt by evaporating the solvent under a gentle stream of nitrogen. The dried sample was dissolved in 10 µL of ice cold distilled water. Diazotization was carried out at 0-5°C by the addition of 5 µL of 0.05M aqueous $NaNO_2$ followed by 5 µL of 0.1M aqueous HCl. The mixture was allowed to react for 5 minutes at which time 100 µL of phosphate buffer (0.2M, pH 7.5) was added. D^{125}ISA was prepared just prior to use as a platelet label.

Suspensions of washed rabbit platelets were prepared in CF-TYR-ALB

solution containing 0.02% EGTA. The platelets were centrifuged at 1500 x g_{max} for 10 minutes and resuspended for 10 minutes in CF-TYR solution without albumin containing 0.02% EGTA. The platelets were washed once more with CF-TYR solution (without albumin) containing 0.02% EGTA before they were suspended in a minimum volume (1 mL per 0.5 mL packed platelets) of CF-TYR solution without albumin containing 0.02% EGTA. The platelets were incubated with ^{51}Cr (100 µCi ^{51}Cr/donor rabbit) for 15 minutes in this solution. D^{125}ISA was added to the platelet suspension at a final concentration of 0.10µM. The platelets were incubated with both radioisotopes for 30 minutes. Labelling was terminated by the addition of a large volume (50 mL) of CF-TYR-ALB solution containing 0.02% EGTA. The doubly-labelled platelets were separated by centrifugation (1500 x g_{max}; 10 minutes). The platelets were washed three times in CF-TYR-ALB solution and once in rabbit PPP to remove unbound ^{51}Cr and D^{125}ISA before they were finally resuspended in rabbit PPP. The concentration was adjusted to 2 x 10^6 platelets per µL with rabbit PPP and 4.0 mL (8 x 10^9 platelets) of this suspension were injected into recipient rabbits. The fraction of free, non-platelet associated ^{51}Cr was less than 1% and free D^{125}ISA was 45.5% of the total

2.2.6 Measurement of Radioactivity

The radioactivity in samples was measured in a Beckman GAM-300 Radiation Counter (Beckman, Instruments Inc., Fullerton, CA) with a NaI (Tl) crystal. The samples were contained in 12 x 75 mm glass tubes and

a constant geometry (volume 2.0 mL) was maintained among all samples which were to be compared to each other. Radioactivity was measured at the 95% confidence limit whenever possible.

The radioactivity in samples containing ^{51}Cr (MeV 0.323, 0.237), ^{111}In (MeV 0.173, 0.247) or ^{131}I (MeV 0.637, 0.363, 0.282, 0.08) was measured with a Variable Discriminator Isoset Module supplied with the instrument with windows set at the appropriate energy spectrum from the Counters Operating Manual (Beckman Instruments Inc., Fullerton, CA 1973).

Samples which contained both ^{125}I and ^{51}Cr were measured with narrow ^{51}Cr window settings and a standard ^{125}I (MeV 0.035) window (Fixed Window Iso-set Module-^{125}I) supplied with the gamma counter. Standard solutions of ^{51}Cr or ^{125}I demonstrated that crossover of ^{125}I into the ^{51}Cr channel was negligible. The 14% crossover of ^{51}Cr into the ^{125}I channel was corrected for in all calculations.

2.2.7 Anesthesia

Rabbits which were to receive aortic catheters or sham operations were anesthetized with an intra-venous injection of sodium pentobarbital (40-50 mg/kg). The anesthetic was administered until the corneal reflex of the animal had subsided.

Animals that were to be prepared for whole body perfusion-fixation were preanesthetized for 15 minutes with Ketamine (100 mg, intramuscularly) and atropine (0.1 mg, subcutaneously). The rabbits then received an intravenous injection of sodium pentobarbital (20-30 mg/kg) until the corneal reflex subsided.

2.2.8 Insertion of Intra-Aortic Catheters in Rabbits

Rabbits were anesthetized with sodium pentobarbital (40–50 mg/kg intra-venously). The right femoral artery was isolated through a 1 cm incision about seven cm below the aortic trifurcation distal to the branches of the deep and lateral circumflex femoral arteries (Figure 2.1A). Two loose sutures were looped around the isolated segment of the femoral artery. The suture farthest from the body (distal) was ligated while the suture closest to the body (proximal) remained loosely tied. A Dieffenbach bulldog clamp (curved, 2.5 cm) was placed on the femoral artery between the two sutures close to the proximal suture, isolating a 0.5 cm segment of the artery. The isolated segment was punctured with a sterile needle (18G-1.5). The sealed tip of the catheter was introduced into the puncture site and inserted as far as the clamp (Figure 2.1B). Holding the catheter in place, the clamp was removed and the catheter was gently threaded into the femoral artery and aorta until the knotted, heat fluted end of the catheter abutted the hole in the femoral artery. The proximal suture was ligated around the femoral artery to prevent blood flow and to hold the catheter *in situ* (Figure 2.1C). The distal suture was ligated again around the fluted end of the catheter to anchor it firmly against the femoral artery. The incision was closed with one suture and the area was cleaned with a 2% solution of sodium hypochlorite.

The tip of a 20 cm catheter was always located 12.0 ± 0.5 (n= 6) cm above the aortic trifurcation and 10.0 ± 0.5 cm below the aortic arch in animals of the size used (2.5-3.8 kg).

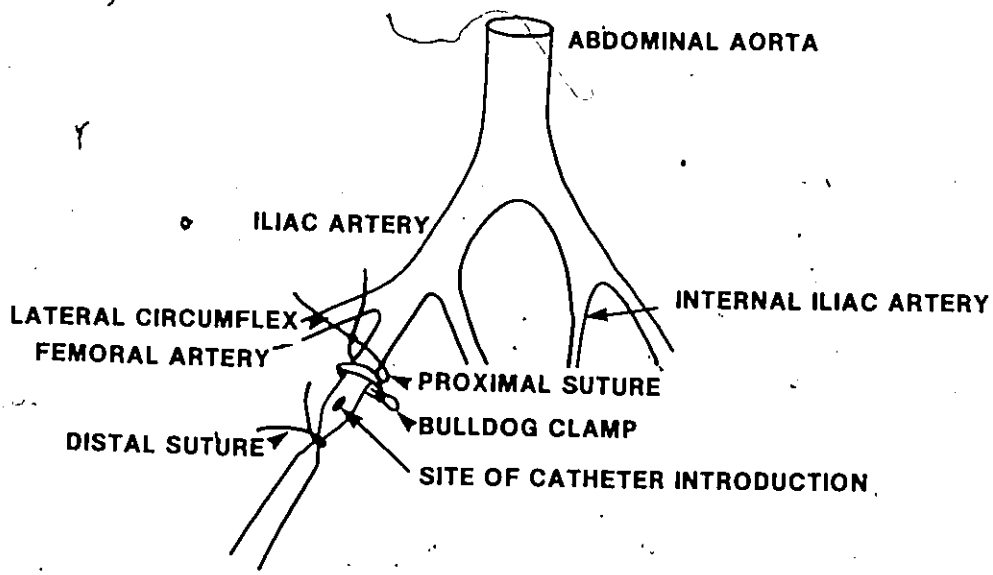
Figure 2.1

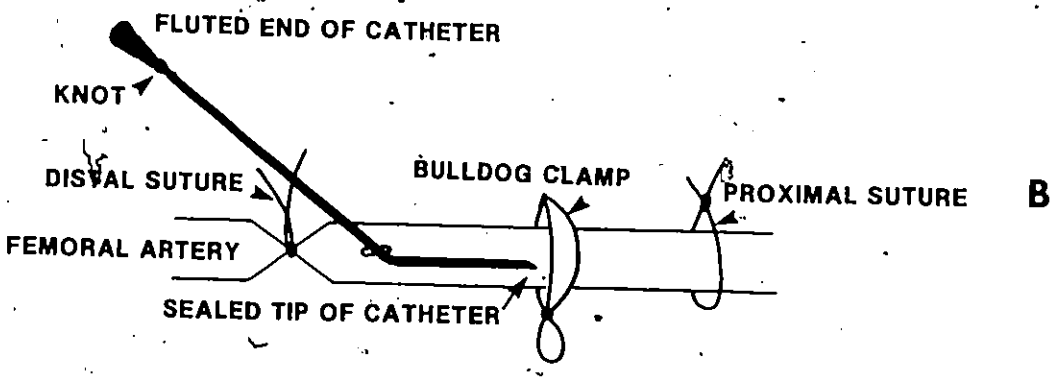
Insertion of Catheters into Aortae of Rabbits

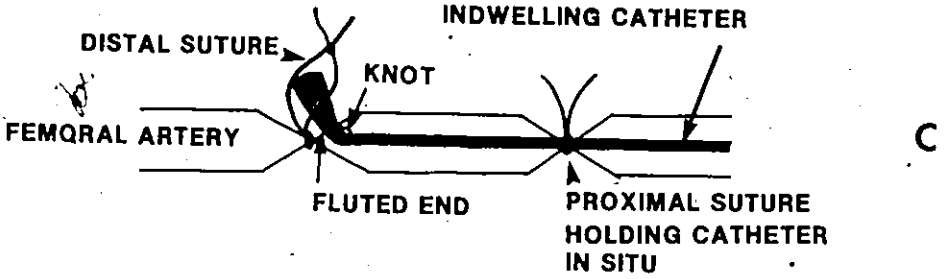
A) The right femoral artery was isolated distal to the lateral circumflex artery. Two sutures (proximal and distal) were applied around the isolated femoral artery. The distal suture was tied and a bulldog clamp was applied close to the loose proximal suture. The isolated arterial segment was then punctured.

B) The sealed, tapered end of the polyethylene catheter was introduced into the puncture site in the femoral artery and gently pushed toward the bulldog clamp. The bulldog clamp was then removed.

C) The total length of the catheter was inserted into the femoral artery until the knot in the catheter abutted the puncture in the vessel. The proximal suture was ligated to hold the catheter *in situ*. The distal suture was tied once more around the sealed, fluted end of the catheter.


ABDOMINAL AORTA
ILIAC ARTERY
LATERAL CIRCUMFLEX
FEMORAL ARTERY
INTERNAL ILIAC ARTERY
PROXIMAL SUTURE
BULLDOG CLAMP
DISTAL SUTURE
SITE OF CATHETER INTRODUCTION
A


FLUTED END OF CATHETER
KNOT
DISTAL SUTURE
BULLDOG CLAMP
PROXIMAL SUTURE
FEMORAL ARTERY
SEALED TIP OF CATHETER
B


INDWELLING CATHETER
DISTAL SUTURE
KNOT
FEMORAL ARTERY
FLUTED END
PROXIMAL SUTURE
HOLDING CATHETER
IN SITU
C

Control animals which received sham operations were treated in an identical manner except for the insertion of the catheter. In these animals, once the isolated femoral artery had been punctured, the proximal suture was ligated and the bulldog clamp was removed. The wound was sutured and cleansed as described.

The total surgical procedure was usually accomplished within 2-3 minutes.

In some experiments, 16 cm and 10 cm catheters were inserted into rabbit aorta. The 10 cm catheter extended 2.5 ± 0.2 cm (n=6) into the abdominal aorta and the 16 cm catheter extended 8 cm (n=7) above the aortic trifurcation.

2.2.9 Perfusion and Fixation of Rabbits

Rabbits were pre-anesthetized with Ketamine (100 mg, intramuscularly) and atropine (0.1 mg, subcutaneously) 15 minutes before they were anesthetized with sodium pentobarbital (approximately 20-30 mg/kg, intravenously). Cannulas (PE-190) were surgically inserted into the left femoral artery and via the left carotid artery into the ascending aorta. Immediately before perfusion, the animals received an intravenous injection of 1000 units of heparin.

Locke's-Ringer solution at 37°C, pH 7.4, oxygenated and containing 1U/mL of heparin, was perfused into the carotid cannula at arterial pressure (70-100 mm mercury). The perfusate flushed blood from the animal via the cannulated femoral (the anesthetized animal remained alive throughout this procedure). When the solution draining from the femoral cannula was almost clear of blood, the Locke's-Ringer solution was

discontinued and immediately replaced by a perfusion of 4% paraformaldehyde fixative in phosphate buffer (pH 7.4) under arterial pressure. This resulted in instant death of the animal. When paraformaldehyde fixative began to drain from the femoral artery, the femoral cannula was tied. The perfusion of paraformaldehyde was continued for approximately 3-5 minutes. The carotid cannula was then tied to keep the fixative in the vessels of the animals. The vessels were allowed to fix *in situ* for about 18 hours at 4°C.

2.2.10 Measurement of Mean Platelet Survival Time in Rabbits

^{51}Cr-labelled rabbit platelets (4.0 mL suspension containing 8×10^9 platelets) suspended in rabbit platelet poor plasma (PPP) were injected through a needle (23G-1) into the marginal ear veins of recipient rabbits. The labelled platelets were allowed to circulate for 2 hours. At this time a sample of venous blood (1.5 mL) was withdrawn into 0.5 mL ACD contained in a 3 mL syringe from the marginal vein of the opposite ear from that used for the platelet infusion.

The percentage recovery of platelets from the circulation was calculated from the ratio of the total platelet-associated radioactivity injected into each animal using the formula:

$$\text{Recovery} = \frac{\text{net cpm/mL blood sample} \times 58 \times \text{Wt. of rabbit (kg)}}{\text{platelet-associated radioactivity injected}} \times 100\%$$

where 58 X Wt. of rabbit (kg) = the conversion factor for rabbits for body weight to blood volume (Reimers *et al.*, 1973).

The 2 hour recovery sample was considered to represent maximum circulating radioactivity and was assigned a value of 100%. Additional blood samples were taken at 2, 19, 22, 43, 67, and 91, hours after the initial recovery (2 h) sample. The radioactivity in all blood samples was measured and expressed as a percentage of the radioactivity measurement at 2 hours (100%).

Mean platelet survival time was calculated using the gamma function described by Murphy et al. (1971a, 1973). The computer program for these calculations was provided by Dr. E.A. Murphy, Johns Hopkins Hospital School of Medicine, Baltimore, MD.

2.2.11 Platelet Survival Time in Rabbits with Aortic Catheters

Mean platelet survival time was determined in rabbits using a variety of experimental conditions.

2.2.11.1 Survival of ^{51}Cr-Labelled Platelets in Rabbits with Indwelling Aortic Catheters

To determine platelet survival time in rabbits with aortic catheters, ^{51}Cr-labelled platelets (4.0 mL suspension containing 8 X 10^9 platelets) were injected into recipient rabbits and allowed to circulate for 2 hours. At this time a venous blood sample (2 hour recovery sample) was obtained. Aortic catheters were inserted into rabbits via the femoral artery immediately after the recovery blood sample was obtained. Control animals received sham operations at this time. Additional blood samples were taken at the predetermined times (2, 19, 22, 43, 67 and 91

hours) after the initial sample to calculate mean platelet survival time.

2.2.11.2 Survival of ^{51}Cr-Labelled Platelets in Rabbits which had Catheter-Induced Thrombi in situ for Various Times before the Survival Studies were begun

In these experiments, catheters were inserted into rabbit aortae and thrombi were allowed to form on and around the areas of catheter induced injury for 3 and 24 hours, 3 and 6 days, 2 weeks and 4 weeks. Control animals had sham operations for the same periods of time.

At these times, ^{51}Cr-labelled platelets (4.0 mL containing 8 X 10^9 platelets) suspended in rabbit PPP were injected into each rabbit. Blood samples were taken at the predetermined times and mean platelet survival time was measured as described in section 2.2.10.

2.2.11.3 Survival in Normal Rabbits of ^{51}Cr-Labelled Platelets Obtained from Rabbits which had Aortic Catheters in situ for Various Times

Platelet survival time was also examined in untreated rabbits using platelets which had been exposed *in vivo* to aortic catheters. In these experiments, catheters were inserted into rabbit aortae and control animals received sham operations. The catheters remained *in situ* for 3 hours, 3 days or 6 days at which time the animals were anesthetized with sodium pentobarbital and the carotid artery was cannulated. Blood was withdrawn into a plastic (50 mL) syringe containing 7.5 mL ACD anticoagulant. The blood from animals with aortic catheters and blood from control animals was processed separately to isolate the platelets in CF-TYR-ALB solution containing 0.02% EGTA . Platelet suspensions were adjusted with CF-TYR-ALB solution to be similar in platelet counts. Each

platelet suspension was labelled with ^{51}Cr as described in section 2.2.5. Four mL of the ^{51}Cr-labelled platelets suspended in PPP were injected into normal untreated rabbits. Blood samples were obtained at the predetermined times and platelet survival was calculated as described previously in section 2.2.10.

2.2.12 Platelet Turnover in the Circulation

Platelet turnover in the circulation was examined in rabbits which had aortic catheters *in situ* 3 hours, 3 days, 2 weeks and 4 weeks. Control animals had sham operations for the same time periods.

At these times, whole blood platelet counts were determined in all animals before they received an injection of ^{51}Cr-labelled platelets (8×10^9 platelets suspended in PPP).

Blood samples (2.25 mL blood into 0.75 mL ACD) were taken from the rabbits at the predetermined times (2 hours after ^{51}Cr-labelled platelet injection; 2, 19, 22, 43, 67 and 91 hours after the initial 2 hour sample). The blood samples were mixed thoroughly. One mL of the blood sample was transferred to a polyethylene test tube to measure platelet counts; the remaining 2.0 mL of sample were transferred to glass (12 X 75 mm) test tubes for radioactivity measurements.

Mean platelet survival (hours) was calculated and the mean platelet count was determined.

Platelet turnover was calculated from the formula:

$$\text{Mean platelet turnover}/\mu L/h = \frac{\text{mean platelet count}/\ \mu L}{\text{mean platelet survival time (h)}}$$

2.2.13 Investigation of Thrombus Formation and Thrombus Reactivity in Rabbits with Indwelling Aortic Catheters

The relation between thrombus formation, thrombus reactivity and platelet survival was examined in rabbits with indwelling aortic catheters using several experimental approaches.

2.2.13.1 Platelet Incorporation into Thrombi Formed in Rabbits with Aortic Catheters

^{51}Cr-labelled rabbit platelets (8×10^9 platelets, suspended in rabbit PPP) were injected intravenously into rabbits and allowed to circulate for 2 hours. At this time, a venous blood sample was obtained to determine the recovery (2h) of circulating radioactivity. Immediately following the blood sampling, catheters were inserted into rabbit aortae. Control animals received sham operations.

The catheters remained *in situ* 1, 3, or 24 hours, 3, 7, or 14 days. At these times the animals were perfused with heparinized Locke's-Ringer solution under arterial pressure to remove the blood from the animal. When the perfusate from the rabbit was free of blood, 4% paraformaldehyde fixative solution in phosphate buffer was infused to fix the vessels and thrombi *in situ*.

The aorta, iliac and femoral arteries from control animals and from animals with catheters and thrombi *in situ* were dissected free from extraneous tissue, removed intact and placed into 4% paraformaldehyde solution. Great care was taken during this procedure to avoid damage to the vessel.

Seven consecutive 2.5 cm segments (Figure 2.2) beginning at the aortic trifurcation and extending toward the aortic arch were cut as representative sections from the vessel. The top of the 5th segment or the bottom of the 6th segment always contained the tip of the indwelling catheter. The 7 vessel segments with catheter and thrombi in situ were individually placed into 12 X 75 mm glass test tubes containing 2.0 mL of 4% paraformaldehyde and the radioactivity associated with the segments was measured in a gamma scintillation counter.

Since the 7th vessel segment was undamaged by the indwelling catheter, the amount of platelet associated radioactivity was negligible and this segment was not included in the calculations. The total radioactivity associated with the first 6 segments was expressed as a percentage of the platelet-associated radioactivity injected into each animal.

Thrombi and catheters were separated from each of the six vessel wall segments. The thrombi from within a vessel were pooled, air dried for 1-2 hours at room temperature and weighed. In some experiments, the six segments of vessel wall, respective catheter pieces and pooled thrombi were recounted separately to determine the amount of radioactivity associated with each fraction.

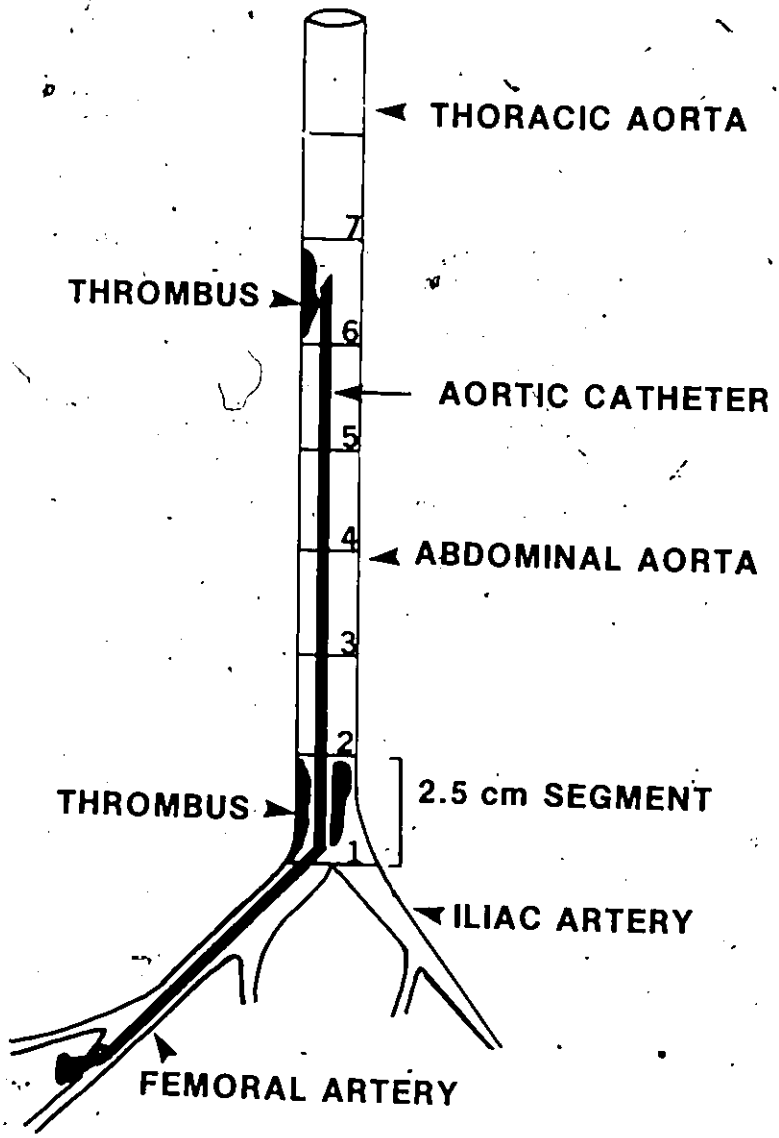
The radioactivity in each fraction (thrombus, vessel wall and catheter) was expressed as a percentage of the radioactivity injected into each animal and as a percentage of the radioactivity associated with the total vessel containing thrombi and catheter.

The radioactivity counts per minute (cpm) per milligram thrombus was also calculated.

Figure 2.2

Measurement of ^{51}Cr-Labelled Platelet Accumulation into Thrombi and on Injured Vessel Walls of Rabbits with Indwelling Aortic Catheters

The aorta with catheter and thrombi *in situ* was cut into six (1-6) consecutive 2.5 cm segments. Each vessel segment containing catheter and thrombi were counted individually. The total amount of radioactivity associated with the six vessel segments was expressed as a percentage of the platelet associated radioactivity injected into each rabbit. In some experiments the radioactivity associated with each fraction (thrombus, vessel wall, and catheter) was expressed as a percentage of the total radioactivity associated with the vessel containing thrombi and catheter.


THORACIC AORTA
7
THROMBUS
6
AORTIC CATHETER
5
4
ABDOMINAL AORTA
3
2
THROMBUS
2.5 cm SEGMENT
1
ILIAC ARTERY
FEMORAL ARTERY

2.2.13.2 Investigation of Thrombus Formation and Dissolution in Rabbits with Aortic Catheters using Platelets Doubly-Labelled with ^{51}Cr and $D^{125}ISA$

The dynamics of platelet incorporation into thrombi, platelet removal from thrombi and platelet lysis within thrombi which formed at sites of catheter injury was investigated using platelets which had their membrane (glyco)proteins labelled with $D^{125}ISA$ and their cytosol labelled with ^{51}Cr.

The ratio of $^{125}I/^{51}Cr$ of doubly-labelled platelets was determined. The doubly labelled platelets were then injected intravenously into rabbits (8×10^9 platelets suspended in PPP) and allowed to circulate for 2 hours. At this time a blood sample was obtained from the rabbits to determine the recovery of platelet-associated ^{125}I and ^{51}Cr from the circulation.

Catheters were immediately inserted into rabbit aortae and control animals received a sham operation. Additional blood samples were taken at 6, 12, and 24 hours, 3 and 6 days following the 2 hour sample.

It had previously been demonstrated that the ^{125}I label may elute from platelet membranes during the first 3 hours after the labelling procedure and that ^{125}I was bound readily to plasma proteins. Therefore, each blood sample containing doubly labelled platelets was centrifuged with Sera Sieve (Hughes & Hughes Ltd., Elms Industrial Estate, Essex, England) an inert gel that layers at the interface of the cells and plasma to facilitate maximum plasma removal before the platelet associated radioactivity was determined.

The platelet-associated ^{125}I and ^{51}Cr of each blood sample was

measured and the ratio of $^{125}I/^{51}Cr$ was calculated.

In addition, it was possible to determine mean platelet survival time in all animals on the basis of ^{125}I or ^{51}Cr concentration in the circulation and to compare the rate of radioisotope removal from the circulation.

At 6 and 24 hours, 3 days and 6 days after insertion of aortic catheters, groups of five animals (4 with aortic catheters; 1 with a sham operation) were killed by perfusion-fixation. The fixed aortae with catheters and thrombi in situ were processed as previously described. The thrombi within the 6 vessel segments beginning at the trifurcation and extending toward the thoracic aorta were removed, pooled, air dried and weighed. The ^{125}I and ^{51}Cr associated with the thrombi was measured and the ratio of $^{125}I/^{51}Cr$ of the thrombi was calculated.

2.2.13.3 Association of ^{51}Cr-Labelled Platelets with Thrombi which had Previously been Formed in Aortae of Rabbits with Indwelling Catheters

The reactivity of thrombi formed for various times in situ was measured by determining their ability to incorporate fresh platelets from the circulation.

In these experiments, catheters were inserted into rabbit aortae and thrombi were allowed to form on the injured vessel wall for 3 hours, 3, 6 and 14 days. Control animals had sham operations for the same periods of time. At these times, ^{51}Cr-labelled platelets (8×10^9 platelets suspended in rabbit PPP) were injected into all control rabbits with sham operations and into rabbits which had aortic thrombi formed in situ. The ^{51}Cr-labelled platelets were allowed to circulate for 2 hours before

whole blood samples were obtained to measure ^{51}Cr-labelled platelet recovery from the circulation. Twenty-four hours following the injection of ^{51}Cr-labelled platelets, the animals were killed by perfusion-fixation.

The aortae containing catheters and thrombi were dissected free from extraneous tissue, removed intact and processed as previously described. The thrombi were removed, pooled and weighed and the ^{51}Cr associated with each thrombus during the 24 hour period in which the labelled platelets circulated was measured and expressed as a percentage of the radioactivity injected into each animal.

2.2.14 Investigation of Vessel Wall Reactivity in Rabbits with Aortic Catheters

Repeated activation of the vessel wall by aortic catheters was examined using several approaches. The extent to which the aorta was injured by the catheter was determined by ^{131}I-labelled albumin accumulation in the vessel wall at various times following insertion of aortic catheters.

The degree to which the repeatedly injured vessel wall remained active toward fresh platelets in the circulation was examined by measuring the association of ^{111}In or ^{51}Cr labelled platelets at various times after the catheters had been inserted.

2.2.14.1 ^{131}I-Labelled Albumin Accumulation in Aortae of Rabbits with Indwelling Catheters

Catheters were inserted into rabbit aortae via the right femoral artery. Control animals received sham operations. The catheters remained *in situ* 3 hours, 3 days or 6 days after which time the rabbits received an injection (intravenous) of ^{131}I-labelled albumin (1mCi/mg/mL) in sterile physiological saline. Each rabbit received 5 uCi ^{131}I-labelled albumin/kg.

The labelled albumin was allowed to circulate for 1 hour before the animals were killed by perfusion-fixation.

The fixed aortae containing catheters and thrombi were dissected free from extraneous tissue, removed intact and placed in 4% paraformaldehyde solution. The vessels were cut into 2.5 cm segments beginning at the trifurcation and extending toward the thoracic aorta. The catheter pieces were gently removed from each vessel segment.

The radioactivity associated with 4 consecutive segments (Figure 2.3) was measured (beginning 2.5 cm above the trifurcation and extending toward the thoracic aorta). These 4 segments excluded the region of the catheter tip and the area of the trifurcation where macrospic thrombi formed.

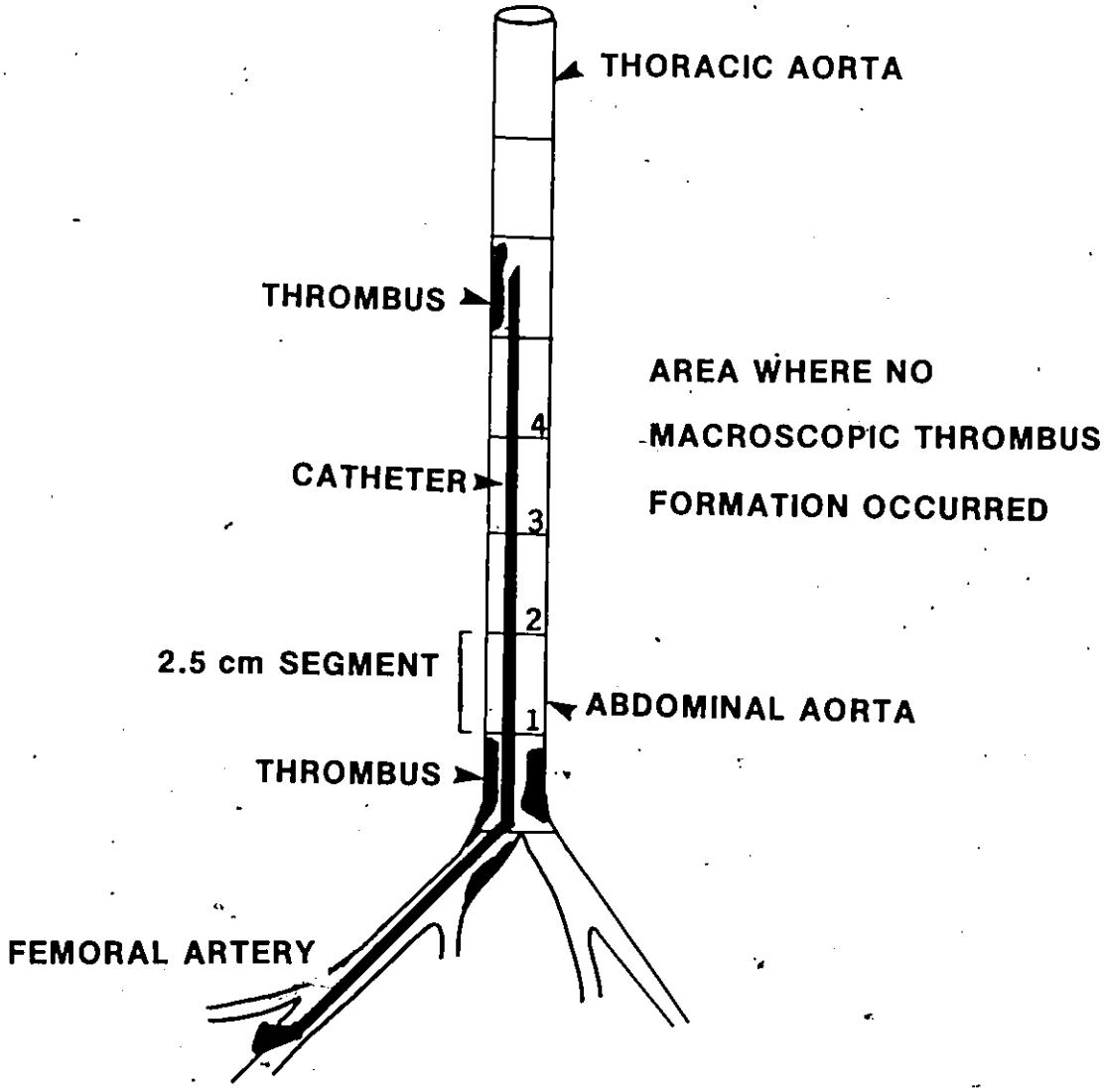
The total area of the 4 vessel segments was determined by summing measurements of the individual segments. The radioactivity associated with the aorta was expressed as counts per minute per square centimetre (cpm/cm^2).

Figure 2.3

Measurement of the Vessel Wall Reactivity in Rabbits with Indwelling Aortic Catheters

The fixed aorta containing catheter and thrombi were removed from the animals intact. The vessel was cut into 4 (1-4), 2.5 cm segments beginning 2.5 cm above the aortic trifurcation and extending toward the thoracic aorta. These 4 segments excluded the region of the catheter tip and the area at the trifurcation where macroscopic thrombi formed.

The 4 segments were considered as the representative area to determine vessel wall reactivity.


THORACIC AORTA
THROMBUS
AREA WHERE NO
MACROSCOPIC THROMBUS
FORMATION OCCURRED
4
CATHETER
3
2
2.5 cm SEGMENT
ABDOMINAL AORTA
1
THROMBUS
FEMORAL ARTERY

2.2.14.2 ^{51}Cr-Labelled Platelet Accumulation on the Vessel Wall of Rabbits with Aortic Catheters

The reactivity of the vessel wall toward circulating platelets was measured in rabbits which had catheters *in situ* 3 hours, 3, 6 and 14 days. Control animals had sham operations for the same time periods. ^{51}Cr-labelled platelets (8 X 10^9 platelets suspended in PPP) were injected into each rabbit and allowed to circulate for 2 hours before a blood sample was obtained to measure recovery of ^{51}Cr-labelled platelets from the circulation.

Twenty-four hours after the injection of ^{51}Cr-labelled platelets the rabbits were killed by perfusion-fixation.

The fixed aortae were processed as described for ^{131}I-labelled albumin accumulation (Section 2.2.14.1; Figure 2.3). The radioactivity associated with 4 consecutive vessel segments beginning 2.5 cm above the trifurcation (to avoid areas of macroscopic thrombus formation) and extending toward the thoracic aorta was measured. The total amount of radioactivity associated in 24 hours with the four segments was expressed as a percentage of platelet-associated radioactivity injected into each animal.

2.2.14.3 ^{111}In-Labelled Platelet Accumulation on the Vessel Wall of Rabbits with Aortic Catheters

Since ^{111}In-oxine has a higher labelling efficiency than ^{51}Cr and labels platelets with a higher specific activity, the reactivity of the vessel wall toward circulating platelets was also measured with ^{111}In-labelled platelets using a shorter (2 h) time period during which

the platelets circulated.

The experimental protocol was similar to that described for ^{51}Cr-labelled platelet accumulation on the vessel wall (Section 2.2.14.2). Briefly, rabbits had aortic catheters in situ 3 hours, 3 days or 6 days (controls had received sham operations) before they received an injection of ^{111}In-labelled platelets (8×10^9 platelets suspended in PPP). The platelets were allowed to circulate for 2 hours before a blood sample was taken to measure recovery of ^{111}In-labelled platelets in the circulation. The rabbits were immediately killed by perfusion-fixation.

The fixed aorta containing catheter and thrombi was processed as described (Figure 2.3). The radioactivity associating with 4 consecutive vessel segments beginning 2.5 cm above the trifurcation and extending toward the thoracic aorta was measured and expressed as a percentage of the platelet-associated ^{111}In injected into each animal.

2.2.15 Distribution in Stractan Density Gradients of Platelets from Rabbits with Indwelling Aortic Catheters

The distribution of platelet density subpopulations in Stractan density gradients was examined in rabbits with aortic catheters and in control animals which had received a sham operation. In these experiments, each rabbit served as its own control since the density distribution of the circulating platelets of each rabbit was examined before and at various times (2 hours to 6 weeks) after the surgical procedure.

2.2.15.1 Preparation of Suspensions of Washed Rabbit Platelets from Small Volumes of Whole Blood

All glassware used to prepare suspensions of washed rabbit platelets was siliconized.

Centrifugations were performed at 23°C in a Sorval RC-3 centrifuge with a swing bucket rotor.

Blood (2.5 mL) was quickly withdrawn from the marginal ear veins of rabbits through a 23G-1 needle into a 5.0 mL plastic syringe containing 0.5 mL ACD anticoagulant solution. The sample was well mixed and transferred to a graduated conical glass centrifuge tube. ACD-CF-TYR-EGTA-ALB solution, pH 6.5, was added to bring the total volume to 5.0 mL. The sample was well mixed and centrifuged at 350 x g_{max} for 1.5 minutes. The platelet rich plasma (PRP) was removed with a pipette to another graduated, conical glass centrifuge tube. The packed red blood cells were reconstituted to a volume of 5.0 mL with ACD-CF-TYR-EGTA-ALB, mixed and centrifuged as before. The supernatant fluid containing platelets was removed and pooled with the PRP from the first spin. This procedure was repeated five times.

The pooled samples (from the multiple wash procedure) containing the platelets, were centrifuged at 1500 x g_{max} for 10 minutes. The supernatant fluid was discarded and the platelet pellet was resuspended in 2.5 mL CF-TYR-ALB containing 0.02% EGTA. The platelet count of the suspension was determined.

In some experiments, additional samples of blood (0.75 mL blood, 0.25 mL ACD) were withdrawn from the rabbits to measure whole blood platelet counts.

The yield of platelets in the CF-TYR-EGTA-ALB suspension was calculated from the formula:

$$\text{platelet yield} = \frac{\text{no. of platelets in suspension}/\mu L \times 10^3 \times \text{vol. of suspension (2.5 mL)}}{\text{no. of platelets}/\mu L \text{ in whole blood} \times 10^3 \times \text{vol. of whole blood from which suspension was prepared (2.5 mL)}} \%$$

The platelet yield using this multiple wash procedure was 107 ± 2.8% (n=8).

2.2.15.2 Separation of Platelets into Density Subpopulations on Discontinuous Stractan Density Gradients

The platelet suspension (2.5 mL) was layered on Stractan density gradients consisting of 3 layers of Stractan solutions of different concentrations (18% bottom layer, 15% middle layer, 13.5% top layer; (Figure 2.4A)

The Stractan density gradients layered with the platelet suspensions were centrifuged at 23°C, at 1700 x g_{max} for 45 minutes (Figure 2.4B). At the end of this time five consecutive 2.0 mL fractions were removed from the Stractan gradients into separate 12 x 75 mm glass test tubes (Figure 2.4C). Great care was taken to avoid disturbing the gradient interfaces during removal of each 2.0 mL fraction. Once the fractions were separated, the top 2.0 mL fraction contained only the CF-TYR-ALB platelet suspending solution (Figure 2.4C). The second 2.0 mL fraction contained the least dense subpopulation of rabbit platelets. The third

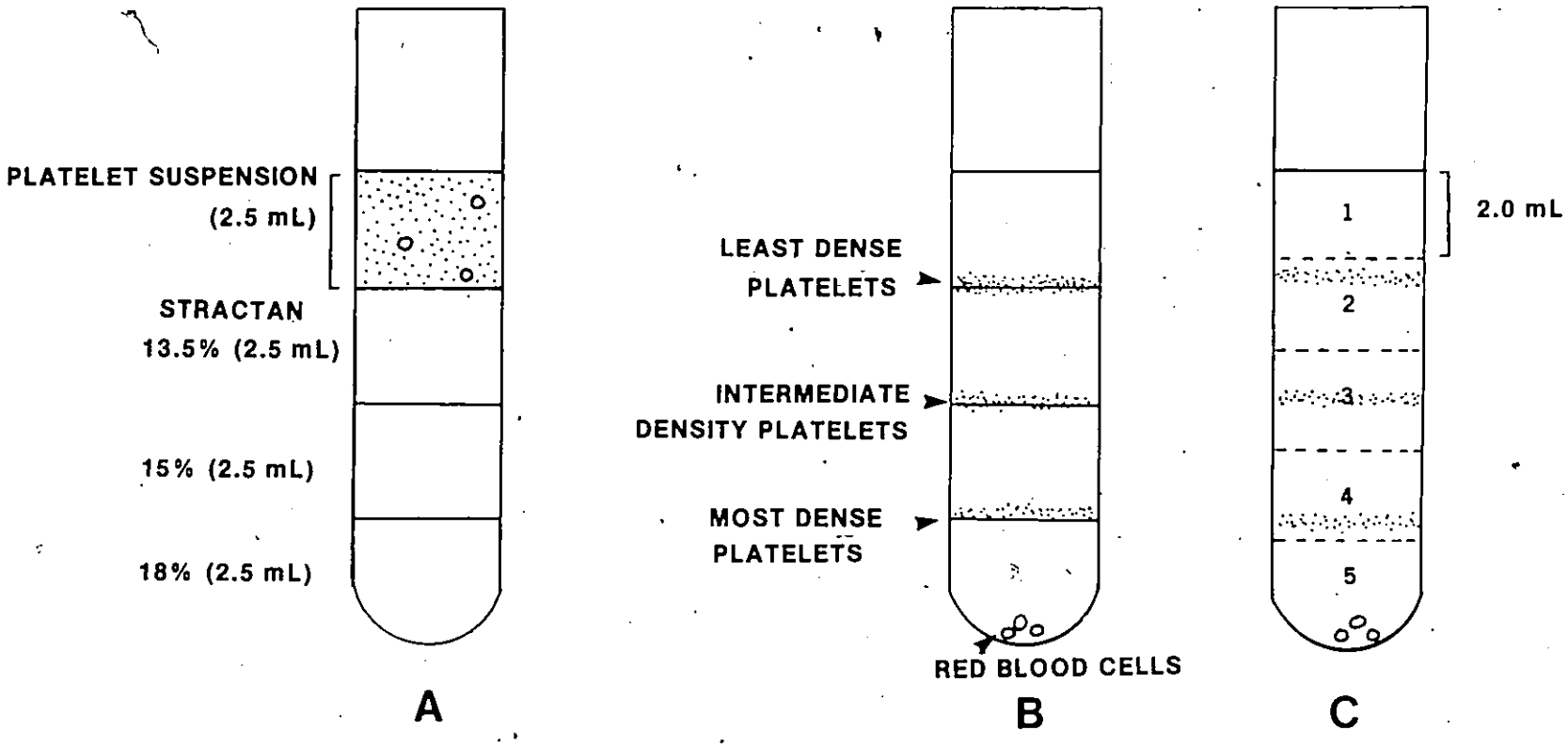
Figure 2.4

Separation of Platelets into Density Subpopulations on Discontinuous Stractan Density Gradients

A) The platelet suspension (2.5 mL) was layered unto a density gradient composed of 3 layers of Stractan solutions (18% bottom, 15% middle 13.5% top).

B) The gradients layered with the platelet suspension were centrifuged at 1700 x g_{max} for 45 minutes at 23°C to separate the platelets into density sub-populations.

C) Five (1-5) consecutive 2.0 mL fractions were removed from the gradients. The top fraction contained only the platelet suspending solution. The second fraction contained the least dense sub-population of platelets while the third and fourth fraction contained intermediate density and most dense platelets respectively. The last 2.0 mL fraction contained contaminating red blood cells (RBC) which passed through the gradients. The number of platelets in each fraction was expressed as a percentage of the number of platelets layered on the gradient.


PLATELET SUSPENSION
(2.5 mL)
STRACTAN
13.5% (2.5 mL)
15% (2.5 mL)
18% (2.5 mL)
A
LEAST DENSE
PLATELETS
INTERMEDIATE
DENSITY PLATELETS
MOST DENSE
PLATELETS
RED BLOOD CELLS
B
1
2
3
4
5
2.0 mL
C

and fourth 2.0 mL fractions contained platelets of intermediate density and most dense platelets respectively. The last 2.0 mL fraction contained contaminating red blood cells which passed through the gradients during centrifugation.

The number of platelets in each fraction was measured and expressed as a percentage of the number of platelets layered on the gradient.

2.2.16 Morphology of Injured Vessel Walls and Arterial Thrombi Formed in Rabbits with Aortic Catheters

Catheters were inserted into rabbit aortae and remained *in vivo* for 3 and 24 hours, 3, 5, and 6 days, 2 and 4 weeks or 4 months. Control animals had sham operations for the same periods of time. At these times, animals from each group were killed by perfusion-fixation. The vessel and thrombus morphology were examined after the vessels had been fixed *in situ* for about 18 hours.

2.2.16.1 Changes in the Permeability of Rabbit Aortae -Evans Blue Dye Studies

In some experiments, animals received an intravenous injection of Evans blue dye (4.5 mg/kg) one hour before they were perfused with Locke's-Ringer solution containing heparin followed by 4% paraformaldehyde fixative solution or 4% gluteraldehyde fixative.

The fixed aortae were dissected free from extraneous tissue and removed intact to a solution of the same fixative used for perfusion-fixation.

In some experiments the vessel was opened longitudinally to expose the catheter and thrombi *in situ*. The vessel was photographed to show the extent of thrombus formation and areas of increased vessel permeability.

2.2.16.2 Light Microscopy

Animals whose vessels were to be processed for light microscopy were perfused with Locke's-Ringer solution containing heparin and 4% paraformaldehyde solution.

The fixed aortae were removed intact and placed in a solution of 4% paraformaldehyde as previously described.

The aorta with catheter and thrombus *in situ* was cross-sectioned at the area of the catheter tip and at the trifurcation where thrombi had formed. The cross-sections containing catheter and thrombi were dehydrated over an 18 hour period through a series of ethanol/water solutions (70%, 95%, 95%) followed by absolute ethanol, chloroform (100%) and chloroform/xylene (50/50). The specimens were then impregnated with and imbedded in wax (Paraplast, Lancer Div. of Sherwood Medical, St. Louis, MO). Sections (7um) were sliced on a microtome (American Optical Corp., Buffalo, NY) and mounted on glass slides coated with albumin.

2.2.16.3 Determination of Thrombus Composition with Lendrum's Martius Scarlet Blue Stain

The relative proportions of, fibrin and red blood cells in thrombi of different ages were determined by staining the thrombus cross sections with Lendrum's Martius Scarlet Blue Stain. This procedure stains

fibrin (red), nuclei (blue-black), connective tissue (blue), erythrocytes (yellow) and muscle (red) (Drury and Wallington, 1967).

Slides of aortic cross-sections were dewaxed in several rinses of xylene and hydrated through 100% ethanol, ethanol/water solutions (100%, 95%, 80%, 70%, 50%) followed by a rinse in tap water. The specimens were fixed for 15 minutes in Bouins fixative at 37°C. The sections were rinsed in running tap water until the excess yellow colouring from the Bouins fixative was gone. Nuclei were stained for 5 minutes with Celestine Blue followed by a rinse in running tap water. The aortic sections were then stained in Mayers Hematoxylin for five minutes and rinsed once more in running tap water followed by 2 minutes in Scott's tapwater. This was followed by a rinse in 95% ethanol/water, 2 minute treatment in Martius Yellow solution and then a rinse with distilled water. The sections were then immersed in Brilliant Crystal Scarlet solution for 10 minutes before they were rinsed with distilled water. The specimens were immersed in 1% phosphotungstic acid for 5 minutes to differentiate the stains and subsequently rinsed with distilled water. The sections were stained in Soluble Blue solution for 10 minutes, rinsed in 1% acetic acid and blotted dry on bibulous paper, dehydrated, cleared and coverslipped.

2.2.16.4 Electron Microscopy

Animals whose vessels were to be processed for electron microscopy were perfused with Locke's-Ringer solution containing heparin followed by 4% gluteraldehyde in sodium cacodylate buffer (0.2M, pH 7.4).

2.2.16.4.1 Scanning Electron Microscopy (SEM)

Gluteraldehyde fixed vessels from which catheters had been removed were cut into 1 cm^2 specimens for scanning electron microscopy. Representative aortic sections were cut from the area of the catheter tip, the abdominal aorta and from the aortic trifurcation.

The vessel sections were post-fixed with a 1% solution of OsO_4 in 0.1M aqueous sodium cacodylate buffer. The specimens were then dehydrated through aqueous graded ethanol (50%, 70%, 95%, 100%, 100%) and dried in a CO_2 critical point dryer (Bomar SPC-900). The dried tissues were mounted on aluminum specimen stubs and coated with gold in a Polaron (Model-5100). The vessel surface was then examined in a Philips 501-B scanning electron microscope and photographed.

2.2.16.4.2 Transmission Electron Microscopy (TEM)

Specimens (1 mm^2) for TEM studies were cut from the area of the catheter tip and the area of the trifurcation of gluteraldehyde-fixed rabbit aortae with thrombi in situ.

The specimens were post fixed with a 1% solution of OsO_4 in 0.1M aqueous sodium cacodylate buffer. The tissues were stained en bloc in 10% aqueous uranyl acetate for 1 hour at room temperature. The specimens were dehydrated through graded ethanol (50%, 70%, 95%, 100%, 100%) followed by 100% propylene oxide. The tissues were impregnated with and embedded in Spurr's firm resin (Spurr, 1969). The samples were sectioned ultra thin on a Reichert Ultra-Microtome and collected on uncoated 200 mesh copper grids. The sections were stained with lead citrate (Reynolds, 1963), examined in a Philips 301 transmission electron microscope at 60 kV accelerating voltage and photographed.

2.2.17 Statistical Analyses

All results are expressed as mean ± standard error (mean ± SE). Logarithmic transformation normalized the data from some experiments for statistical analyses. Where appropriate, Student's 't' test for independent means, regression analysis, single factor analysis of variance (ANOV) and repeated measures analysis of variance and co-variance (ANOV) were used for statistical comparisons. The Biomedical Computer Program Statistical Package (previously known as BMDP, 1982) was used to calculate the repeated measures ANOV. Statistical significance was defined at a p value of <0.05. Data which showed significant differences by ANOV were further analyzed by Student-Newman-Keuls (SNK) multiple range test (Zar, 1974) to compare all pairs of means, or, by Dunnet's test (Zar, 1974) to compare means to control means.

CHAPTER 3

RESULTS

3. RESULTS

These studies examined the relation among vessel injury, thrombus formation and platelet survival in rabbits which had polyethylene catheters surgically implanted into their aortae. The catheter extended from the femoral artery to the trifurcation and along the length of the aorta to the lower thoracic region. Movement of the catheter within the aorta facilitated repeated interaction with the vessel endothelium.

The effect of the indwelling catheter on the aortic morphology was examined. The dynamics of thrombus growth at sites of vessel injury was determined and the relation between thrombus formation and platelet survival was examined.

In addition, the role of the repeatedly activated vessel wall in the reduction of mean platelet survival time was established.

3.1 Effects of Indwelling Aortic Catheters in Rabbits on the Morphology of the Vessel Wall

3.1.1 Accumulation of Evans Blue Dye into Aortae of Rabbits with Indwelling Catheters

The extent to which the aortic endothelium was injured by indwelling catheters was examined in rabbits after catheters had been *in situ* for various lengths of time. The animals received an injection of Evans blue dye (4.5 mg/kg) one hour before they were killed by perfusion fixation. Since the dye binds to albumin in the circulation (Packham, 1967), in areas where endothelial cell injury has occurred, the vessel wall becomes permeable to macromolecules including the albumin-bound dye resulting in enhanced blueing of the aorta at sites of injury.

Accumulation of Evans blue in aortae of animals with indwelling catheters showed that movements of the catheter repeatedly damaged endothelial cells along the length of the vessel to alter their permeability for as long as four months after the insertion of the catheter (Figures 3.1, 3.2, 3.3, 3.4). When the catheters had been *in situ* for 24 hours to 6 days, (Figures 3.1, 3.2, 3.3) the most intensive accumulation of dye occurred in the area of the catheter tip and at the abrtic trifurcation where the catheter curved against the vessel wall. Within four months after the insertion of the catheter (Figure 3.4) the intensity of dye accumulation was more evenly dispersed over the entire area of the vessel wall where the catheter had extended.

Significant amounts of dye did not accumulate in the aortic regions examined (thoracic aorta to trifurcation) in animals which had received

Figure 3.1

Accumulation of Evans Blue Dye in Abdominal and Thoracic Aorta of a Rabbit with an Indwelling Catheter

A) Rabbit which had an aortic catheter *in situ* for 24 hours.

B) Rabbit which had received a sham operation 24 hours before it was killed by perfusion-fixation.

In each animal in this experiment and in the following experiments, the catheter was inserted via the right femoral artery. The rabbits were given an injection of Evans blue dye (4.5 mg/kg) one hour before they were killed by perfusion-fixation. Areas of blueing indicates the regions of increased vessel permeability as a result of the catheter induced injury.

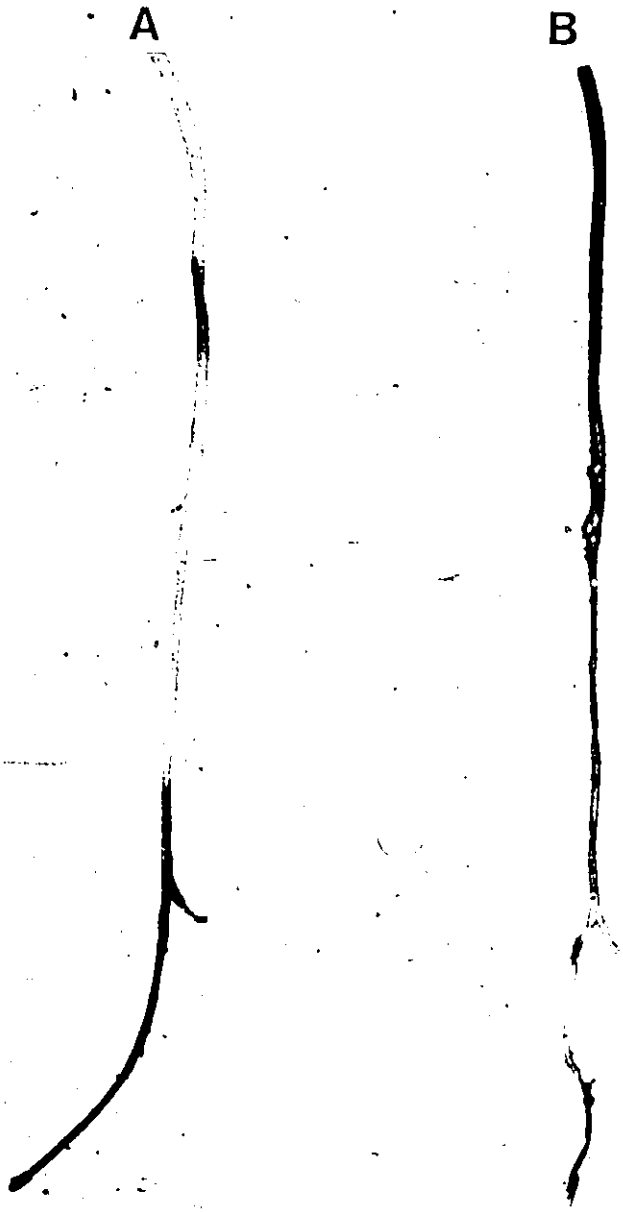

A
B

Figure 3.2

Accumulation of Evans Blue Dye in the Abdominal and Thoracic Aorta of a Rabbit with an Indwelling Catheter

A) Rabbit which had an aortic catheter *in situ* for 3 days.

B) Rabbit which had received a sham operation 3 days before it was killed by perfusion-fixation.

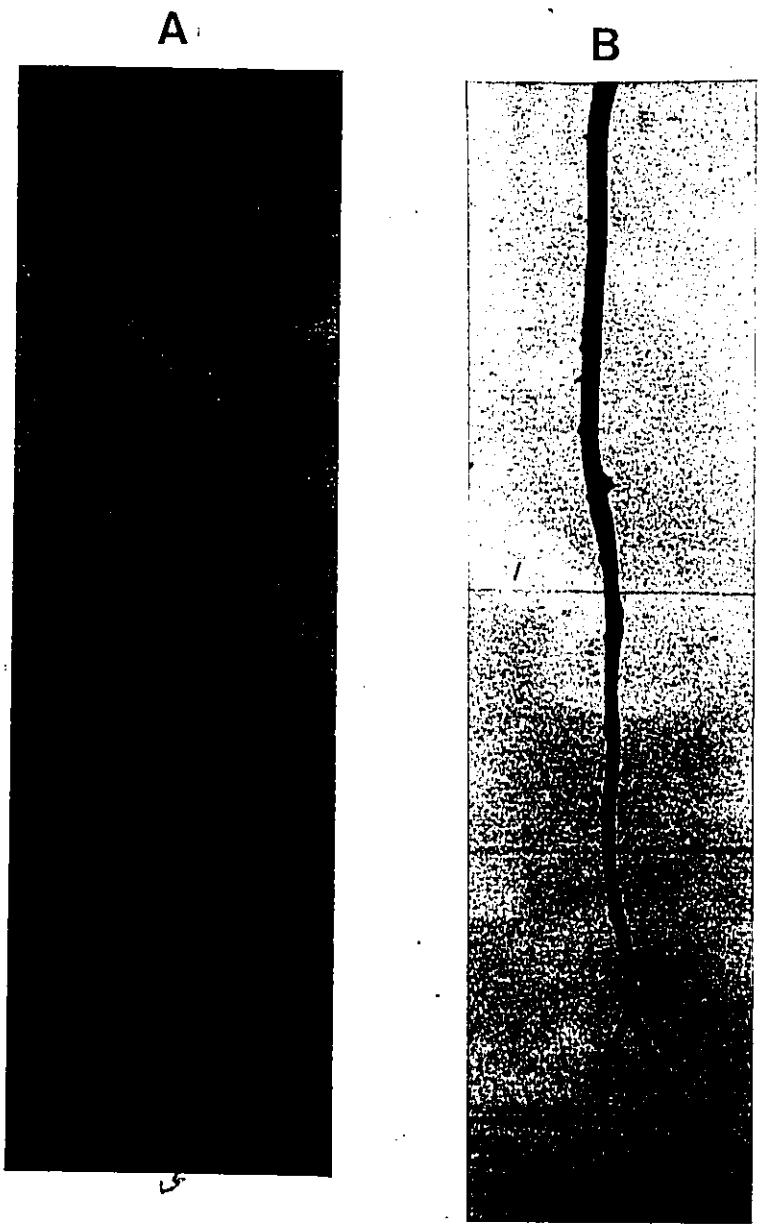

A
B

Figure 3.3

Accumulation of Evans Blue Dye in the Abdominal and Thoracic Aorta of a Rabbit with an Indwelling Catheter

A) Rabbit which had an aortic catheter *in situ* for 6 days

B) Rabbit which had received a sham operation 6 days previously.

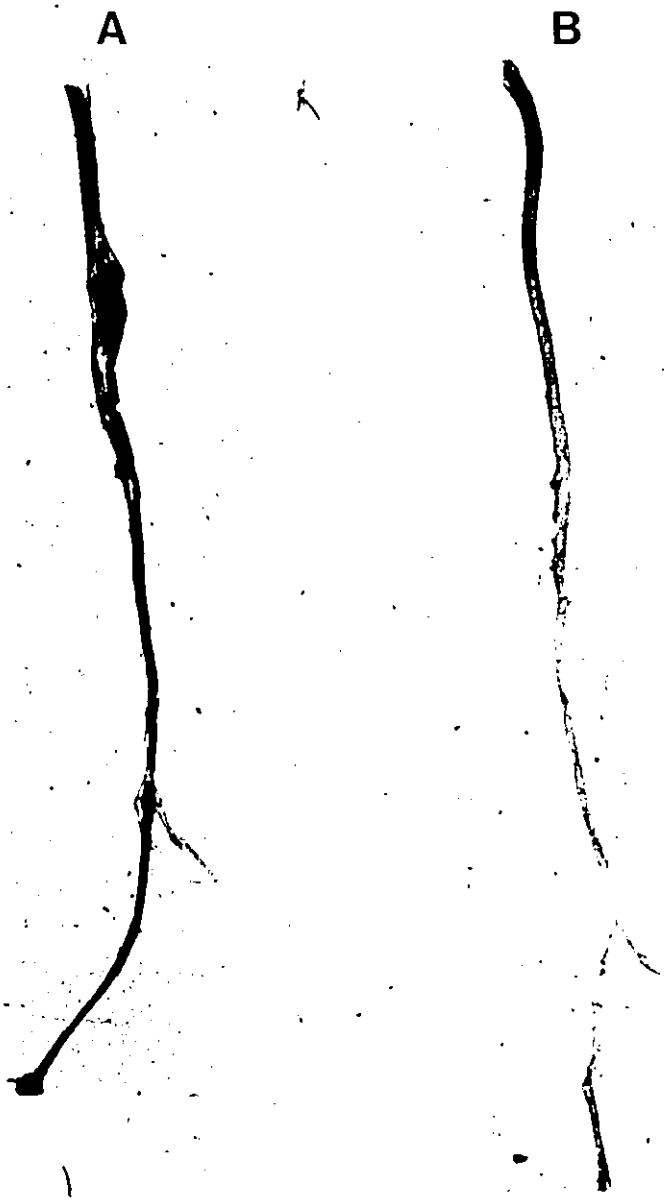

A
B

Figure 3.4

Accumulation of Evans Blue Dye in the Abdominal and Thoracic Aorta of a Rabbit with an Indwelling Catheter

A) Rabbit which had an aortic catheter *in situ* for 4 months.

B) Rabbit which had received a sham operation 4 months previously.

A

B

sham operations although there was evidence of minimal dye accumulation around orifices of branch vessels (Figure 3.1B).

3.1.2 Gross Morphology and Light Microscopy of Thrombi Formed in Aortae of Rabbits with Indwelling Catheters

Examination of fixed aortae from rabbits with indwelling catheters showed that the catheters remained free within the aortae for at least 4 weeks and it was assumed and later demonstrated (see section 3.1.3.2) that movements of the catheter during this time would result in repeated injury to the endothelium (Figure 3.5).

Macroscopic thrombi formed primarily in the region of the aortic trifurcation and in the thoracic aorta where the catheter tip was located. Thrombi that formed at the catheter tip were irregular in shape and were attached to the side of the vessel wall (Figure 3.6). In contrast, thrombi that formed at the trifurcation (Figure 3.7) extended around the circumference of the vessel and around the catheter. These thrombi were attached to the vessel wall in several places.

Thrombi from both areas were friable and mottled in appearance (Figures 3.6, 3.7). Distinct bands of grey-white material were usually observed (Figure 3.7).

Examination by light microscopy of thrombi that had formed *in situ* for 3 hours, 3 days or 6 days (Figure 3.8 A, B, C) showed that the thrombus composition was similar. Homogeneous bands of varying width consisting of layers of platelets and dense zones of fibrin were prominent. Masses of red blood cells enmeshed in fibrin were also present. Leucocytes were visible at the margins of the platelet zones among the masses of red blood cells.

Figure 3.5

Fixed Aorta from a Rabbit which had a Catheter in situ for 5 Days

The catheter remained free within the vessel. Macroscopic thrombi were observed in the region of the catheter tip and at the aortic trifurcation. In all of these experiments the thrombus extended into the right femoral artery where the catheter was inserted.

T - thrombus

C - catheter

ThA - thoracic aorta

AbA - abdominal aorta

Trf - trifurcation

FA - femoral artery

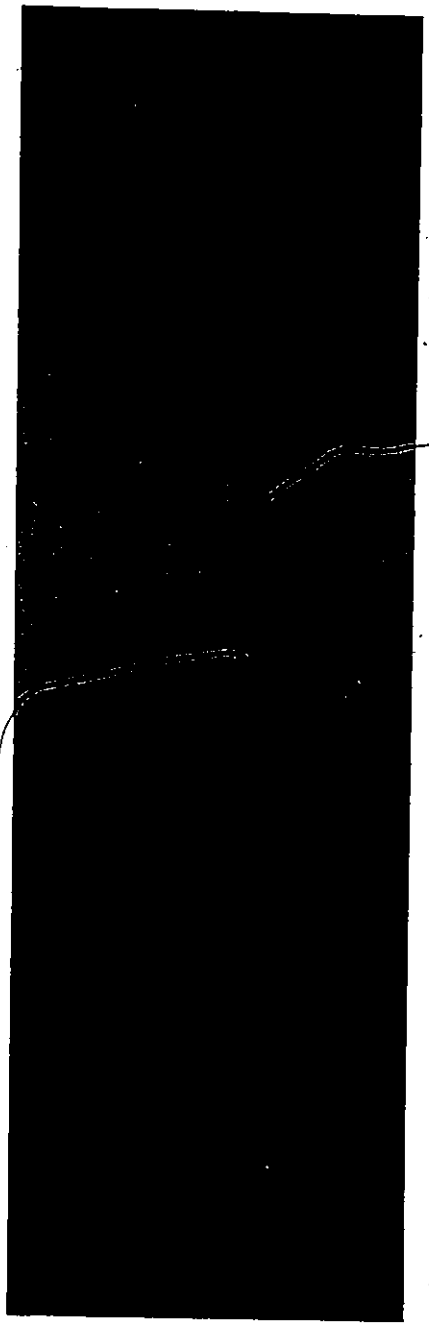
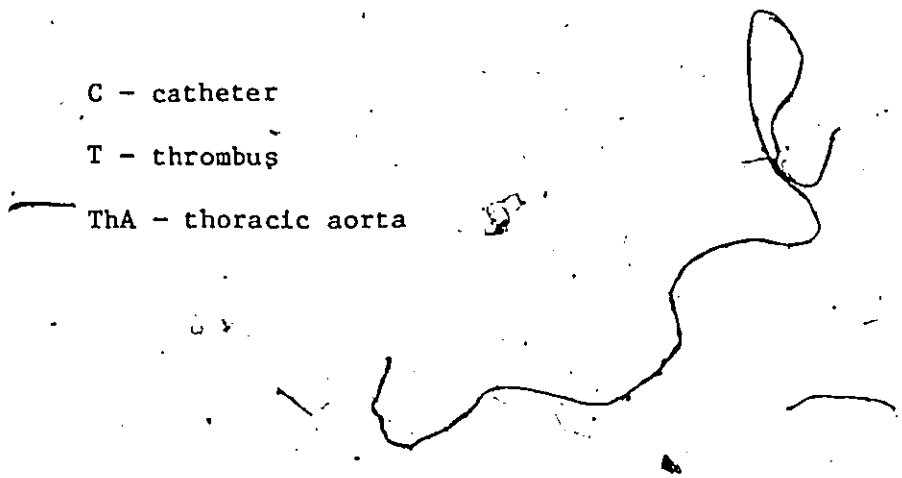


Figure 3.6

Macroscopic Thrombus Formation in the Thoracic Aorta of a Rabbit which had a Catheter in situ for 6 Days

The thrombus was irregular in shape and had a grey and red mottled appearance. The thrombus adhered to the side of the injured vessel wall.

The catheter remained free within the vessel and areas of blueing indicate regions of increased vessel permeability where the endothelium had been injured by the catheter.



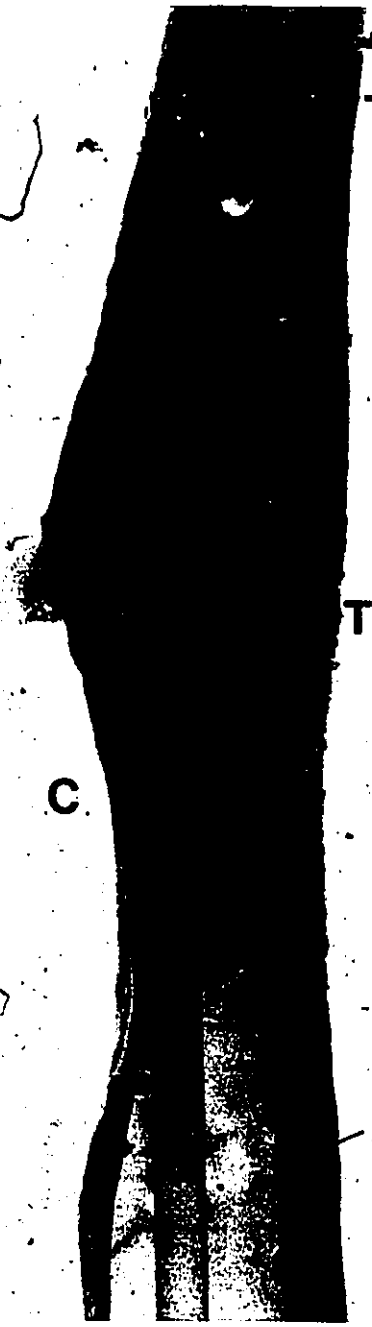

ThA
T
C

Figure 3.7

Macroscopic Thrombus Formation in the Area of the Aortic Trifurcation in a Rabbit which had an Aortic Catheter in situ for 6 Days

Mottled red and grey-white thrombi formed around the catheter in the aortic trifurcation. A distinct band of grey-white material was visible within the thrombus. The thrombus was attached to the vessel wall in numerous places and extended into the femoral artery where the catheter was inserted.

IA - iliac artery (right)

Trf - trifurcation

T - thrombus

C - catheter

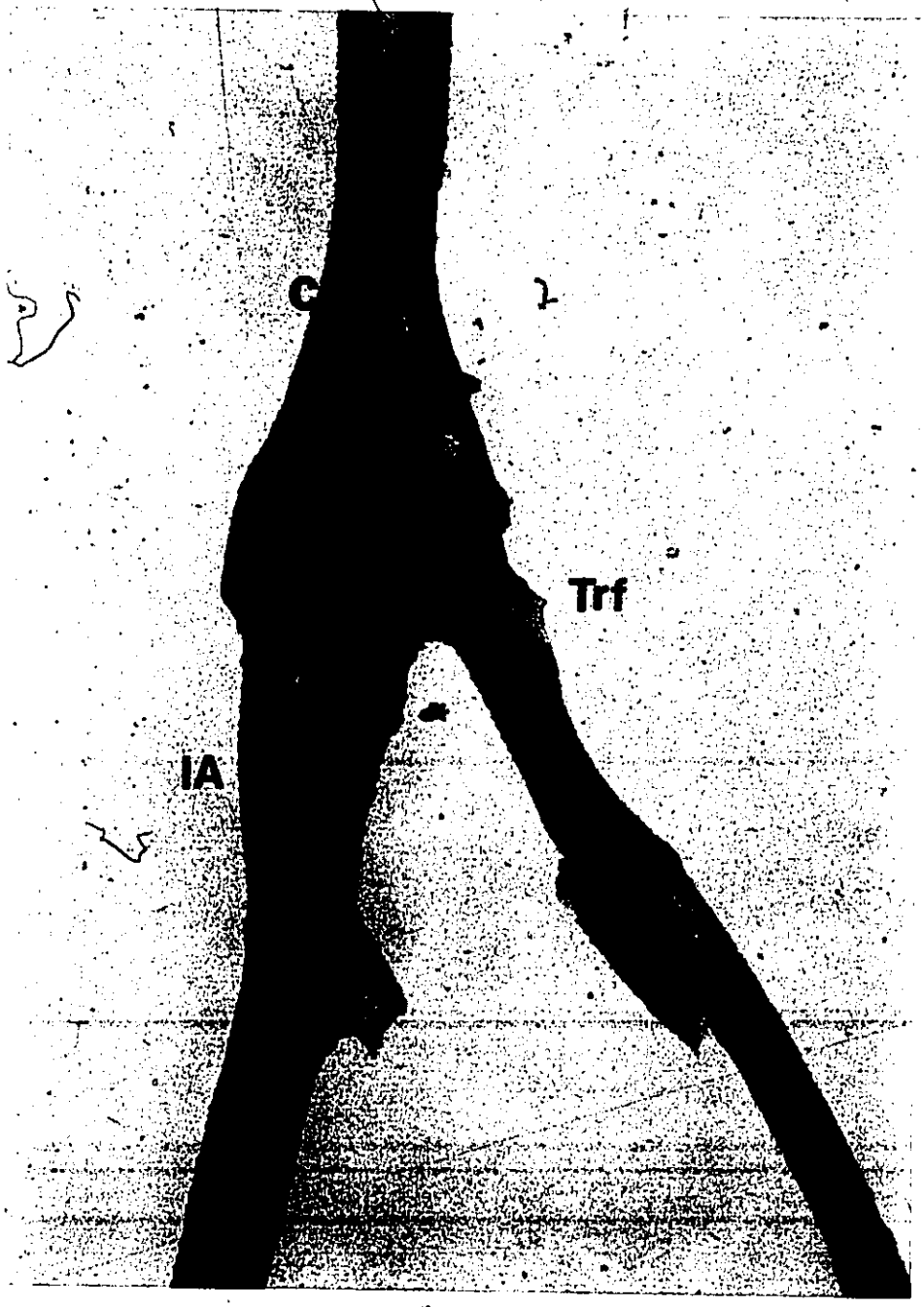

C
Trf
IA

Figure 3.8

Light Micrographs of Thrombi Obtained from Aortae of Rabbits which had Catheters in situ for Various Time Periods

A) The thrombus was obtained from the trifurcation area of the aorta of a rabbit which had a catheter in situ for 3 hours. The thrombus was composed primarily of platelets (PLT), white blood cells (WBC) and red blood cells (RBC). X 468

B) The thrombus was obtained from the region of the catheter tip in the aorta of a rabbit which had a catheter in situ for 3 days. The thrombus was attached to the vessel wall and was composed of bands of platelets and fibrin (F). White blood cells (WBC) and red blood cells (RBC) were also present. X 360

C) The thrombus was obtained from the trifurcation area of the aorta of a rabbit which had a catheter in situ for 6 days. The thrombus was composed of bands of platelets and fibrin (F) and masses of red blood cells (RBC). White blood cells (WBC) were prominent at margins of platelet zones. X 360

As noted from the yellow stained erythrocytes, all specimens were stained with Lendrum's Martius Scarlet Blue Stain which stains fibrin (red), nuclei (blue-black), connective tissue (blue), erythrocytes (yellow), muscle (red).

WBC
A
B
C

3.1.3 Scanning Electron Microscopy (SEM)

The rabbit aortae prepared for SEM were obtained from animals which had aortic catheters *in situ* 3 hours (n=3), 3 days (n=3) or 5 days (n=3), and from rabbits which had sham operations for the same time periods (n=3).

Six sections of aorta, each 2.5 cm in length including the area from the trifurcation to the lower portion of the thoracic aorta were examined from each rabbit. Each segment of the vessel was opened longitudinally and the catheter was carefully removed to avoid further damage to the vessel wall and to prevent the thrombus from being dislodged from the damaged aorta.

3.1.3.1 Endothelium from Normal Rabbit Aorta

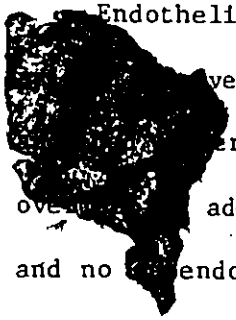
SEM of views of the endothelial surface of rabbit thoracic aortae (Figure 3.9) showed a continuous layer of flattened cells with tapering ends. The endothelial cells were oriented along the longitudinal axis of the aorta. The centre of all cells exhibited nuclear bulges. Individual cells were closely apposed and scalloped margins of adjacent cells often overlapped. The cell margins were continuous and no interruptions between cells were evident. Regular, longitudinal endothelial folds were present. Platelets and other formed elements from the blood were not seen on the surface of the endothelial cells.

3.1.3.2 Scanning Electron Micrographs of the Luminal Surface of Rabbit Aortae which had Indwelling Catheters *in situ* for Various Times

Three hours after the insertion of aortic catheters, endothelial cells had detached from the vessel wall in the area of the catheter tip

Figure 3.9

Scanning Electron Micrograph of Endothelium from a Normal Rabbit Aorta

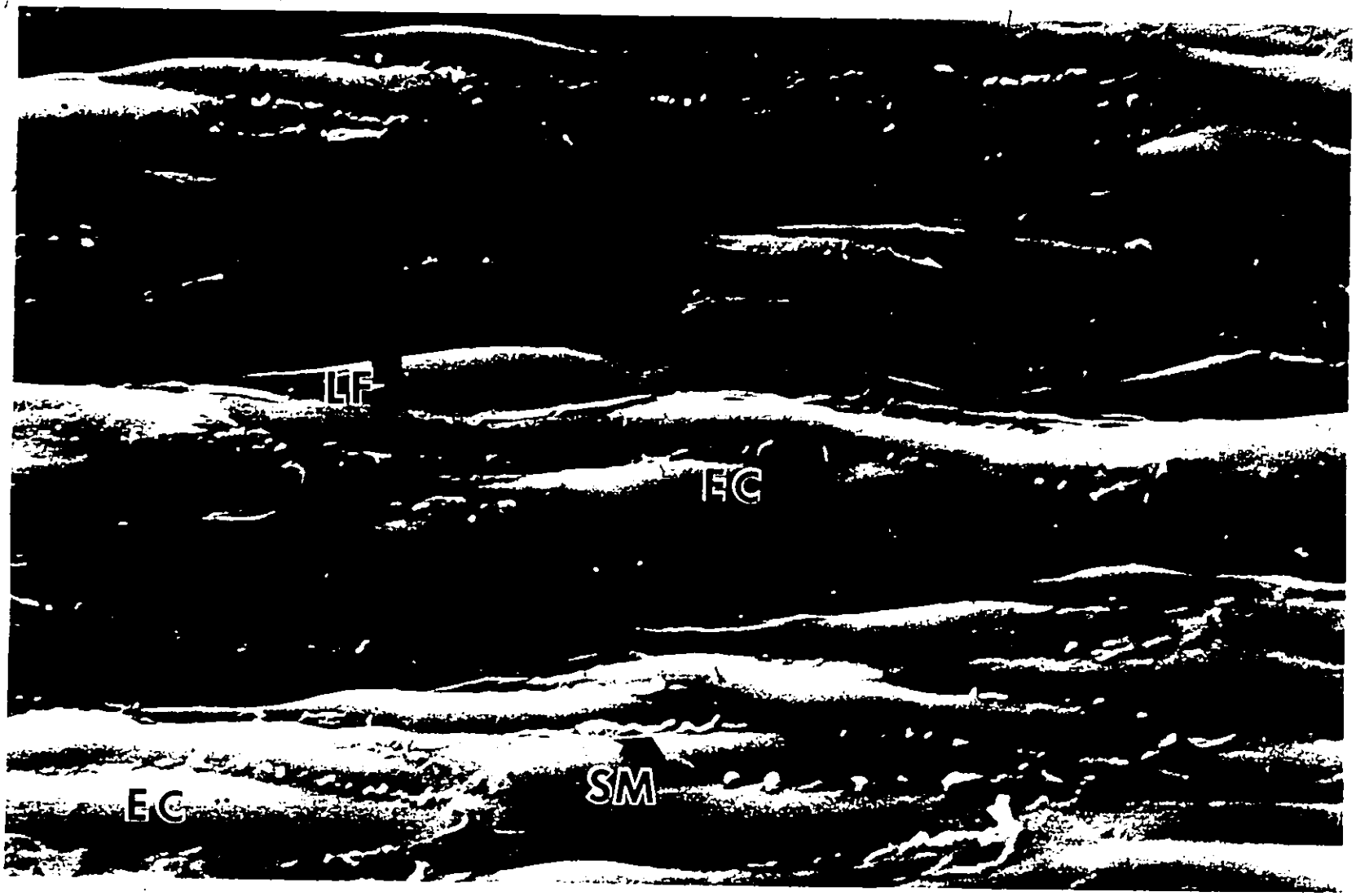


Endothelial cells formed a continuous layer along the longitudinal vessel. Individual cells were rhomboidal in shape, flattened red ends. Scalloped cell margins can be readily seen ov adjacent endothelial cells. The cell margins were continuous and no endothelial structures could be observed between cells. Some of the endothelial cells had microvilli on their surfaces. Formed elements of the blood (red blood cells, white blood cells and platelets) did not adhere to normal endothelial cells. X 2500

EC - endothelial cells

LF - longitudinal folds

SM - scalloped margins


LF
EC
EC
SM

(Figure 3.10 A) and along the length of the vessel (Figure 3.10B). Platelets covered most of the damaged surface although in some areas the surface appeared to be non-reactive to platelets (Figure 3.10 C). Most of the platelets had spread on the surface and had extended pseudopods; others remained rounded. In other areas, the endothelial cells appeared to be normal (Figure 3.10 A). Occasional strands of fibrin were found on the surface of the vessel at this time. Some polymorphonuclear leucocytes adhered to the exposed subendothelium.

Microscopic thrombi (Figure 3.10 A) formed at sites of endothelial cell injury primarily in the regions surrounding the catheter tip in the thoracic aorta and along the abdominal aorta (Figure 3.10 B).

Electron micrographs taken at 3 days (Figure 3.11 A, B) and 5 days (Figure 3.12 A, B, C) after insertion of the aortic catheters showed platelets on the injured surface. In some areas the subendothelium appeared to be non-reactive to circulating platelets while other areas accumulated significant numbers of platelets. At these times, a network of polymerized fibrin stands was present on the exposed subendothelium.

3.1.4 Transmission Electron Microscopy (TEM)

Thrombi were obtained from rabbits which had aortic catheters *in situ* for 24 hours (n=3), 3 days (n=3) or 4 weeks (n=3). At these times the rabbits were killed by perfusion-fixation with Locke's-Ringer solution (pH 7.4, 37°C) followed by 4% gluteraldehyde fixative in sodium cacodylate buffer. The aortae were removed intact. The vessel wall, including the attached thrombus, was diced into sections (1 mm^2) and processed for TEM.

Figure 3.10

Scanning Electron Micrograph of a Rabbit Aorta 3 Hours after the Insertion of an Indwelling Catheter

A) Endothelial cell injury and endothelial cell detachment were evident in the thoracic aorta at the area of the catheter tip. Undamaged endothelium was present in regions adjacent to the injury sites. Adherent platelets completely covered the exposed endothelium and platelet thrombi were visible in many areas. X 560

B) Areas along the abdominal aorta were also injured by the indwelling catheter. Adherent platelets covered most of the subendothelial tissues. The majority of adherent platelets had extended pseudopodia and had spread on the surface; some remained rounded. A thrombus had also formed on the surface. X 2200

C) Endothelial cells had sloughed from areas along the throacic aorta exposing the subendothelial tissue. Platelets accumulated on the subendothelium while in other regions exposed subendothelium appeared to be non reactive to platelets. X2200

EC - endothelial cells

T - thrombus

PLT - platelets

WBC - white blood cells

SE - subendothelium

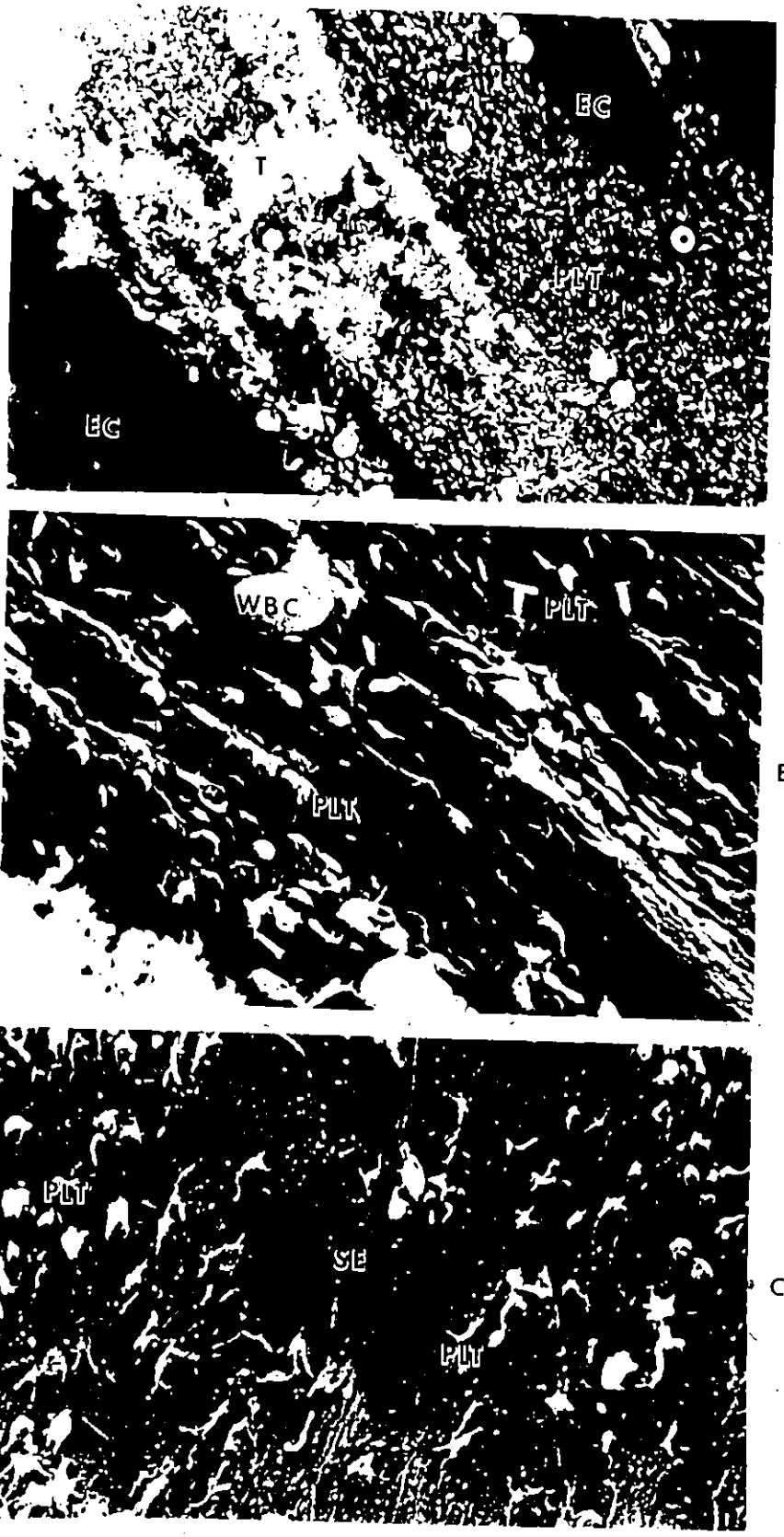

EC
T
PLT
EC
A
WBC
PLT
PLT
B
PLT
SE
PLT
C

Figure 3.11

Scanning Electron Micrographs of a Rabbit Aorta 3 Days after the Insertion of an Indwelling Catheter

The vessel section was obtained from the thoracic aorta in the region of the catheter tip.

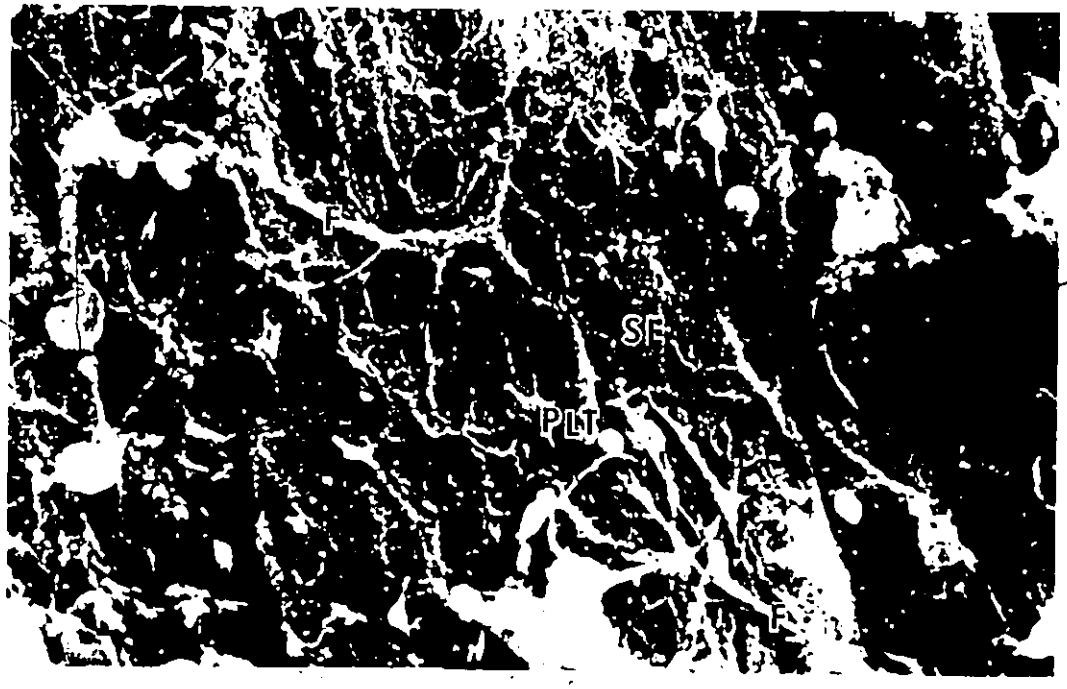
A) Endothelial cells were lost from the surface. In many areas exposed subendothelium did not appear to be reactive to platelets, whereas in other areas there was extensive platelet accumulation. Strands of fibrin were evident on the surface. X2500

B) Adherent platelets had spread on the surface. Strands of polymerized fibrin were also present on the surface. X2500

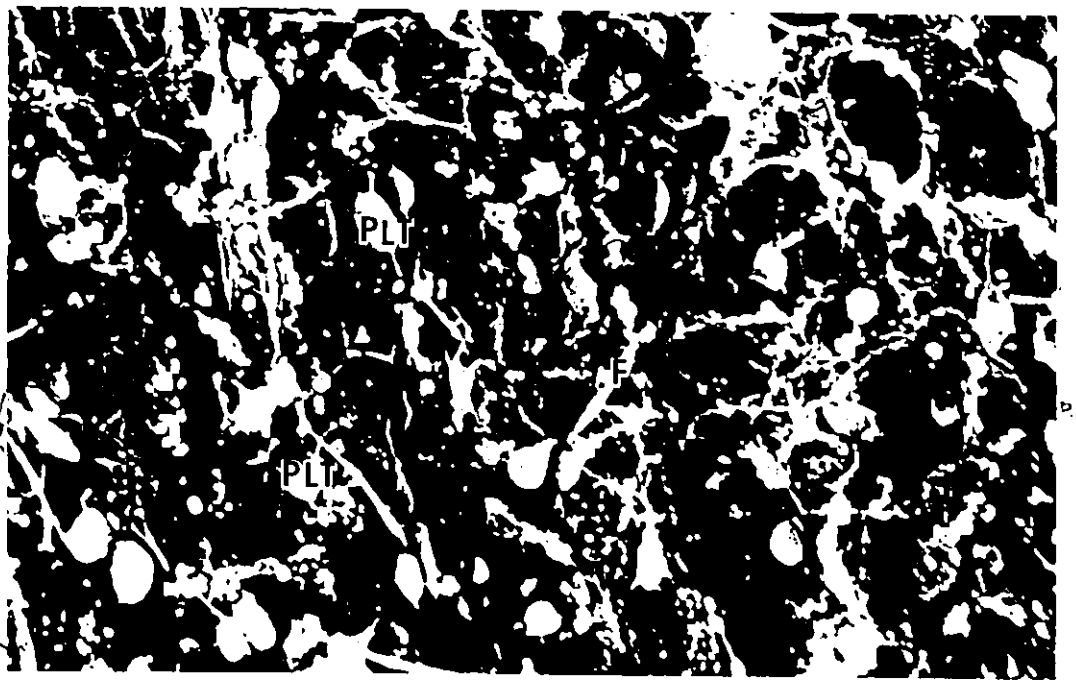
PLT - platelets

F - fibrin

SE - subendothelium



A



B

Figure 3.12

Scanning Electron Micrographs of the Abdominal Aorta of a Rabbit which had a Catheter in situ for 5 Days

A) In some areas of the abdominal aorta the endothelial cells were not damaged and retained a normal appearance. X 1100

B) In other regions, sites of vessel injury were completely covered with a monolayer of adherent platelets. Many of the platelets had extended pseudopods; others remained rounded. X 2200

C) Exposed subendothelial tissue free of adherent platelets was also present along the length of the vessel. Numerous white cells were often observed on the injured surface. X 2200

EC - endothelial cells

PLT - platelets

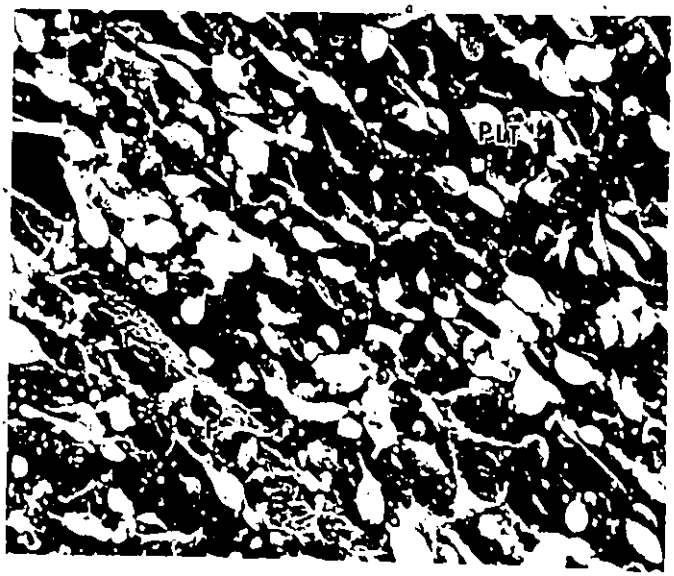
F - fibrin

WBC - white blood cells

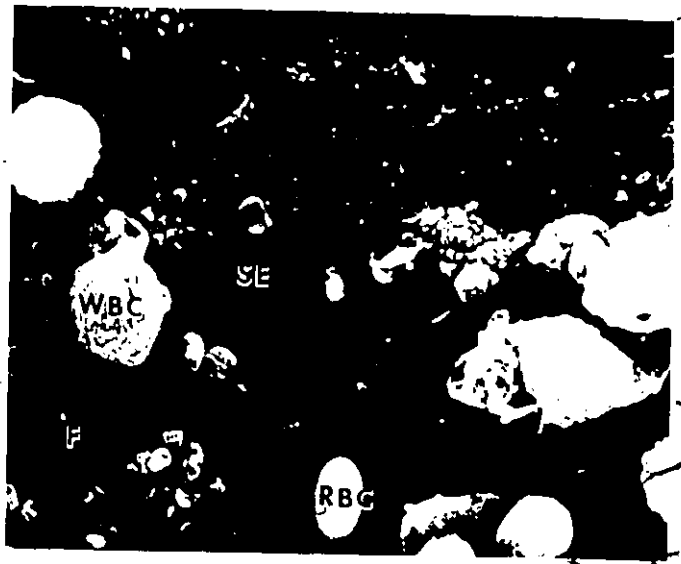
SE - subendothelium

RBC - red blood cells

A



B



C

3.1.4.1 24 Hour Thrombi

Thrombi which had formed in vivo for 24 hours were composed primarily of platelets interspersed between fibrin strands (Figure 3.13 A). Most of the platelets retained intact plasma membranes and had released the constituents of their cytoplasmic granules (Figure 3.13 C). Less densely packed platelets in various stages of activation, some with pseudopods extended, others which had released their cytoplasmic constituents, were present on the luminal surface of the thrombus (Figure 3.13 B). Leucocytes were evident on the surface of the thrombus and within the interstices.

3.1.4.2 3 Day Old Thrombi

Thrombi which had formed in vivo for 3 days were also composed primarily of platelets between distinct layers of fibrin (Figure 3.14 A) Many platelets had intact plasma membranes although considerable cellular debris was also present. The presence of many intact, degranulated platelets on the luminal surface of the thrombus (Figure 3.14 B) suggested that the surface had remained active to platelets in the circulation. The amount of fibrin did not appear to be different from that which was present in thrombi that had formed in vivo for 24 hours (Figure 3.14 A, B). There were numerous leucocytes present within these thrombi.

3.1.4.3 4 Week Thrombi

Thrombi which had formed in vivo for 4 weeks also had a distinctly layered appearance consisting of cells and granular debris between fibrin

Figure 3.13

Transmission Electron Micrographs of a Thrombus formed in the Aorta of a Rabbit which had a Catheter in situ for 24 Hours

A) The thrombus had a distinct layered appearance consisting of masses of platelets between fibrin strands. White blood cells were prominent within these thrombi. X 2500

B) Many platelets with extended pseudopods and degranulated cytoplasm were associated with the luminal surface of the thrombus. X 5700

C) The platelets in the centre of the thrombus were degranulated. Most of these platelets retained an intact plasma membrane. Fibrin was prominent within the thrombus. X 5700

PLT - platelets

PM - plasma membrane

PS - pseudopodia

WBC - white blood cell

F - fibrin

A

B

C

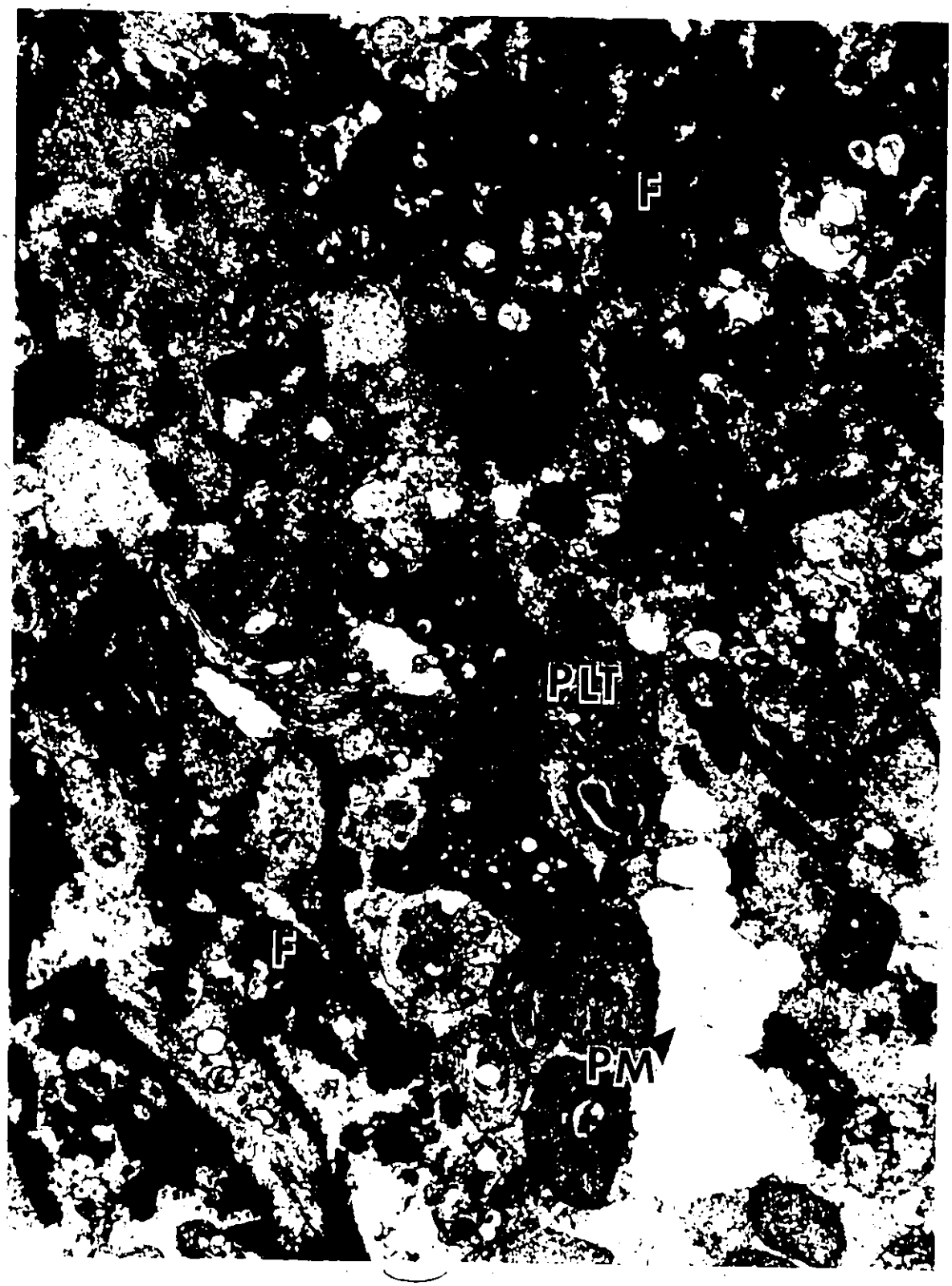


Figure 3.14

Transmission Electron Micrograph of a Thrombus Formed in the Aorta of a Rabbit which had a Catheter in situ for 3 Days

A) The thrombus had a distinct layered appearance composed of platelets and cellular debris between fibrin strands. Many of the platelets remained intact although they had lost the contents of their cytoplasmic granules. White blood cells were prominent within the thrombus. X 3030

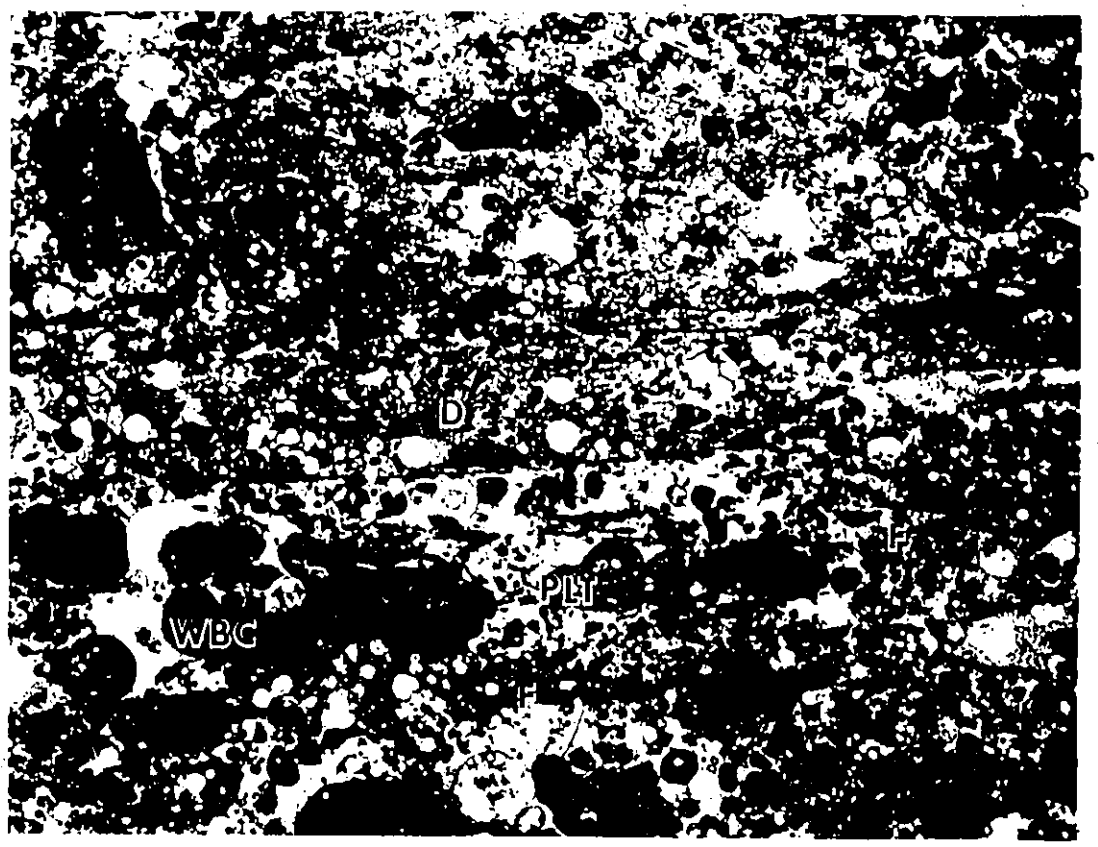
B) Intact, degranulated platelets were present on the luminal surface of the thrombus. X 24,000

PLT - platelets

D - debris

WBC - white blood cells

F - fibrin



A

B

strands (Figure 3.15A). Intact, degranulated platelets were still apparent although they were considerably altered in appearance. Many leucocytes were present at this time (Figure 3.15B). Smooth muscle cells (Figure 3.15C) and macrophages were noted in many sections. In some areas collagen was observed in close relation to the smooth muscle cells. Some of the smooth muscle cells appeared to be lipid-laden. Evidence of a few fresh platelets on the luminal surface indicated the possibility of continued thrombus reactivity (Figure 3.15A). The amount of fibrin appeared to be similar to that which was present in thrombi that had formed in vivo during the shorter periods of time.

Thus, the morphological evidence showed that thrombi were composed primarily of layers of platelets stabilized by strands of fibrin. During the acute phase of thrombus formation (first 24 hours) the luminal surface of the thrombus appeared to be very reactive to fresh platelets in the circulation but this reactivity diminished as the thrombus aged in vivo. Cellular debris, probably representative of lysed platelets also became apparent as thrombi aged in vivo. Leucocytes were evident in and around thrombi during the early period of formation and continued to be present. By 4 weeks following the insertion of aortic catheters, proliferating smooth muscle cells containing lipid droplets were apparent in increasing numbers.

3.2 Survival of ^{51}Cr-Labelled Platelets in Rabbits with Indwelling Aortic Catheters

The effect of indwelling aortic catheters on platelet survival was

Figure 3.15

Transmission Electron Micrograph of a Thrombus Formed in the Aorta of a Rabbit which had a Catheter in situ for 4 Weeks

A) The thrombus was composed of cells and granular debris between layers of fibrin. Fresh platelets were adherent to the luminal surface. X 2925

B) Intact, degranulated platelets and numerous white blood cells were present between the fibrin layers. X 5400

C) Collagen formation was extensive around the smooth muscle cells which had proliferated in the area of thrombus formation. Many of the smooth muscle cells contained accumulations of lipid. X 8520

PLT - platelets

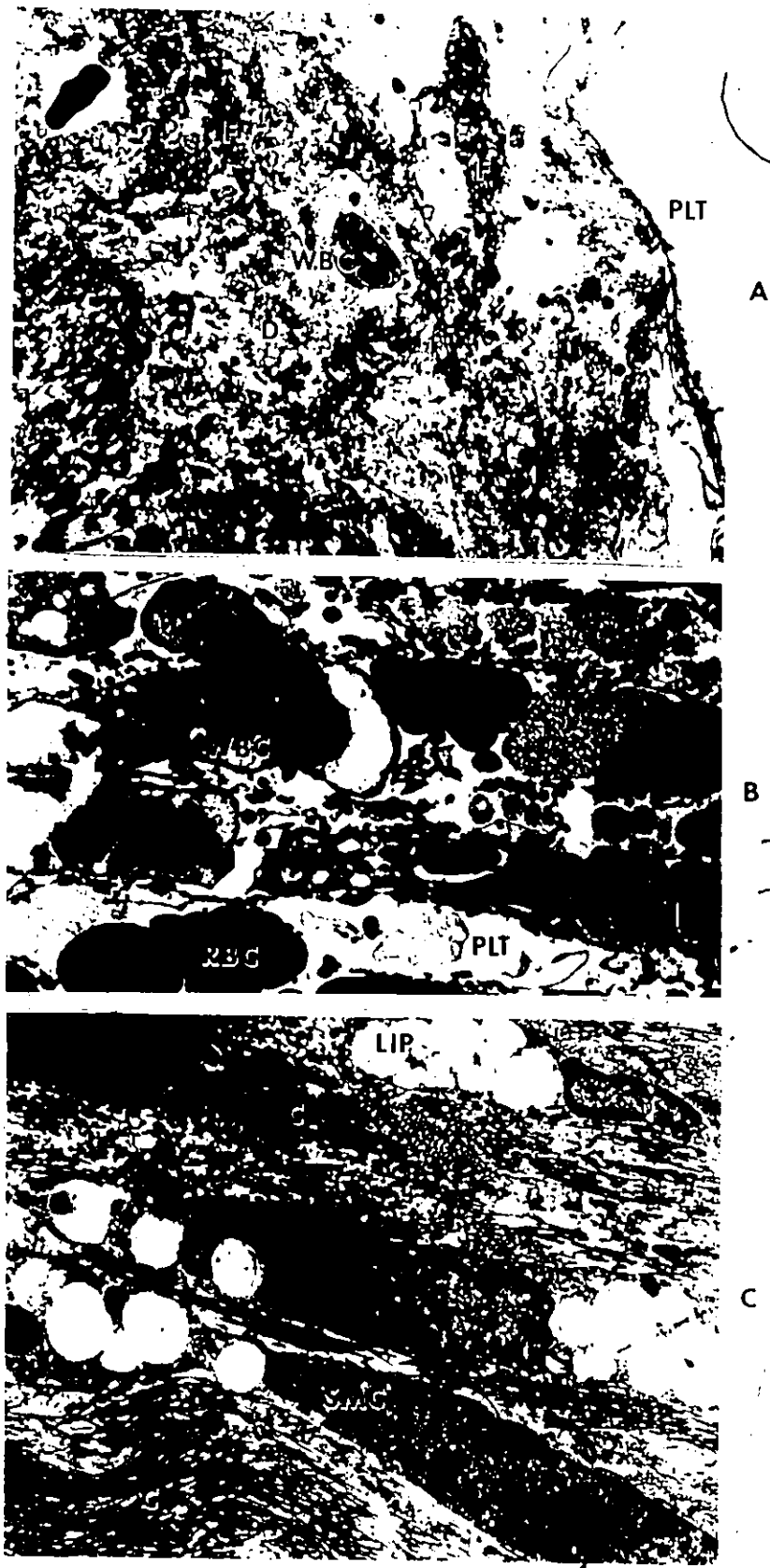
SMC - smooth muscle cells

C - collagen

F - fibrin

LIP - lipid

RBC - red blood cells


PLT
W.B.C.
A
RBC
PLT
B
LIP.
C
S.M.C.
C

examined in rabbits which had catheters inserted two hours after they had received an infusion of ^{51}Cr-labelled platelets. Control animals received a sham operation at the same time.

To ensure that the percentage of ^{51}Cr-labelled platelets in the circulation was similar in all animals at the beginning of the survival study and prior to any surgical procedure, the amount of platelet associated radioactivity in the circulation was measured 2 hours after their infusion and expressed as a percentage of the radioactivity injected into each animal to give the platelet recovery value.

The recovery of ^{51}Cr-labelled platelets (Table 3.1) from the circulation of rabbits which were to receive aortic catheters (Group I), was 81.8 ± 2.0% (mean ± standard error; Mean ± SE). Animals which were to receive a sham operation (Group II) had 78.4 ± 2.5% of injected radioactivity in the circulation at 2 hours. These differences were not statistically significant.

Based on previous experience (Kinlough et al., 1966), the amount of radioactivity in the 2 hour sample was considered to represent maximum circulating radioactivity (100%). At predetermined times (2, 19, 22, 43, 67, and 91 hours) following the insertion of aortic catheters or sham operations, the amount of circulating radioactivity was measured and expressed as a percentage of the 2 hour (100%) sample (taken just prior to the surgical procedure) to calculate platelet survival time.

Two hours after the surgical procedure (Figure 3.16), the percentage of ^{51}Cr in the circulation of rabbits with aortic catheters was not significantly different from that of animals which had received sham operations (Figure 3.16). Between 19 and 91 hours after the surgery, the

Table 3.1

Recovery of ^{51}Cr-Labelled Platelets 2 Hours Following Their Injection into the Circulation of Rabbits

Group	Number of Animals	Recovery of ^{51}Cr-Labelled Platelets (% of injected ^{51}Cr)
I	16	81.8 ± 2.0[a]
II	12	78.4 ± 2.5

[a]Mean ± SE
Group I: Animals which would receive aortic catheters
Group II: Control animals which would receive a sham operation

Results of Student's 't' Test:

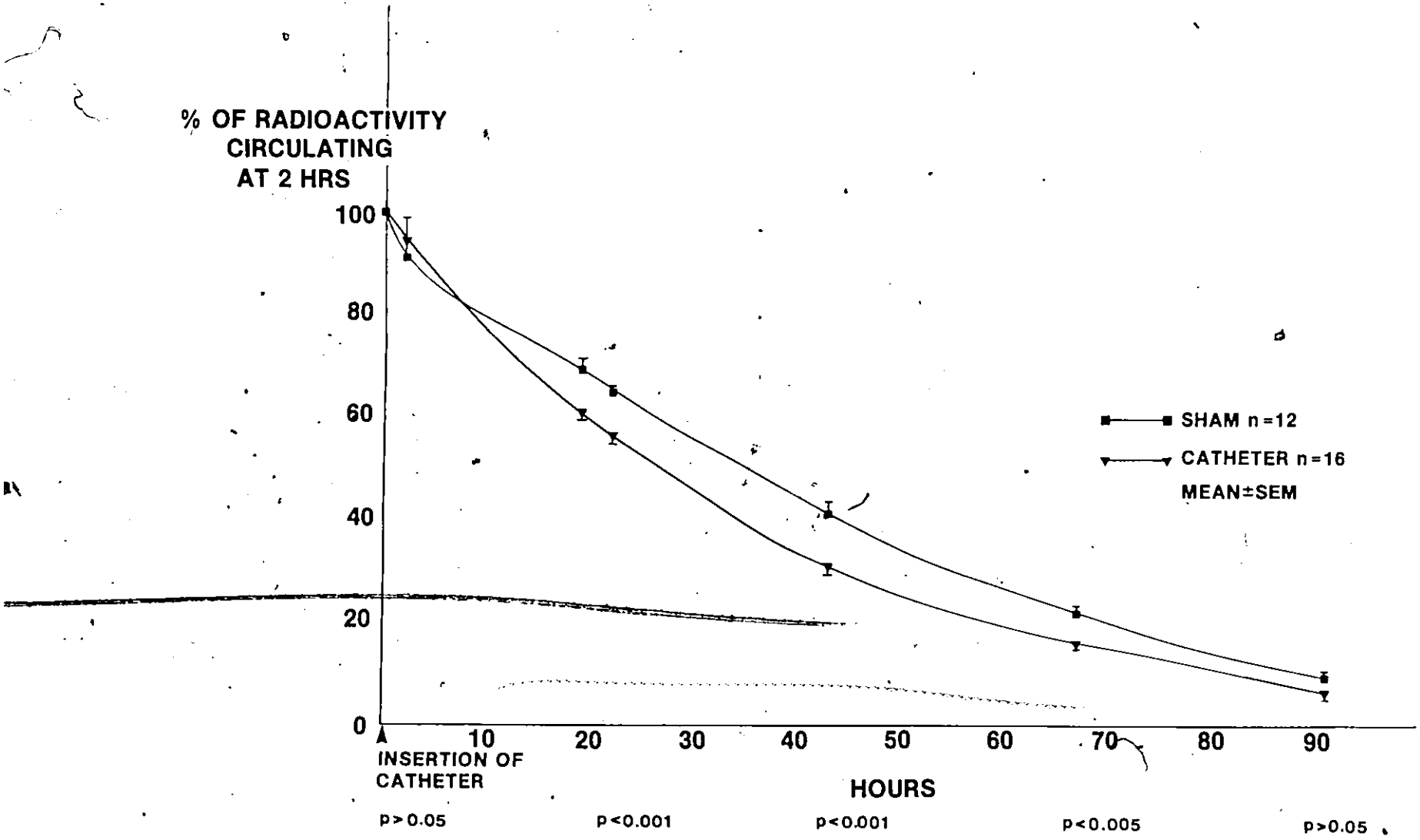
Recovery of ^{51}Cr-labelled platelets, Group I vs Group II, $p>0.10$

^{51}Cr-labelled platelets (8×10^9 platelets) were injected into the circulation of rabbits via the marginal ear vein. Two hours after the injection, a blood sample was taken from the marginal vein of the contralateral ear. The radioactivity of the blood sample was measured. The recovery of ^{51}Cr-labelled platelets in the circulation was calculated as a percentage of platelet associated radioactivity injected into each animal.

Figure 3.16

Percentage ^{51}Cr-Labelled Platelets in the Circulation of Rabbits at Various Times After Insertion of Indwelling Aortic Catheters or After Sham Operations

^{51}Cr-labelled platelets were injected into rabbits. A blood sample taken at 2 hours after the injection was considered to represent maximum recovery of circulating radioactivity (100%). Indwelling aortic catheters were inserted into rabbit aortae; control animals received sham operations. Circulating ^{51}Cr was determined at 2, 19, 22, 43, 67, and 91 hours after the surgical procedure. Each measurement was expressed as a percentage of the 2 hour (100%) value. The data points on the survival curve represent the mean of 12 (sham operated, control animals) or 16 (animals with aortic catheters) measurements.


% OF RADIOACTIVITY CIRCULATING AT 2 HRS
100
80
60
40
20
0
10
20
30
40
50
60
70
80
90
INSERTION OF CATHETER
HOURS
SHAM n=12
CATHETER n=16
MEAN±SEM
p>0.05
p<0.001
p<0.001
p<0.005
p>0.05

percentage of circulating radioactivity in catheter bearing animals was significantly less than the percentage of ^{51}Cr in the circulation of animals with sham operations.

Platelet survival curves were analyzed using three mathematical approaches:

a) Gamma function (Multiple-hit)

b) Linear

c) Exponential.

As might be expected, the same data produced very different values for platelet survival time dependent upon the method of analysis (Table 3.2). Furthermore, the comparison of data between studies is not valid unless the method of analysis is similar.

All three methods of analysis demonstrated that the presence of a catheter in the aorta of a rabbit shortens mean platelet survival time (Table 3.2). As indicated by the values of the residual sum of squares, the curves which best fitted the data were generated by the gamma function. Therefore, all subsequent platelet survival values described in this study were derived using this method.

3.3 Effect of Aortic Catheters on Whole Blood Platelet Counts in Rabbits

Whole blood platelet counts were determined in rabbits before and at various times after the introduction of aortic catheters and in rabbits at the corresponding times before and after sham operations.

Table 3.2

Mean Platelet Survival Time In Rabbits with Indwelling Aortic Catheters Analysed by Different Mathematical Approaches

Treatment	Number of Animals	Mean Platelet Survival Time (hours)		
		γ Function	Linear	Exponential
Sham Operation	12	61.1 ± 3.0[a]	91.9 ± 1.5	39.6 ± 1.8
Aortic Catheter	16	44.2 ± 2.7*	86.1 ± 0.8**	34.1 ± 1.0**

Average Residual Sum of Squares

	γ Function	Linear	Exponential
sham	41.9	374.5	205.6
catheter	30.7	723.8	137.1

[a]Mean ± SE

Results of Student's 't' Test:

Mean platelet survival, catheter vs respective sham: *p 0.001; **p 0.02

^{51}Cr-labelled platelets were injected into the circulation of rabbits. A sample of blood was obtained from each rabbit 2 hours after the injection of labelled platelets. Catheters were inserted into rabbit aortae and control animals received a sham operation. Additional blood samples were taken at 2, 19, 22, 43, 67 and 91 hours after the surgical procedure. The amount of ^{51}Cr in each sample was measured and mean platelet survival time was analysed with the gamma function, linear or exponential mathematical models using the computer program provided by Murphy et al.(1973).

There were no differences between whole blood platelet counts of animals in the catheter group and animals in the control group before the experimental procedure (Table 3.3). An injection of 4.0 mL of ^{51}Cr-labelled platelets (about 8 x 10^9) platelets increased the mean platelet count in both groups of rabbits (Table 3.3). The surgical procedure resulted in a decrease in platelet counts of all rabbits during the first 19 hours. In animals with aortic catheters, the decrease in circulating platelet numbers was evident within the first 2 hours after insertion of the catheter.

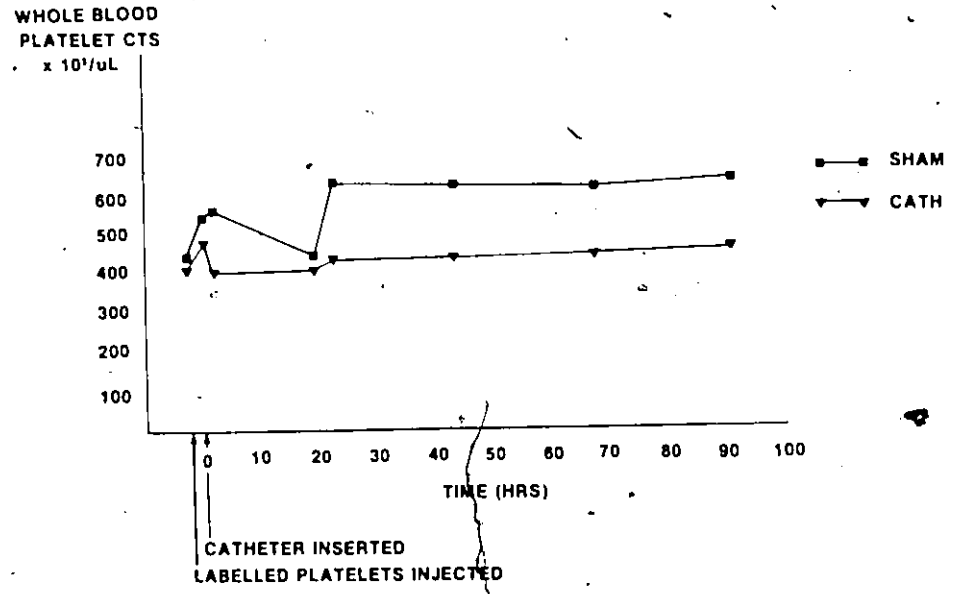
Between 19 and 22 hours after the surgery, the number of circulating platelets in animals with sham operations increased above the values obtained before surgery. During the same time, the platelet counts did not rise appreciably in animals with aortic catheters so that by 22 hours after surgery, these animals had significantly fewer platelets in the circulation (Table 3.3). This difference was evident for as long as 91 hours after insertion of the catheter.

3.4 Contribution of Thrombus Formation to Shortened Platelet Survival in Rabbits with Indwelling Aortic Catheters

Since thrombi formed in aortae of rabbits with indwelling catheters, it was likely that continued platelet incorporation into thrombi where they may be lysed or phagocytosed was one of the mechanisms responsible for shortened platelet survival. Alternatively, turnover of platelets in thrombi as they undergo formation and dissolution may expose platelets to proteolytic enzymes generated by leucocytes, activated fibrinolytic

Table 3.3

Effect of Indwelling Catheters on Whole Blood Platelet Counts of Rabbits



Time After Insertion of Catheter or Sham Operation (h)	Catheter Animals n	Catheter Animals platelet counts x $10^3/\mu L$	Sham Animals n	Sham Animals platelet counts x $10^3/\mu L$	*p
0	6	472.5 ± 87.8[a]	2	545.0	>0.05
2	6	405.0 ± 84.2	4	553.5 ± 45.9	>0.05
19	8	403.0 ± 57.6	7	440.3 ± 44.0	>0.05
22	6	438.7 ± 68.6	4	631.3 ± 39.2	<0.05
43	11	441.4 ± 50.8	7	624.0 ± 37.7	<0.05
67	10	437.7 ± 68.6	7	610.4 ± 62.4	<0.05
91	10	486.7 ± 51.6	7	631.7 ± 47.1	<0.05

[a]Mean ± SE

Results of Statistical Analyses:

ANOV; platelet counts, catheter vs sham $p<0.001$
*Student-Newman-Keuls, comparing catheter to sham at each time period

factors or platelets themselves at sites of thrombus formation. Proteolytic enzymes can modify platelet surface membrane glycoproteins thereby contributing to premature platelet clearance from the circulation (Greenberg et al., 1979). Thus, thrombus formation could shorten platelet survival time through platelet incorporation, lysis, phagocytosis or through platelet modification.

To establish the importance of thrombus formation to shortened platelet survival we examined:

a) the extent to which ^{51}Cr-labelled platelets incorporated into thrombi that had formed for various times in situ and whether platelet incorporation persists
b) whether whole platelets escaped from thrombi
c) whether platelets lysed in thrombi
d) whether thrombi that had formed in situ retained their ability to incorporate fresh platelets from the circulation over a period of time, and if so,
e) whether continued reactivity of the thrombi affected platelet survival
f) and, whether the platelets that had turned over in thrombi returned to, and were detectable in the circulation.

3.4.1 Incorporation of ^{51}Cr-Labelled Platelets into Thrombi and Sites of Endothelial Injury in Rabbits with Indwelling Aortic Catheters

To determine whether reduced platelet survival in animals with indwelling aortic catheters was due to continued incorporation of platelets into thrombi and areas of injury around the catheter,

preliminary experiments examined the extent to which platelets were incorporated into these sites.

Catheters were inserted into rabbit aortae 2 hours after they had received an injection of ^{51}Cr-labelled platelets. Control animals received sham operations at the same time. The catheters remained *in situ* 1, 3, or 24 hours, 3, 7, or 14 days following which the animals were killed by perfusion-fixation. The ^{51}Cr associated with the vessel containing the catheter and thrombi was measured and expressed as a percentage of the platelet-associated ^{51}Cr injected into each animal.

The weight of the thrombi that had formed within the vessel during these times was also measured.

Maximum numbers of platelets were incorporated into thrombi and areas of endothelial injury during the first 24 hours (Table 3.4). During this time 1.11 ± 0.14% (Mean ± SE) of injected ^{51}Cr became associated with the thrombi and the injured vessel wall. After this time, the amount of ^{51}Cr that was associated with the thrombi and the vessel wall gradually diminished. By 3 days after the insertion of the catheter, the ^{51}Cr associated with the vessels containing the catheter and thrombi was only half of that present at 24 hours. This rate of ^{51}Cr loss from the thrombus and vessel wall closely paralleled the rate of platelet associated ^{51}Cr loss from the circulation (refer to Figure 3.16) and suggested that that the thrombi may be losing ^{51}Cr as a result of platelet turnover.

The weights of the thrombi that formed at sites of vessel injury gradually increased during the first 24 hours following insertion of the catheter. After this time, there was no significant change in thrombus weight.

Table 3.4

Accumulation of ^{51}Cr-Labelled Platelets into Thrombi and Sites of Endothelial Injury in Rabbits Following Insertion of Indwelling Aortic Catheters

	Time Catheter Remained in Vessel	Number of Animals	^{51}Cr Incorporated into Thrombi and Vessel Wall (% of Injected)	Thrombus Weight (mg)
A	1 h	10	0.09 ± 0.04[a]	0.96 ± 0.33
B	3 h	8	0.94 ± 0.19	23.6 ± 5.7
C	24 h	21	1.11 ± 0.14	45.9 ± 6.3
D	3 d	14	0.53 ± 0.08	44.1 ± 10.9
E	7 d	19	0.19 ± 0.02	33.6 ± 4.7
F	14 d	16	0.15 ± 0.02	43.6 ± 6.5
	Sham	16	0.0	0.0

[a]Mean ± SE

Results of Student-Newman-Keuls Test:

^{51}Cr Accumulation

AEF not significantly different $p>0.05$
B vs C $p>0.05$
C vs DEF $p<0.001$
(ANOV: $p<0.001$)

Thrombus Weight

A vs BC $p<0.001$
C vs DEF $p>0.05$

(ANOV: $p<0.001$)

Catheters were inserted into rabbit aortae 2 hours after they had received an infusion of ^{51}Cr-labelled platelets. At the specified times, the animals were killed by perfusion-fixation. The ^{51}Cr associated with the aorta and thrombus was determined and expressed as a percentage of the ^{51}Cr infused. The weight of the thrombi that formed during the same time was measured. Logarithmic transformation normalized the thrombus weight data and ^{51}Cr accumulation data for statistical analyses.

3.4.2 Incorporation of ^{51}Cr-Labelled Platelets into Thrombi that Form at Sites of Vessel Injury in Rabbits with Aortic Catheters

^{51}Cr-labelled platelets were incorporated into the injured vessel wall and thrombi that formed for about 24 hours after the insertion of aortic catheters. After this time, ^{51}Cr was lost from the injured vessel containing catheter and thrombi. The amount of ^{51}Cr associated with the vessel wall, the thrombus and the catheter was therefore examined to determine the specific site at which ^{51}Cr-labelled platelets accumulated and the sites from which the ^{51}Cr was lost.

The majority of the total radioactivity (about 80-95%) that accumulated in the vessel containing thrombi and catheter was found associated with the thrombus (Table 3.5). Thus the loss of ^{51}Cr from the vessel containing catheter and thrombi (refer to Table 3.4) was primarily due to loss of ^{51}Cr from the thrombus since there was a significant decrease in the specific radioactivity of the thrombus (cpm/mg) as the thrombus aged *in vivo*.

The remainder of the ^{51}Cr was associated with the vessel wall; the polyethylene catheter accumulated little radioactivity (<2% of the total activity associated with the vessel containing the thrombus and the catheter).

Thus, at sites of repeated vessel injury radioactive platelets accumulate into thrombi primarily during the acute phase of formation. As the thrombus aged *in vivo*, the total amount of associated radioactivity decreased suggestive of platelet lysis or platelet loss from the thrombus. Simultaneously, the proportion of total radioactivity associated with the vessel wall increased suggesting that the vessel wall

Table 3.5

Incorporation of ^{51}Cr-Labelled Platelets into Thrombi Formed at Sites of Vessel Injury

	Time Catheter Remained in Vessel	Number of Animals	cpm ^{51}Cr/mg Thrombus	Radioactivity in thrombus[b]	Thrombus Wt. (mg)
A	24 h	4	810.2 ± 85.0[a]	82.6 ± 4.2	19.5 ± 10.9
B	3 d	4	332.5 ± 48.6	94.4 ± 2.1	24.1 ± 8.9
C	6 d	4	132.9 ± 19.8	94.5 ± 1.6	24.4 ± 8.8
D	14 d	6	18.0 ± 3.1	79.3 ± 3.4	24.7 ± 8.1

[a]Mean ± SE

[b]Radioactivity in thrombus expressed as a percentage of the total ^{51}Cr on vessel, catheter and thrombus.

Results of Student-Newman-Keuls Test:

cpm ^{51}Cr/mg thrombus, A vs BC $p<0.01$
C vs D $p>0.05$

^{51}Cr-labelled platelets were injected into the circulation of rabbits. Catheters were inserted into rabbit aortae at 2 hours after the injection of labelled platelets. The catheters remained in situ for 24 hours, 3, 6, or 14 days before the animals were killed by perfusion-fixation. The amount of ^{51}Cr associated with the thrombi was measured and expressed as cpm/mg thrombus. The amount of ^{51}Cr associated with the thrombus only was also expressed as a percentage of the total ^{51}Cr associated with the vessel, catheter and thrombus.

continued to accumulate platelets. Based on the very low values of radioactivity associated with the catheter, the interaction between catheter and platelets appeared to be negligible.

3.4.3 Contribution of Platelet Loss and Platelet Lysis to the Decrease in ^{51}Cr in Thrombi Formed in Aortae of Rabbits with Indwelling Catheters-Experiments Using Platelets Doubly-Labelled with D^{125}ISA and ^{51}Cr

Several mechanisms could explain the loss of ^{51}Cr from the thrombus in vivo:

a) ^{51}Cr-labelled platelets which had become incorporated into thrombi may have lysed and the ^{51}Cr (which associates largely with cytoplasmic constituents) would have been lost from the thrombus.

b) Alternatively, since thrombi continually undergo formation and dissolution, whole platelets may escape thereby decreasing the amount of ^{51}Cr associated with the thrombi as platelets with higher specific activity were replaced by platelets with lower specific activity.

c) Combination of a and b.

To examine these mechanisms, doubly-labelled platelets were prepared in which membrane (glyco)proteins were labelled with diazotized ^{125}I-iodosulfanilic acid and the platelet cytosol was labelled with ^{51}Cr. Thrombi composed of doubly-labelled platelets in which platelet

lysis occurred would preferentially lose ^{51}Cr and retain ^{125}I with trapped membranes providing proteolytic enzymes are not clearing labelled glycoproteins. Escape of whole, doubly-labelled platelets from the thrombus would result in simultaneous loss of ^{125}I and ^{51}Cr from the thrombus. Therefore, the ratio of $^{125}I/^{51}Cr$ of doubly-labelled platelets was measured before and after their injection into the circulation of rabbits, and, at various times after insertion of aortic catheters or after sham operations. The ratio of $^{125}I/^{51}Cr$ in thrombi that had formed for various times in situ was also determined.

In addition, in these studies it was possible to measure platelet survival on the basis of circulating ^{125}I or ^{51}Cr and to compare the rates of removal of the two isotopes from the circulation.

Because ^{125}I elution from the platelet surface was a potential source of error, the plasma was removed from all blood samples to ensure that the measured ^{125}I and ^{51}Cr values represented platelet associated radioactivity.

The ^{51}Cr recovered from the circulation of rabbits which were to receive aortic catheters was 91.9 ± 2.8%, (Table 3.6) at 2 hours after the infusion of platelets doubly-labelled with ^{51}Cr and D^{125}ISA. Similar ^{51}Cr values of 85.8 ± 0.6%, were obtained from animals which were to have a sham operation. The ^{125}I recovery calculated from the same blood samples as those used for ^{51}Cr calculations was similar in both groups of rabbits (28.1 ± 1.2% and 26.9 ± 0.6%, respectively). However, the percentage ^{125}I recovery in the circulation at 2 hours was significantly reduced compared to the amount of ^{51}Cr recovery.

Figure 3.17 shows the recovery of the two isotopes in the

Table 3.6

Recovery of Radioactivity from the Circulation of Rabbits 2 Hours After Injection of Platelets Doubly-Labelled with ^{51}Cr and D^{125}ISA

Isotope	Group Ia n	Group Ia Recovery (% of injected radioactivity)	Group IIb n	Group IIb Recovery (% of injected radioactivity)
^{125}I	16	28.1 ± 1.2c	4	26.9 ± 0.6
^{51}Cr	16	91.9 ± 2.8	4	85.8 ± 0.6

[a]Group I: Rabbits which were to receive aortic catheters
[b]Group II: Rabbits which were to receive sham operations
[c]Mean ± SE

Results of Student's 't' Test:

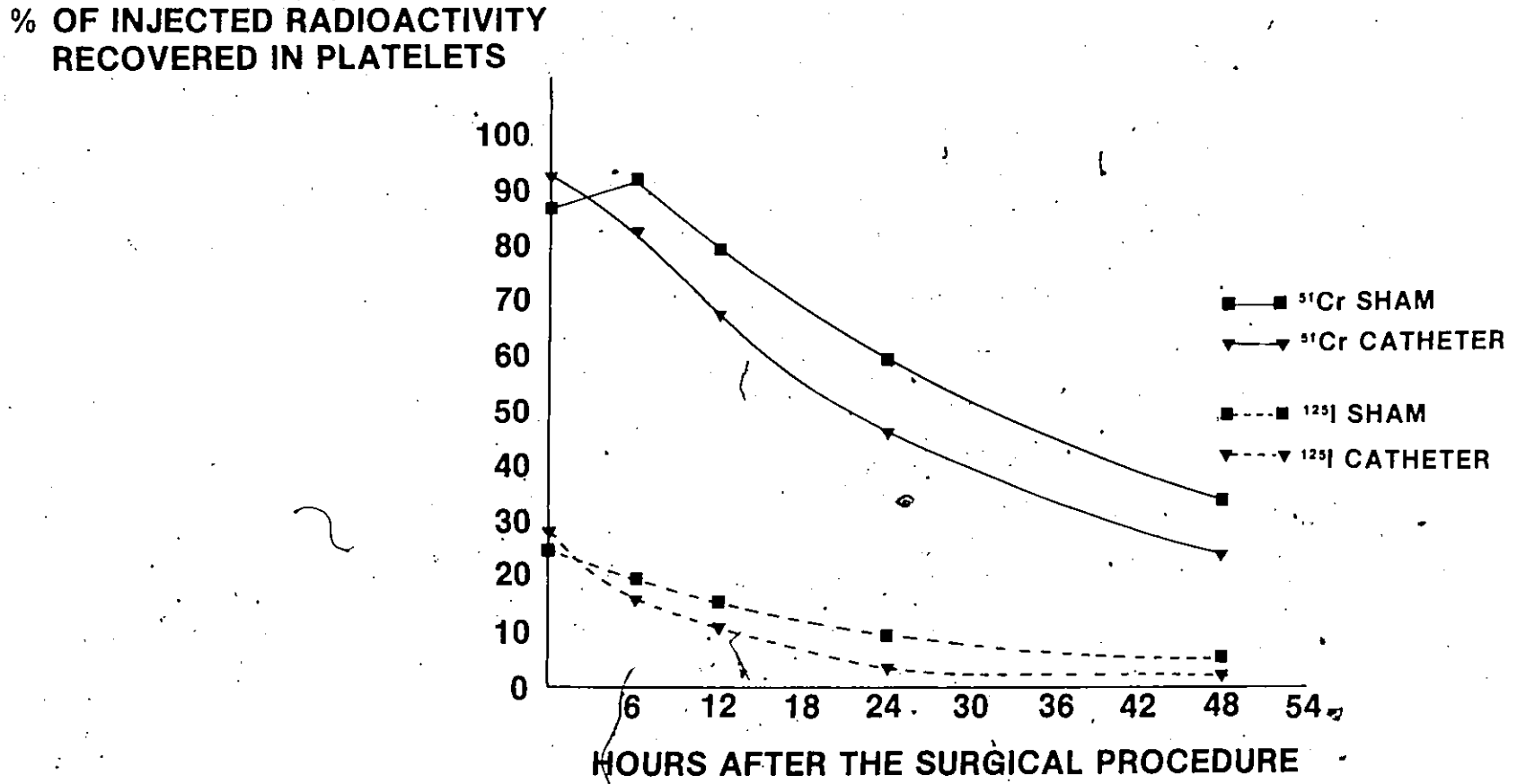
Recovery of radioactivity; ^{125}I Group I vs Group II; $p>0.10$
^{51}Cr Group I vs Group II; $p>0.05$

Platelets doubly-labelled with ^{51}Cr and D^{125}ISA were injected into the circulation of rabbits via the marginal ear vein. Two hours after the injection blood samples were taken and the amount of ^{125}I or ^{51}Cr was measured and expressed as a percentage of the platelet associated ^{125}I or ^{51}Cr injected into the animal.

Figure 3.17

^{125}I and ^{51}Cr in Circulating Platelets from Rabbits Following Infusion of Platelets Doubly-labelled with ^{51}Cr and D^{125}ISA

Platelets doubly-labelled with ^{51}Cr and D^{125}ISA were injected into the circulation of rabbits. Two hours after the injection, catheters were inserted into rabbit aortae. Control animals received sham operations. Blood samples were taken at the times specified. The plasma was removed and the platelet associated ^{125}I and ^{51}Cr was measured on the same blood sample and was expressed as a percentage of injected ^{125}I or ^{51}Cr.


% OF INJECTED RADIOACTIVITY RECOVERED IN PLATELETS
100
90
80
70
60
50
40
30
20
10
0
6
12
18
24
30
36
42
48
54
HOURS AFTER THE SURGICAL PROCEDURE
51Cr SHAM
51Cr CATHETER
125I SHAM
125I CATHETER

circulation at various times after injection of doubly-labelled platelets expressed as a percentage of the amount of infused radioactivity.

The mean survival time of platelets labelled with ^{125}I and ^{51}Cr was measured by loss of ^{51}Cr or loss of ^{125}I from the circulation (Figure 3.18). When mean platlet survival time was calculated on the basis of circulating ^{51}Cr, the survival time was always longer in animals with sham operations or in animals with indwelling catheters than the survival time calculated on the basis of ^{125}I content of the same sample.

These data raised the possibility that the ^{125}I membrane (glyco)protein label was lost more rapidly from the circulating platelets than ^{51}Cr, a cytosol label.

Extensive work by other investigators (George et al., 1976a, b; Rand, 1982) had demonstrated that elution of ^{125}I from the platelet surface may contribute to the more rapid loss of this isotope. However, both George (1976a, b) and Rand (1982) had demonstrated that when platelet membranes were labelled with D^{125}ISA or its analogue ^{125}I-diazotized di-iodosufanilic acid, elution occurred only during the first 2-3 hours after the labelling procedure. After this time, the preferential loss of ^{125}I from the platelet surface was not a result of elution.

Since platelet survival calculations using the 2 hour recovery sample as 100% (Figure 3.18 A) may have been complicated by in vivo ^{125}I elution, platelet survival was also calculated using the 6 hour circulating radioactivity measurement as the initial 100% recovery value (Figure 3.18 B). Mean platelet survival time calculated by this method showed similar results as platelet survival times calculated using the 2

Figure 3.18

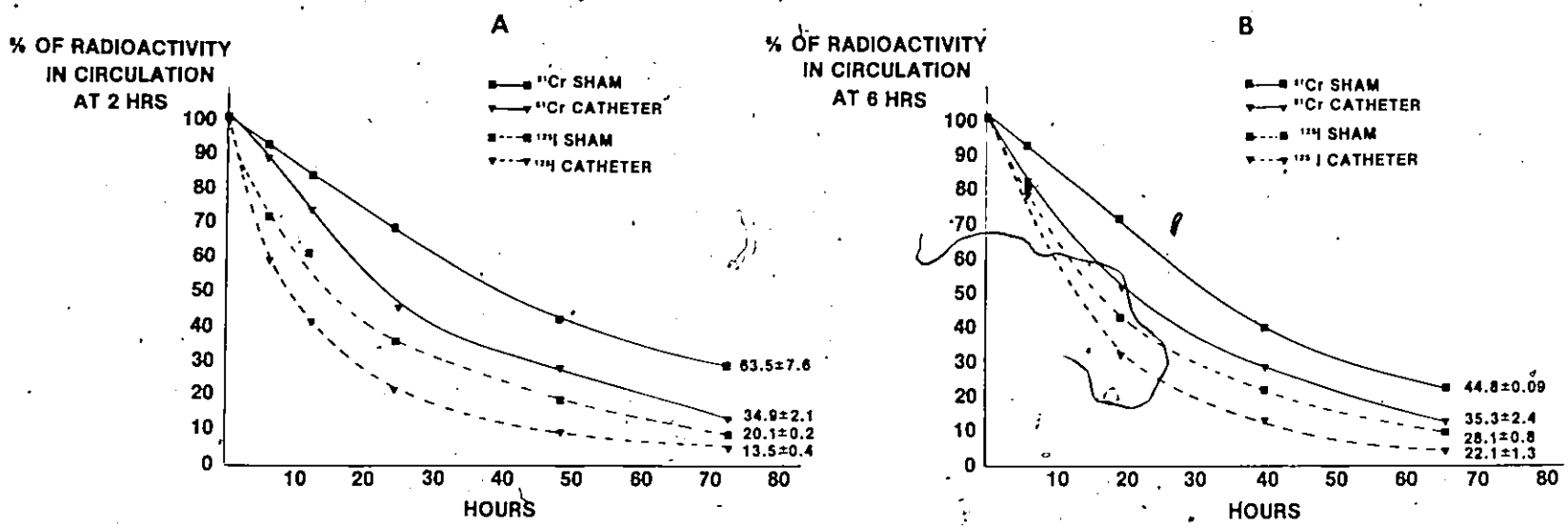
Percentage of Radioactivity Recovered in Doubly-Labelled Platelets from the Circulation of Rabbits with Aortic Catheters or Sham Operations

Platelets doubly-labelled with ^{51}Cr and D^{125}ISA were injected into the circulation of rabbits. A blood sample was taken at 2 hours following the infusion of labelled platelets. Catheters were inserted into rabbit aortae and control animals received sham operations. Additional blood samples were obtained at 6, 12, 24, 48, and 72 hours after the surgical procedure. The platelet associated ^{125}I or ^{51}Cr was measured in all blood samples and expressed as a percentage of the ^{125}I of ^{51}Cr in the circulation at 2 hours (A) or at 6 hours (B) to calculate platelet survival time.

A) 2 hour value as 100%

B) 6 hour value as 100%

The number given after each survival curve is the mean platelet survival time calculated by Murphy's Gamma Function.


A
% OF RADIOACTIVITY IN CIRCULATION AT 2 HRS
51Cr SHAM
51Cr CATHETER
125I SHAM
125I CATHETER
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
HOURS
63.5±7.6
34.9±2.1
20.1±0.2
13.5±0.4
B
% OF RADIOACTIVITY IN CIRCULATION AT 6 HRS
51Cr SHAM
51Cr CATHETER
125I SHAM
125 I CATHETER
HOURS
44.8±0.09
35.3±2.4
28.1±0.8
22.1±1.3

hour circulating radioactivity value as 100%. These data demonstrated that the ^{125}I label was lost more rapidly from circulating platelets than ^{51}Cr as a result of events in the circulation and not due to elution. This observation was compatible with the fact that the increased rate of ^{125}I loss was most evident during the first 24 hours after the surgical procedure when platelet-vessel wall interactions would be expected to be increased.

With either isotope, mean platelet survival time in animals with aortic catheters was always significantly shorter compared to platelet survival times in animals which had received sham operations (Figure 3.17; Table 3.7).

The ratio of $^{125}I/^{51}Cr$ in doubly-labelled platelets injected into the circulation of rabbits was 3.15 (Table 3.8). Due to the preferential loss of ^{125}I from the platelet surface membranes, this ratio decreased significantly during the first 2 hours in the circulation and continued to decrease significantly during the following 3 days. There was no significant difference between the ratio observed in circulating platelets from animals with aortic catheters and animals with sham operations but it appeared that during the early period following the surgical procedure, the ratio of $^{125}I/^{51}Cr$ in the animals with aortic catheters decreased to a greater extent. Although not conclusive, this observation raised the possibility that the preferential loss of platelet membrane label was accelerated in animals with aortic catheters.

The ratio of $^{125}I/^{51}Cr$ of the thrombi that formed during the first 6 hours after insertion of the catheter was 1.09 ± 0.14 (Mean ± SE, Table 3.9). This was not significantly different from the ratio of $^{125}I/^{51}Cr$

Table 3.7

Survival of Platelets Doubly-Labelled with ^{51}Cr and D^{125}ISA in the Circulation of Rabbits with Aortic Catheters or Sham Operations

Treatment	Mean Platelet Survival Time (h)				
	2 h Recovery as 100%			6 h Recovery as 100%	
	n	^{51}Cr	^{125}I	n	^{51}Cr	^{125}I
Sham Operation	4	63.5 ± 7.6[a]	20.1 ± 0.2	3	44.8 ± 0.1	28.1 ± 0.8
Aortic Catheter	16	34.9 ± 2.1	13.5 ± 0.4	12	35.3 ± 2.4	22.1 ± 1.3

[a]Mean ± SE

Results of Student's 't' Test:

Mean platelet survival time, catheter vs respective sham: $p<0.05$ for each isotope

Platelets doubly-labelled with ^{51}Cr and D^{125}ISA were injected into the circulation of rabbits. A blood sample was taken from each animal 2 hours after the injection and 6, 12, 24, 48 and 72 hours after the the insertion of aortic catheters or after sham operations. The ^{125}I or ^{51}Cr circulating 2 hours or 6 hours was taken as the 100% recovery value and all subsequent measurements were expressed as a percentage of the 2 hour or the 6 hour recovery values to calculate mean platelet survival time.

Table 3.8

Ratio $^{125}I/^{51}Cr$ in Doubly-Labelled Platelets Obtained from the Circulation of Rabbits Before and After the Insertion of Aortic Catheters or After Sham Operations

Time $^{125}I/^{51}Cr$ Ratio Measured	Catheter Animals		Sham Animals	
	n	$^{125}I/^{51}Cr$ Ratio in platelets	n	$^{125}I/^{51}Cr$ Ratio in platelets
2 h After Infusion[a] (before surgery)	16	0.96 ± 0.02[b]	4	0.98 ± 0.02
Post Catheter or Sham Operation				
6 h	16	0.63 ± 0.01	4	0.71 ± 0.07
12 h	12	0.54 ± 0.01	3	0.55 ± 0.01
24 h	12	0.46 ± 0.01	3	0.47 ± 0.02
48 h	8	0.37 ± 0.01	2	0.41
72 h	4	0.35 ± 0.01	1	0.38

[a]Ratio $^{125}I/^{51}Cr$ of platelets infused into all animals was 3.15
[b]Mean ± SE

Results of ANOV:

Change in $^{125}I/^{51}Cr$ ratio over time: p 0.001 in both groups
Difference in $^{125}I/^{51}Cr$ ratio between groups: p 0.05

The amount of ^{125}I and ^{51}Cr in doubly-labelled platelets was determined before they were injected into the circulation of rabbits. Blood samples were taken from all rabbits at 2 hours after the injection. Catheters were then inserted into rabbit aortae and control animals received sham operations. Additional blood samples were taken at the specified times after the surgical procedure. The amount of platelet associated ^{125}I and ^{51}Cr of each blood sample was measured and the ratio $^{125}I/^{51}Cr$ was calculated.

Table 3.9

Ratio of $^{125}I/^{51}Cr$ of Platelets Incorporated into Thrombi

Duration of Thrombus Formation	Number of Animals	$^{125}I/^{51}Cr$ Ratio in Thrombus
6 h	4	1.09 ± 0.14[a]
24 h	4	1.25 ± 0.05
3 d	4	1.23 ± 0.06
6 d	4	1.21 ± 0.14

[a]Mean ± SE

Results of ANOV:

Change in $^{125}I/^{51}Cr$ ratio over time: $p>0.05$

Rabbits received an injection of platelets doubly-labelled with D^{125}ISA and ^{51}Cr. At 2 hours after the injection, catheters were inserted into the aortae of rabbits. The catheters remained *in situ* for 6, and 24 hours, 3 days or 6 days, before the animals were killed by perfusion-fixation. The macroscopic thrombi were dissected from the vessel. The amount of ^{125}I and ^{51}Cr associated with the thrombi was measured and the ratio of $^{125}I/^{51}Cr$ in the thrombi was calculated.

(0.96 ± 0.02) of platelets in the circulation at the time the catheter was introduced (refer to Table 3.8). In sharp contrast to platelets in the circulating blood, the ratio of $^{125}I/^{51}Cr$ in thrombi that had formed for 24 hours, 3 or 6 days after the insertion of the catheter was not significantly different from the ratio measured in thrombi formed for 6 hours ($p > 0.05$, ANOV).

Based on the assumption that membrane glycoproteins were not lost from thrombi (if platelets lysed), and, all of the ^{125}I which initially incorporated into the thrombus remained there with trapped platelet membranes, the constant ratio of $^{125}I/^{51}Cr$ observed in the thrombus, despite a decreasing ratio in the circulating platelets, is consistent with any of the following interpretations:

1) All the platelets which initially incorporated into a thrombus remained there unchanged (no loss of ^{51}Cr; no loss of ^{125}I; no change in $^{125}/^{51}Cr$ ratio).

2) Whole platelets were removed from the thrombus (simultaneous loss of ^{125}I and ^{51}Cr) and these platelets were <u>not</u> replaced by platelets from the circulation with a lower ratio.

3) A proportion of platelets in the thrombus turns over (simultaneous loss of ^{125}I and ^{51}Cr) to be replaced by new platelets from the circulation which have less ^{125}I associated with them. At the same time, some of the platelets within the thrombus lyse thereby selectively losing ^{51}Cr. ^{51}Cr loss through

lysis and loss of whole platelets, balanced by influx of platelets with a lower $^{125}I/^{51}Cr$ ratio, would maintain the constant ratio in the thrombus.

The first possibility was excluded since it was demonstrated in this and in previous experiments (see results Table 3.5) that the total radioactivity of thrombi formed in the presence of radiolabelled platelets decreased significantly as thrombi aged in vivo.

The second possibility was also ruled out by previous experiments (see Table 3.4) in which we demonstrated that the thrombus weight was maintained despite the loss of radioactivity and that thrombi continued to incorporate fresh platelets from the circulation (see next Section 3.4.4.) Furthermore, based on morphological criteria, the constant thrombus weight could not be explained by increased accumulation of fibrin or cellular elements other than platelets.

The third possibility was consistent with all the experimental evidence. Previous experiments had demonstrated that the rate at which ^{51}Cr was lost from thrombi was similar to the rate at which ^{51}Cr-labelled platelets were removed from the circulation suggesting that an exchange of whole platelets was occuring within thrombi. The fact that the total amount of ^{125}I and ^{51}Cr in the thrombus decreased simultaneously, and the observation that the ratio did not increase also suggested that whole platelets were lost from the thrombus. However, since any platelets which might enter the thrombus from the circulation had a lower ratio of $^{125}I/^{51}Cr$, there must have been some selective ^{51}Cr loss from the thrombus to maintain the constant ratio. Thus, it was most likely that

the loss of radioactivity from the thrombus was a consequence of platelet turnover on the thrombus in addition to some degree of lysis.

On the basis of the data available, it was not possible to calculate the extent of ^{51}Cr loss that occured as a result of lysis.

3.4.4 Thrombus Reactivity and Exchange of Platelets in Thrombi Formed in Rabbits with Aortic Catheters

During the first 24 hours following the insertion of aortic catheters, platelets were primarily incorporated into thrombi that formed on the injured vessel surface. Twenty-four hours after the thrombus had formed, some platelets escaped from the thrombus although the weight of the thrombus did not change significantly during the time when platelets were being lost. Since it has been demonstrated by numerous investigators (Bizzozero, 1882; Woolf, 1982) that thrombi continually undergo dissolution and reformation, it was likely that fresh platelets from the circulation were turning over in thrombi thereby maintaining the thrombus weight.

Dissolution of thrombi can occur as a result of platelet deaggregation. Alternatively, proteolytic enzymes such as plasmin can digest stabilizing fibrin strands and the glycoproteins by which platelets adhere to surfaces. Shear forces can then dislodge platelets and thrombi from the vessel wall. As previously described, proteolytic enzymes can also modify platelet surface membrane glycoproteins that are important to platelet survival *in vivo* (Greenberg *et al.*, 1979). Therefore, exchange of circulating platelets in thrombi could contribute to shortened platelet survival time.

Since the evidence suggested (Section 3.4.3) that whole platelets escape from thrombi, it was necessary to establish whether thrombi which had preformed *in vivo* continued to remain reactive and incorporate fresh platelets from the circulation.

Catheters were inserted into rabbit aortae and control animals received a sham operation. Three hours, 3, 6 or 14 days after the surgery, ^{51}Cr-labelled platelets were infused into all animals. The recovery of labelled platelets from the circulation was determined 2 hours after injection. The labelled platelets were then allowed to circulate for 24 hours before the animals were killed by perfusion-fixation. The ^{51}Cr that became associated with the thrombi during this 24 hour period was measured and expressed as a percentage of the ^{51}Cr injected into each animal.

The recovery of ^{51}Cr-labelled platelets from the circulation of rabbits in which the only previous procedure was the infusion of labelled platelets was 81.9 ± 2.8% (Table 3.10). Animals which had received sham operations 3 and 24 hours, and 3 or 6 days previous to the infusion of ^{51}Cr-labelled platelets had platelet recovery values which were similar to the values observed in untreated animals.

In those rabbits which had aortic catheters inserted 24 hours, 3 days or 6 days prior to the infusion of radioactive platelets the recovery values were not significantly different from untreated animals.

In contrast, the recovery of labelled platelets from the circulation of rabbits which had received aortic catheters 3 hours before the injection of radioactive platelets was significantly lower than that of control or untreated animals (Table 3.10). Thus, the freshly injured

Table 3.10

Recovery of ^{51}Cr-Labelled Platelets 2 Hours After Their Injection into the Circulation of Rabbits that had Aortic Catheters in Place for Different Periods

Untreated Animals (no surgery)	n	^{51}Cr in Circulation (% of ^{51}Cr injected)
	13	81.9 ± 2.8

	Aortic Catheters		Sham Operation	
Duration of injury (before ^{51}Cr-labelled platelets infused)	n	^{51}Cr in Circulation (% of ^{51}Cr injected 2 h previously)	n	^{51}Cr in Circulation (% of ^{51}Cr injected 2 h previously)
3 h	8	69.7 ± 3.0[a]	8	78.6 ± 2.8*
24 h	6	84.3 ± 1.8	6	88.5 ± 4.5
3 d	13	83.9 ± 2.7	13	84.5 ± 2.9
6 d	7	84.5 ± 2.4	8	80.9 ± 1.7

[a]Mean ± SE

Results of Student's 't Test:

Recovery of ^{51}Cr from the circulation, catheter vs respective sham: *$p<0.05$; all others $p>0.05$

Untreated animals received only an infusion of ^{51}Cr-labelled platelets. In other animals, catheters inserted into the aorta remained *in situ* for the times specified. Control animals had sham operations for the same time periods. ^{51}Cr-labelled platelets were infused into all animals and at 2 hours following the injection of labelled platelets blood samples were obtained from each animal. The radioactivity in the sample was measured and expressed as a percentage of the platelet associated radioactivity injected into each animal.

vessel wall and thrombus caused approximately 10-15% of the labelled platelets to be removed from the circulation within 2 hours of the infusion.

During a 24 hour period following the injection of ^{51}Cr-labelled platelets (Table 3.11), 1.33 ± 0.09% of injected radioactive platelets became associated with the thrombus that had formed during the preceding 3 hours. Regression analysis of the ^{51}Cr accumulation showed that as thrombi aged *in vivo* from 3 hours to 14 days, the extent to which ^{51}Cr-labelled platelets became incorporated during a 24 hour period decreased significantly. However, it was apparent that thrombi which had formed *in vivo* for 14 days continued to accumulate some fresh platelets from the circulation.

3.4.5 Survival of ^{51}Cr-labelled Platelets in the Circulation of Rabbits with Pre-existing Thrombi of Various Ages

The previous data showed that platelets accumulated to a greater extent on thrombi that had formed *in vivo* for 3 hours than on thrombi which had formed for 14 days. To determine whether the apparent reduction in reactivity influenced mean platelet survival time (assuming that the vessel wall effects were constant), platelet survival was examined in rabbits in which aortic catheters had been *in situ* for different times.

Catheters were implanted into rabbit aortae and control animals received sham operations. The catheters remained *in situ* for 3 hours, 24 hours, 3, and 6 days, 2 and 4 weeks. At these times, all animals received an infusion of ^{51}Cr-labelled platelets and platelet survival

Table 3.11

Accumulation in 24 Hours of ^{51}Cr-Labelled Platelets into Thrombi that had Formed for Different Periods of Time in Rabbits with Indwelling Aortic Catheters

Time After Catheter Insertion (when ^{51}Cr-labelled platelets injected)	Number of Animals	^{51}Cr Associated with Thrombi (% of injected ^{51}Cr)
3 h	8	1.33 ± 0.09[a]
3 d	3	0.82 ± 0.29
6 d	7	0.61 ± 0.08
14 d	8	0.71 ± 0.14
Sham	4	0

[a]Mean ± SE

Results of ANOV and Regression Analysis:

Change in ^{51}Cr Association over time: ANOV, $p<0.005$
Regression, $r=0.5$; $p<0.01$

Catheters were inserted into rabbit aortae. Control animals received a sham operation. At various times after thrombi had formed in situ, ^{51}Cr-labelled platelets were injected into each animals and allowed to circulate for 24 hours before the animals were killed by perfusion-fixation. The ^{51}Cr accumulating on the preformed thrombi during 24 hours was measured and expressed as a percentage of the platelet associated ^{51}Cr injected.

time was measured.

Mean platelet survival time was significantly shortened in animals in which indwelling catheters were in place for 3 hours to 2 weeks before the survival study was begun (Table 3.12). When studies were started 4 weeks after insertion of aortic catheters, platelet survival time was not significantly different from that in control animals. Nevertheless, there was a tendency for it to remain shortened.

Regression analysis of the difference in mean platelet survival time between catheter-bearing animals and control animals versus time, showed that the extent to which platelet survival was reduced, decreased significantly as time after insertion of the catheter increased ($r=0.69$; $p<0.05$).

3.4.6 Density Distribution of Circulating Platelets from Rabbits with Indwelling Aortic Catheters or Sham Operations

The experimental evidence indicated that some platelet exchange occurred in thrombi that formed at sites of vessel injury. It was possible that some of the platelets which had been freed from thrombi were sufficiently modified to be cleared from the circulation. Alternatively, some of the freed platelets might continue to circulate.

As indicated in the introduction (Section 1.2), some agonists which stimulate platelets (thrombin, ADP, plasmin) decrease platelet density. It was predicted that platelets which had participated in thrombus formation and were exposed to these agonists, would have their density altered. Accordingly, the density distribution of circulating platelets from rabbits with aortic catheters and animals with sham operations was

Table 3.12

Survival of ^{51}Cr-Labelled Platelets in Rabbits with Pre-existing Thrombi

Time After Surgery when ^{51}Cr Platelets Injected	Catheter Animals		Sham Animals		
	n	Mean Platelet Survival Time (h)	n	Mean Platelet Survival Time (h)	p[b]
3 h	8	33.8 ± 2.2[a]	8	69.5 ± 3.9	<0.001
24 h	8	53.4 ± 3.7	8	77.8 ± 6.2	<0.01
3 d	13	51.7 ± 4.5	13	65.9 ± 4.5	<0.05
6 d	8	49.9 ± 6.5	8	71.3 ± 6.5	<0.05
2 Weeks	10	43.5 ± 5.9	11	62.6 ± 4.5	<0.05
4 Weeks	8	49.9 ± 4.9	11	60.6 ± 4.3	>0.10

[a]Mean ± SE
[b]Results of Student's 't' test, mean platelet survival time, catheter vs respective sham

Results of Regression Analysis:

Difference between catheter and sham vs time; $r=0.69$; $p<0.05$

Catheters were inserted into rabbit aortae and control animals received sham operations. The catheters remained *in situ* for 3 hours, 24 hours, 3 and 6 days, 2 and 4 weeks. ^{51}Cr-labelled platelets were then injected into each animal and a blood sample was taken 2 hours following the injection (2 hour recovery) and assigned a value of 100%. Additional blood samples were taken 2, 19, 22, 43, 67, and 91 hours after the initial 2 hour (recovery) sample. The amount of radioactivity in each sample was measured and expressed as a percentage of the 2 hour (recovery) sample to calculate mean platelet survival time.

examined for evidence of platelets that had participated in thrombus formation.

In these experiments each animal served as it own control as the distribution of circulating platelets in Stractan density gradients was determined before, and at various times after insertion of aortic catheters or sham operations.

Before these experiments were begun, it was necessary to ensure that the platelet suspension applied to and recovered from the Stractan density gradients included the total platelet population and not a selected platelet population. Thus, the platelet yield from the whole blood samples obtained from rabbits was measured. In addition, the recovery of platelets from the gradients was also determined.

The platelet yield from whole blood samples taken from rabbits was 107.3 ± 2.8% (mean ± SE, n=8) using the multiple wash procedure. Of the platelets applied to the gradients, between 96.7 to 100.9 % (Table 3.13) were recovered in the density subpopulations. There was no difference in the percentage of platelets recovered from gradients containing platelets from sham operated animals or from animals with catheters.

Table 3.14 shows the distribution of platelets separated in Stractan density gradients prior to, and during the first 3 days after insertion of the catheter or sham operation. The proportion of platelets separating as the least dense fraction of Stractan density gradients decreased by about 10% in both groups of animals during the first 3 days after surgery.

During the same time, the proportion of most dense platelets in the circulation of animals with aortic catheters did not change significantly

Table 3.13

Percentage of Applied Platelets Recovered in Density Subpopulations Separated on Discontinuous Stractan Density Gradients

Platelets obtained from rabbits with:	Number of Gradients	Recovery from Gradient (% of Applied Platelets)
Sham Operation	89	96.7 ± 2.2[a]
Aortic Catheters	69	100.9 ± 2.2

[a]Mean ± SE

Results of Student's 't' test:

Recovery of applied platelets, catheter vs sham; $p>0.10$

Whole blood was obtained from rabbits which previously had sham operations and from rabbits which had aortic catheters *in situ* for the same time periods. The platelets were isolated and separated into density subpopulations on discontinuous Stractan density gradients. The number of platelets recovered from the gradients was measured and expressed as a percentage of the platelets applied to the gradients.

compared to pre-surgery percentages (Table 3.14). In contrast, by 24 hours after the surgery, a significantly greater proportion of most dense platelets was observed in the circulation of animals with sham operations. This increased proportion of most dense platelets was present in the circulation for as long as 3 days after the sham operation.

Based on the results from animals with sham operations, it was recognized that studies of platelet density distribution during the first three days after surgery, may be complicated by the effects of surgery. Moreover, since most dense, young platelets have been shown to be hemostatically more active (Hirsh et al., 1968; Blajchman et al., 1981), the incorporation of platelets into enlarging thrombi during the early period (24 hours) after insertion of the catheter may affect specific density subpopulations of platelets. Thus, further experiments were performed to examine the density distribution of circulating rabbit platelets at 6 days to 6 weeks after the insertion of aortic catheters or after sham operations. At these times, the effects of surgery were minimal and thrombus growth had stopped. However, as previously mentioned, there were continued significant effects on platelet survival for at least 4 weeks.

A significantly greater proportion of less dense (Table 3.15) platelets was present in the circulation of rabbits with aortic catheters between 7 and 9 days after the surgery. By fourteen days after insertion of the catheter, the percentage of less dense platelets was the same as that observed before the catheter was inserted. During the same time, there were no significant changes in the proportion of less dense

Table 3.14

Effect of Indwelling Aortic Catheters on Platelet Density Distribution

(% of Platelets Applied to Gradient)

Time of Sample	Platelets in Least Dense Fraction				Platelets in Most Dense Fraction	
	n	Catheter	n	Sham	Catheter	Sham
Pre-surgery	18	31.3 ± 1.9[a]	19	25.1 ± 2.5	20.5 ± 2.8	24.4 ± 2.9
Post-surgery						
2 h	14	22.3 ± 2.5	15	14.8 ± 1.2	28.0 ± 3.7	30.1 ± 3.1
24 h	11	20.9 ± 1.9	11	13.2 ± 1.2	24.3 ± 2.1	35.2 ± 1.8
48 h	7	22.1 ± 2.9	7	15.6 ± 3.8	29.9 ± 2.3	39.5 ± 4.8
72 h	14	20.8 ± 2.2	15	14.4 ± 1.2	23.4 ± 2.9	34.9 ± 2.2

[a]Mean ± SE

Results of ANOV:

Platelets in least dense fraction, catheter vs sham, $p<0.05$
catheter/sham vs time, $p<0.02$

Platelets in most dense fraction, catheter vs sham, $p<0.05$
sham vs time, $p<0.05$

Platelets were isolated from whole blood obtained from rabbits before and at various times after the surgical procedure. The isolated platelets were separated into density subpopulations on discontinuous Stractan density gradients. The number of platelets in each density fraction were counted and expressed as a percentage of the platelets applied to the gradient.

Table 3.15

Effect of Indwelling Aortic Catheters on Platelet Density Distribution

(% of Platelets Applied to the Gradient)

Time of Sample	Platelets in Least Dense Fraction				Platelets in Most Dense Fraction	
	n	Catheter	n	Sham	Catheter	Sham
Pre-surgery	11	33.8 ± 2.3[a]	12	30.8 ± 2.5	20.9 ± 4.3	17.6 ± 1.8
Post-surgery						
6 d	10	39.4 ± 4.8	12	32.4 ± 4.6	13.1 ± 2.6	16.7 ± 2.2
7 d	9	44.5 ± 3.9	11	35.5 ± 3.1	10.9 ± 1.3	16.4 ± 1.6
9 d	6	46.7 ± 4.1	8	40.1 ± 5.5	10.1 ± 1.7	14.3 ± 2.8
10 d	6	39.7 ± 3.1	7	33.4 ± 5.0	13.2 ± 1.9	19.2 ± 4.2
14 d	6	32.3 ± 5.3	7	25.1 ± 4.1	17.4 ± 4.3	18.5 ± 4.8
4 Weeks	4	32.3 ± 3.9	4	27.9 ± 2.2	16.9 ± 1.4	16.4 ± 1.3
6 Weeks	4	34.4 ± 5.1	4	26.6 ± 2.7	17.6 ± 3.7	19.6 ± 1.6

[a]Mean ± SE

Results of ANOV:

Platelets in least dense fraction, catheter vs sham, $p<0.05$
catheter vs time, $p<0.02$

Platelets in most dense fraction, catheter vs sham, $p>0.05$
catheter/sham vs time, $p>0.05$

Platelets were isolated from whole blood obtained from rabbits before and at various times after the surgical procedure. The isolated platelets were separated into density subpopulations on discontinous Stractan density gradients. The number of platelets in each density fraction was measured and expressed as a percentage of the platelets applied to the gradient.

platelets in the circulation of animals with sham operations.

There were no significant changes observed in the proportion of platelets in the most dense fraction of either group of animals (Table 3.15) between 6 days to 6 weeks after the surgical procedure.

3.4.7 Survival of ^{51}Cr-labelled Platelets from Rabbits with Indwelling Aortic Catheters after Injection into Normal Rabbits

It was established in the previous studies that the circulating platelet population from rabbits with indwelling aortic catheters contained an increased proportion of least dense platelets. This observation is compatible with the return to the circulation of platelets which had previously interacted with the thrombus and had their density modified by exposure to agents such as ADP, thrombin or plasmin. An alternative interpretation of the data may be that rabbits with indwelling aortic catheters have an increased proportion of older (less dense) platelets.

Accordingly, cross-over experiments were performed to examine the survival time in normal rabbits of platelets obtained from rabbits with aortic catheters.

In these experiments, platelets were harvested from rabbits at various times after aortic catheters had been introduced or after sham operations. The harvested platelets were labelled with ^{51}Cr, reinjected into normal animals and their survival in the circulation was measured.

As with the previously described density experiments, the platelet yield from animals with aortic catheters was measured as the possibility existed that modified platelets may be lost during the isolation procedure.

The platelet yield in the plasma from animals which had no treatment was 82.8 ± 1.3% after the first centrifugation (Table 3.16). This was not significantly different from yields (78.6 ± 2.5%) obtained from blood of animals which had catheters *in situ* for 3 days.

Similarly, when platelets from animals with aortic catheters or sham operations were washed, labelled and resuspended in platelet poor plasma, there was no difference in the yield of platelets (Table 3.17) in the final suspension.

Platelets obtained from animals which had catheters *in situ* for short time intervals (3 h) survived for the same length of time after infusion into normal animals as platelets obtained from animals which had received a sham operation 3 hours previously (Table 3.18).

However, it was apparent that interpretation of this early data might be complicated by the effects of the surgical procedure since the platelet count data (Section 3.3) provided evidence that the platelet population had changed in all animals. This was consistent with the observation that platelets harvested from animals with a fresh sham operation survived considerably longer in normal animals than platelets from animals which had sham operations 3 days or 6 days previously.

Platelets obtained from rabbits which had catheters *in situ* for 3 or 6 days survived significantly longer in normal animals than platelets from animals which had sham operations for the same time periods (Table 3.18). These data demonstrated that the presence of an aortic catheter in rabbits induced an increased proportion of younger platelets in the circulation. Since the previous density data (see Section 3.4.3) had also shown that these platelets were less dense, it was apparent that the

Table 3.16

Yield of Platelets in Plasma (PRP) from Whole Blood of Untreated Rabbits and Rabbits with Aortic Catheters in situ

Treatment	Number of Animals	Platelet Yield in PRP (% of platelets in whole blood)
No treatment	3	82.8 ± 1.3[a]
Catheter in situ 3 days	3	78.6 ± 2.5

[a]Mean ± SE

Results of Student's 't' Test:

Yield in PRP from animals with catheters in situ vs non-treated animals; $p>0.05$

Platelet Yield was determined by the formula:

$$\% \text{ Yield} = 100\% - \left[\frac{\text{no. of platelets in packed red cells}}{\text{no. of platelet in whole blood}} \times 100\% \right]$$

Whole blood was collected from rabbits which had no previous treatment and from rabbits which had aortic catheters in situ for 3 days. The whole blood platelet count was determined before platelet rich plasma was removed. The number of platelets which remained in the packed red cells was measured and platelet yield was determined.

Table 3.17

Final Yield of Platelets Obtained from Whole Blood of Untreated Rabbits and Rabbits with Aortic Catheters

Treatment	Number of Animals	Platelet Yield in Final Suspension (% of platelets in whole blood)
No treatment	6	67.1 ± 9.1[a]
Catheter *in situ* for 3 days	6	65.7 ± 3.3

[a]Mean ± SE

Results of Student's 't' Test:

Platelet yield, untreated animals vs catheter animals, p 0.10

The number of platelets recovered in the final platelet suspension was determined and expressed as a percentage of the number of platelets in the whole blood sample from which the suspension was made.

Table 3.18

Survival of Platelets from Rabbits With Indwelling Aortic Catheters After Injection into Normal Animals

Platelets from Animals with:		Number of Animals	Platelet Survival (Hours)	p^b
3 h	Catheter *in situ*	13	68.1 ± 4.5[a]	>0.10
	Sham operation	6	79.3 ± 6.7	
3 d	Catheter *in situ*	10	84.9 ± 5.2	<0.01
	Sham operation	10	65.1 ± 2.9	
6 d	Catheter *in situ*	14	73.2 ± 4.9	<0.05
	Sham operation	15	57.7 ± 4.5	

[a]Mean ± SE

[b]Results of Student's 't Test:

Platelet survival time, catheter vs respective sham

Platelets were isolated from the blood of rabbits which had aortic catheters *in situ* for the times specified. Control animals had sham operations for the same periods of time. The platelets were labelled with ^{51}Cr and re-infused into normal animals. Blood samples were taken 2 hours following the injection (2 hour recovery) and assigned a value of 100%. Additional samples were taken 2, 19, 22, 43, 67 and 91 hours after the initial (recovery) sample. The amount of radioactivity in each sample was measured and expressed as a percentage of the 2 hour value to calculate mean platelet survival time.

young (normally most dense) platelets had their density modified probably as a result of participation in thrombus formation.

3.5 Turnover of ^{51}Cr-labelled Platelets in the Circulation of Rabbits in which Vessel Injury had been Induced and Thrombi Allowed to Form for Specific Times before Platelet Turnover was Measured

The turnover of platelets in the circulation of animals which had catheters *in situ* for various times before ^{51}Cr-labelled platelets were injected, was determined at the time of platelet survival studies.

Platelet turnover was calculated from the formula:

$$\text{platelets}/\mu L/\text{hour} = \frac{\text{whole blood platelets counts}/\mu L}{\text{mean platelet survival time (hours)}}$$

The whole blood platelet count used to calculate platelet turnover was the average of daily platelet counts obtained on each of 5 successive days of the platelet survival study.

The turnover of platelets in the circulation depends upon the extent to which platelets are removed from the circulation and the extent to which new platelets (produced by the bone marrow or freed from sites of sequestration) appear in the circulation.

In rabbits which had catheters *in situ* for 6 days to 4 weeks, the total platelet population was in equilibrium since the platelet counts in whole blood remained fairly constant after 91 hours.

The turnover of platelets in animals with indwelling aortic catheters was significantly increased during this time compared to animals which had sham operations (Table 3.19).

During the first 3 days following insertion of the catheter or after sham operations, changes in circulating platelet counts were observed. There was an initial increase in circulating platelet numbers that could be attributed, at least in part, to the injection of radio-labelled platelets. Thereafter, there was a reduction in platelet counts in both the animals with aortic catheters and also in control animals with sham operations. By the third post operative day, the platelet counts had increased in relation to pre-surgery numbers in both groups of animals but remained lower in animals with catheters in situ (see Table 3.3).

In rabbits with aortic catheters, the fluctuation in platelet count and the reduction in mean platelet survival during this period resulted in considerable variation in the calculated values of platelet turnover in the circulation. For example, animals with catheters in situ for 3 hours before radio-labelled platelets were injected had reduced platelet counts and a minimum observed value for mean platelet survival time. In these animals, platelet turnover was significantly increased compared to sham operated controls in which the fall in platelet count was less extensive and in which platelet survival was unaltered.

In animals with catheters in situ for 24 hours to 3 days before survival studies were begun, whole blood platelet counts were significantly reduced compared to animals with sham operations. The extent of reduction in mean platelet survival time was not as great as that observed when survival studies included the period immediately after

Table 3.19

Turnover of ^{51}Cr-Labelled Platelets in the Circulation of Rabbits with Vessel Injury and Thrombi Induced by Indwelling Aortic Catheters

Catheters were inserted into rabbit aortae and remained *in situ* for the times specified. Control animals had sham operations for the same periods of time. ^{51}Cr-labelled platelets (8×10^9) were injected into each animal. Blood samples for radioactivity measurements and platelet counts were obtained at 2 hours after the infusion of labelled platelets to determine platelet recovery. Additional blood samples were obtained at 2, 19, 22, 43, 67 and 91 hours after the initial (2 h) sample. Platelet counts were determined on all samples and averaged. The radioactivity in each sample was measured and mean platelet survival time was determined. Platelet turnover in the circulation was calculated from the formula:

$$\text{Platelets}/\mu L/h = \frac{\text{mean platelet count } (\mu L)}{\text{mean platelet survival time } (h)}$$

Turnover of ^{51}Cr-Labelled Platelets in the Circulation of Rabbits with Vessel Injury and Thrombi Induced by Indwelling Aortic Catheters

Time After Insertion of Catheter or Sham Operation when ^{51}Cr-Labelled Platelets Injected	Catheter Animals		Sham Animals		p[b]
	n	Turnover of Platelets (platelets/μL/h)	n	Turnover of Platelets (platelets/μL/h)[a]	
3 h	6	12,970.0 ± 2019.6[a]	6	7,342.1 ± 651.8	<0.005
24 h	6	7,356.1 ± 404.0	6	5,985.9 ± 365.6	>0.05
3 d	11	8,225.1 ± 382.0	11	6,838.9 ± 701.2	>0.05
6 d	6	11,895.6 ± 1,625.7	6	7,653.8 ± 704.6	<0.005
2 Weeks	10	9,603.9 ± 1,195.7	11	7,112.7 ± 605.0	<0.025
4 Weeks	3	9,511.4 ± 749.7	5	5,693.5 ± 247.8	<0.025

[a]Mean ± SE

Results of Statistical Analyses:

ANOV Catheter or sham vs time 3 h to 4 weeks, $p<0.01$
Catheter or sham vs time 3 days to 4 weeks, $p>0.05$
Catheter vs sham, $p<0.001$

[b]Student-Newman-Keuls - catheter vs respective sham

the introduction of the aortic catheter. The net effect of reduced whole blood platelet counts and moderately reduced platelet survival time was that platelet turnover was not significantly different from that obtained for animals that had a sham operation.

It was apparent from the shifting platelet counts in the circulation that platelet production and platelet consumption were altered during the first 3 days following the insertion of the aortic catheter. However, these observations were not always reflected in increased platelet turnover values until 6 days following the insertion of the catheter at which time circulating platelet counts had stabilized.

3.6 Contribution of Injured Vessel Wall to Reduced Platelet Survival in Rabbits with Aortic Catheters

In rabbits, aortic catheters cause repeated endothelial cell injury resulting in thrombus formation and reduced platelet survival. In rats, aortic catheters have also been shown to cause repeated endothelial injury and reduced platelet survival (Winocour et al., 1982). However, in contrast to rabbit aortae, macroscopic thrombi do not form in rat aortae with indwelling catheters of the size and type used by Winocour. These studies raised the possibility that platelets which had interacted with the vessel wall were freed from the surface and returned to the circulation from where they were rapidly cleared. It was likely that this process was mediated by proteolytic enzymes which can digest the glycoproteins involved in platelet adhesion. Thus, shortened

platelet survival may be a consequence of continued platelet turnover and modification on the injured vessel wall. Therefore, the contribution of vessel wall injury to shortened platelet survival in rabbits was examined using several approaches:

a) The extent of vessel injury caused by the aortic catheter was measured by examining changes in the permeability of the aorta to ^{131}I-labelled albumin.

b) Next, the extent to which the injured vessel wall could accumulate ^{51}Cr or ^{111}In-labelled platelets from the circulation was also examined.

c) In addition, the effect on platelet survival of varying the extent of vessel wall injury in relation to thrombosis was determined in rabbits using aortic catheters of different lengths.

3.6.1 Accumulation of ^{131}I-Labelled Albumin in Aortae of Rabbits which had Catheters in situ for Various Times

The degree to which the aortic endothelium was either stimulated, damaged or removed by aortic catheters was determined by measuring the amount of ^{131}I-labelled albumin that accumulated during a one hour period at various times after insertion of the catheter. The area of the vessel wall that was examined for the accumulation of ^{131}I-labelled albumin excluded the regions around the catheter tip and at the trifurcation

where thrombus formation normally occurs. In these areas, the vessel wall may have been modified by vasoactive components generated around the thrombus or released from platelets and leucocytes incorporated into the thrombus.

The amount of ^{131}I-labelled albumin that became associated with the vessel wall (Table 3.20) was directly related to the length of time the catheter had been in place. Rabbit aortae which had catheters in situ for 3 hours before the radio-labelled albumin was infused, accumulated the same amount of ^{131}I as vessels from animals that had a sham operation 3 hours previously. Although there was no difference between the ^{131}I-albumin that accumulated at 3 and 6 days after insertion of the catheters, the vessels at these times accumulated significantly more ^{131}I-labelled albumin during a one hour period than the vessels which had catheters in situ for shorter (3 h) time intervals.

3.6.2 Accumulation of ^{51}Cr or ^{111}In-labelled Platelets on the Injured Vessel Wall of Rabbits with Aortic Catheters in situ

The extent to which the vessel wall remained reactive to fresh platelets in the circulation was examined using two experimental approaches:

a) the association of ^{51}Cr-labelled platelets during a 24 hour period

b) the association of ^{111}In-labelled platelets during a 2 hour period.

The labelled platelets were infused into rabbits which had aortic

Table 3.20

Accumulation in One Hour of ^{131}I-Labelled Albumin into Rabbit Aortae that had Catheters in situ for Various Times

Time Catheters in Place before ^{131}I-Albumin Injected	Number of Animals	^{131}I-Albumin Accumulation in Vessel Wall[b] (cpm/cm^2)
3 h	4	13.3 ± 1.9[a]
3 d	4	38.7 ± 9.9
6 d	4	44.4 ± 5.8
Sham	3	13.1 ± 5.4

[a]Mean ± SE
[b]Vessel segments where macroscopic thrombus formation was absent

Results of Statistical Analysis:

^{131}I-Albumin accumulation in catheter animals vs time; ANOV, $p<0.05$

^{131}I-Albumin accumulation; Student-Newman-Keuls, 3 h vs 3 d $p<0.05$
3 h vs 6 d $p<0.05$
3 vs 6 d $p>0.05$

Catheters were inserted into rabbit aortae and remained in situ for the times specified. Control animals had sham operations for the same time periods. ^{131}I-albumin was then injected into each rabbit and allowed to circulate for 1 hour before the animals were killed by perfusion-fixation. The amount of ^{131}I-albumin that incorporated into the vessel wall where macroscopic thrombus formation did not occur was measured and expressed as cpm/cm^2.

catheters *in situ* for various times. When the platelets had circulated for the specified times, the animals were killed by perfusion-fixation. Areas of the vessel wall where macroscopic thrombus formation did not occur, were examined for platelet accumulation. The amount of ^{51}Cr or ^{111}In that associated with the vessel was expressed as a percentage of the ^{51}Cr or ^{111}In-labelled platelets injected into each animal.

Both experimental procedures (Table 3.21, Table 3.22) demonstrated that 3 days and 6 days (and 14 days in the ^{51}Cr experiments) after the insertion of aortic catheters, the vessel wall was as reactive to platelets as a freshly injured (3 h) surface. However, the absolute values for ^{51}Cr accumulation were larger than those observed for ^{111}In accumulation. Since the ^{51}Cr experiments measured accumulation over a 24 hour period and the ^{111}In studies measured accumulation over a 2 hour period, the higher ^{51}Cr values may simply reflect differences in the rate of platelet removal from an injured surface.

Nevertheless, unlike the significant loss of thrombus reactivity *in vivo*, the vessel wall maintained its ability to interact with platelets and thus may contribute significantly to shortened platelet survival.

3.6.3 Platelet Survival in Rabbits with Varying Extents of Vessel Injury

Since the vessel wall of rabbits with aortic catheters maintained its capacity to interact with circulating platelets, it was necessary to determine if this continued reactivity contributed to shortened platelet survival.

In preliminary studies performed in our laboratory, we had observed

Table 3.21

Accumulation During 24 Hours of ^{51}Cr-Labelled Platelets on the Injured Vessel Wall of Rabbits with Aortic Catheters in situ for Various Times

Time Catheters in Place Before ^{51}Cr-Platelets Injected	Number of Animals	^{51}Cr-Platelets Associated with Vessel Wall[b] in 24 h (% of ^{51}Cr injected)
3 h	8	1.06 ± 0.10[a]
3 d	3	0.71 ± 0.36
6 d	8	0.61 ± 0.09
14 d	8	0.67 ± 0.15

[a]Mean ± SE
[b]Vessel segments where macroscopic thrombus formation did not occur

Results of ANOV:

^{51}Cr association in 24 hours over time; $p>0.05$

Catheters were inserted into rabbit aortae and remained *in situ* for the specified times. ^{51}Cr-labelled platelets were then infused into the rabbits and allowed to circulate for 24 hours before the animals were killed by perfusion-fixation. The ^{51}Cr associated with the vessel wall where macroscopic thrombus formation did not occur was determined and expressed as a percentage of the ^{51}Cr injected into each animal.

Table 3.22

Accumulation During 2 Hours of ^{111}In-Labelled Platelets on the Injured Vessel Wall of Rabbits which had Aortic Catheters in situ for Various Times

Time Catheters in Place Before ^{111}In-Platelets Injected	Catheter Animals		Sham Animals	
	n	^{111}In Associated with vessel wall[b] (% of injected)	n	^{111}In Associated with vessel wall[b] (% of injected)
3 h	6	0.028 ± 0.003[a]	2	0.006
3 d	6	0.025 ± 0.003	2	0.006
6 d	6	0.040 ± 0.005	2	0.008

[a]Mean ± SE
[b]Vessel segments where macroscopic thrombus formation was absent

Results of ANOV:

^{111}In association, catheter vs sham; $p<0.001$
^{111}In association, catheter/sham over time; $p>0.05$

Catheters were inserted into rabbit aortae and remained *in situ* for the specified times. ^{111}In-labelled platelets were then infused and allowed to circulate for 2 hours before the animals were killed by perfusion-fixation. The ^{111}In associated with the vessel wall where macroscopic thrombus formation did not occur was measured and expressed as a percentage of the ^{111}In injected into each animal.

that all aortic catheters induced similar patterns of thrombus formation in rabbits (at the area of the trifurcation and at the area of the catheter tip; Figure 3.19). Thus, if thrombus formation and platelet turnover in thrombi were similar, by varying the extent of vessel injury using long or short catheters, the influence of the vessel wall on platelet survival could be examined independent of the influence of the thrombus.

Evans blue dye accumulation in the aortae of animals with indwelling catheters of varying length (10 cm, 16 cm, 20 cm) confirmed that the longer catheters damaged a larger area of the vessel wall than the shorter catheter (Figure 3.19).

Platelet survival studies were performed in the usual manner by injecting ^{51}Cr-labelled platelets into rabbits followed 2 hours later by insertion of aortic catheters of varying length or by a sham operation. These survival studies would include the acute phase of thrombus formation which had previously been shown to be maximal during the first 24 hours (refer to Table 3.4).

Results from these studies (Table 3.23) demonstrated that the short indwelling catheters (10 cm, 16 cm) reduced platelet survival time to the same extent as the long (20 cm) catheters.

However, the shortest (10 cm) catheter resulted in the formation of a significantly larger thrombus (Table 3.23) than that formed by the longer (16 cm, 20 cm) catheters without additional shortening of platelet survival time.

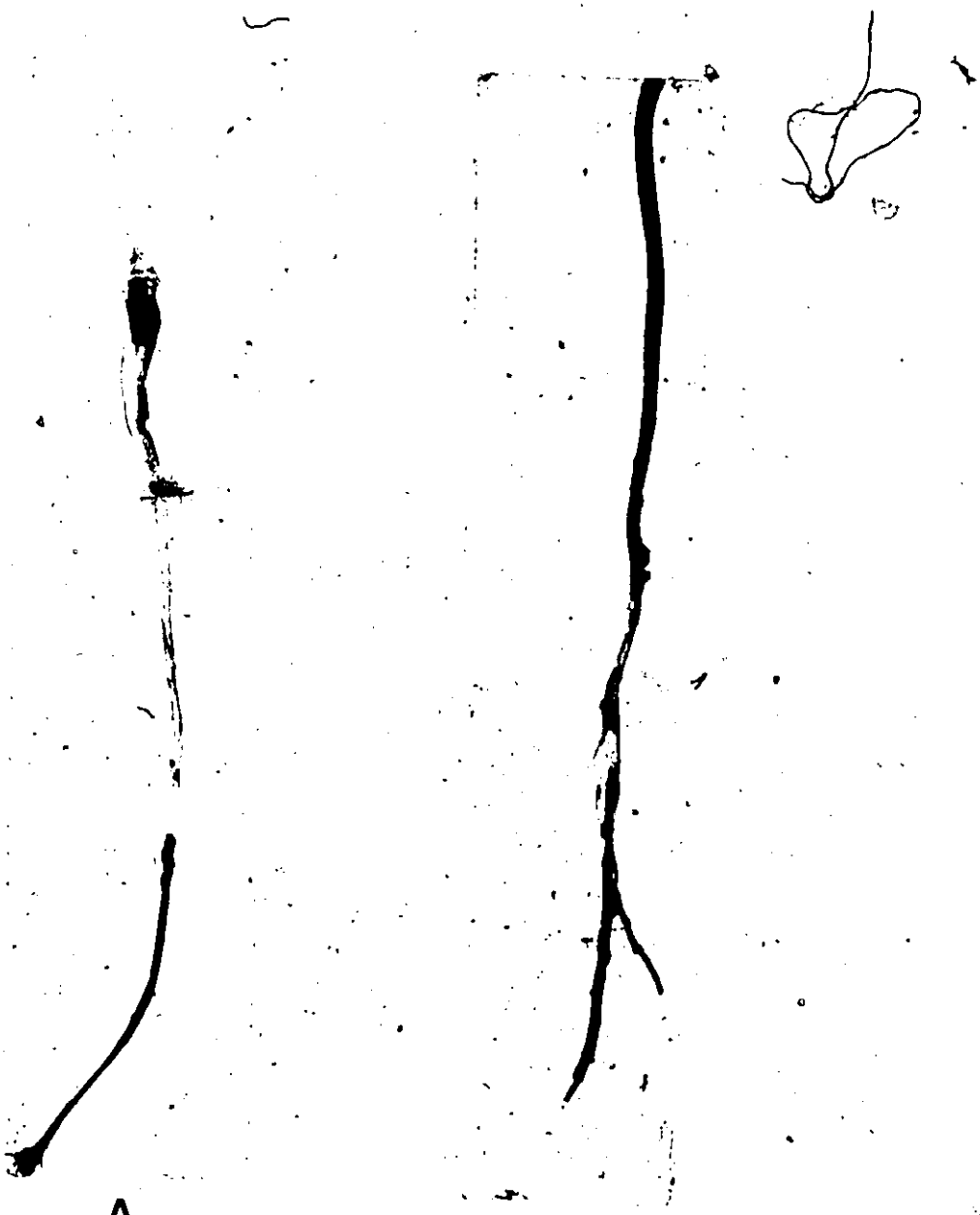
Figure 3.19

Extent of Vessel Injury and Thrombus Formation in Aortae of Rabbits with Indwelling Aortic Catheters of Varying Length

A) Aorta from a rabbit which had a 20 cm catheter *in situ* for 6 days.

B) Aorta from a rabbit which had a 10 cm catheter *in situ* for 6 days.

Thrombus formation occurred in similar areas in both vessels; at the aortic trifurcation and in the area of the catheter tip. Accumulation of Evans Blue dye along the length of the vessel containing the 20 cm catheter indicates areas of increased permeability where the vessel wall was damaged by the indwelling catheter.


A
B

3.6.4 Platelet Survival in Rabbits in which Different Extents of Vessel Injury were Induced for 3 Days Before Platelet Survival Studies were Begun

The previous experiments with long and short catheters examined platelet survival immediately following the introduction of aortic catheters. As indicated on Tables 3.4 and 3.5, platelet incorporation into thrombi was maximized during the first 24 hours after catheter insertion. As well, the thrombi which formed in response to the short catheter were significantly larger (Table 3.23). It was postulated that platelet incorporation into thrombi of different sizes masked the influence of varying extents of vessel injury on platelet survival.

Therefore, the survival of ^{51}Cr-labelled platelets was measured in animals which had varying extents of vessel injury induced for 3 days by long (20 cm) or short (10 cm) catheters before labelled platelets were infused.

Previous experiments (refer to Table 3.11) had also demonstrated that at this time, thrombus reactivity toward circulating platelets was significantly reduced and therefore extensive platelet incorporation into thrombi was less of a complicating factor.

Under these experimental conditions (Table 3.24), the long (20 cm) catheter which continued to damage a larger area of the vessel wall caused a significant reduction in platelet survival time.

Although platelet survival time in rabbits with short (10 cm) catheters tended to be less than in sham operated control animals, the differences were not statistically significant.

This experimental evidence suggested that once the acute phase of

Table 3.23

Survival of ^{51}Cr-Labelled Platelets in Rabbits with Indwelling Aortic Catheters of Varying Length

	Length of Catheter (cm)	Number of Animals	Mean Platelet Survival Time (hours)	Thrombus Wt. (mg at 5 days)
A	20	10	44.2 ± 2.7[a]	16.8 ± 4.7
B	16	7	42.5 ± 2.7	18.2 ± 5.1
C	10	9	44.6 ± 4.2	56.0 ± 19.0
	Sham	10	61.1 ± 3.0	0

[a]Mean ± SE

Results of Statistical Analyses:

Mean platelet survival time of each group compared to sham; $p<0.01$ in each case (Dunnet's test)

Thrombus Weight:

Student-Newman-Keuls, A vs B, $p>0.10$
AB vs C, $p<0.05$

(ANOV; $p<0.05$)

A blood sample was taken at 2 hours after injection of labelled platelets. The amount of radioactivity in this sample was considered to represent maximum (100%) circulating radioactivity. Catheters were inserted into rabbit aortae. Control animals received sham operations. Additional blood samples were taken at predetermined times (2, 19, 22, 43, 67, 91 hours) after the insertion of the catheter or after sham operations. The amount of radioactivity in each blood sample was measured and expressed as a percentage of the 2 hour value to calculate platelet survival time. The animals were killed by perfusion -fixation when the survival study was terminated (5 days). The aortae were removed and the thrombus weight was determined. Logarithmic transformation normalized the thrombus weight data for statistical analyses.

Table 3.24

Platelet Survival in Rabbits in which Different Extents of Vessel Injury were Induced for 3 Days before Platelet Survival Studies were Begun

Length of Catheter which Induced Vessel Injury	Number of Animals	Mean Platelet Survival Time (hours)
20 cm	5	45.6 ± 4.1[a]
10 cm	5	60.5 ± 4.4
Sham	5	69.3 ± 2.5

[a]Mean ± SE

Results of Dunnet's Test

Mean platelet survival time in animals with 20 cm catheter vs sham; $p<0.01$
Mean platelet survival time in animals with 10 cm catheter vs sham; $p>0.05$

Catheters (20 cm, 10 cm) were inserted into rabbit aortae and remained *in situ* for 3 days. Control animals had sham operations for the same periods of time. At this time ^{51}Cr-labelled platelets were injected into all animals and a blood sample was obtained at 2 hours following the infusion of labelled platelets. Additional blood samples were taken at 2, 19, 22, 43, 67, and 91 hours after the initial sample. The amount of radioactivity in each sample was measured and expressed as a percentage of the 2 hour value to calculate mean platelet survival time.

thrombus formation had ceased, there was a direct relationship between the extent of vessel injury and shortened platelet survival time.

However, before any firm conclusions could be drawn, it was necessary to confirm that the thrombi formed by either long or short catheters were equally reactive to fresh platelets in the circulation.

3.6.5 Accumulation of ^{51}Cr-labelled Platelets in 24 Hours on Thrombi Formed for 6 Days by Indwelling Aortic Catheters of Varying Length - Measurement of Thrombus Reactivity

The thrombi formed in response to long (20 cm) and short (10 cm) catheters were significantly different in size. Previous experiments had also shown that thrombi formed *in vivo* remained reactive to circulating platelets. It was possible that variation in thrombus reactivity may have contributed to the differences in platelet survival observed in animals which had long or short aortic catheters *in situ* for 3 days before survival studies were started.

If platelets interacted to a similar extent with these thrombi, the observed shortened platelet survival in animals with long catheters must then relate primarily to the extent of vessel injury.

For these experiments, long (20 cm) or short (10 cm) catheters were inserted into rabbit aortae. The catheters remained *in situ* for 6 days during which time thrombi formed on the injured vessel wall. ^{51}Cr-labelled platelets were infused into the animals and allowed to circulate for 24 hours before the animals were killed by perfusion-fixation. The amount of ^{51}Cr associated with the thrombus was measured and expressed as a percentage of the ^{51}Cr injected into the

rabbit.

Of the injected radioactivity, 0.61 ± 0.08% (mean ± SE; Table 3.25) accumulated in 24 hours on thrombi which had been formed in vivo for 6 days by 20 cm aortic catheters. The amount of ^{51}Cr which accumulated during 24 hours on thrombi preformed for 6 days by 10 cm catheters (0.47 ± 0.07%) was not significantly different from that which accumulated on thrombi formed by long catheters.

It was apparent that thrombi formed in vivo by long or short aortic catheters remained equally reactive to circulating platelets. Thus, any effects on platelet survival after the acute phase of thrombus formation was primarily a result of platelet interaction with the injured vessel wall.

Table 3.25

Accumulation During 24 Hours of ^{51}Cr-Labelled Platelets on Thrombi Formed in vivo for 6 Days by Indwelling Aortic Catheters of Varying Length

Length of Catheter which induced Thrombus Formation	Number of Animals	^{51}Cr Associated with Thrombi (% of injected)
20 cm	7	0.61 ± 0.08[a]
10 cm	6	0.47 ± 0.07

[a]Mean ± SE

Results of Student's 't' Test:

^{51}Cr associated with thrombus induced by 10 cm catheter vs ^{51}Cr associated with thrombus induced by 20 cm catheter, $p>0.05$

Catheters (20 cm, 10 cm) were inserted into rabbit aortae. Thrombi were formed *in situ* for 6 days at which time ^{51}Cr-labelled platelets were infused. The radiolabelled platelets circulated for 24 hours before the animals were killed by perfusion-fixation. The amount of ^{51}Cr associated with the thrombus was measured and expressed as a percentage of the ^{51}Cr injected into each rabbit.

CHAPTER 4

DISCUSSION

4.0 Discussion

The objective of this study was to examine the relative contribution of thrombus formation and of the repeatedly injured vessel wall to shortened platelet survival time in experimentally induced thromboembolic vascular disease.

Several key conclusions were reached.

1. Thrombus formation, especially during the acute phase of growth, contributes significantly to shortened platelet survival.

2. The contribution of the thrombus to shortened platelet survival decreases as the thrombus aged *in vivo* since thrombus reactivity toward circulating platelets diminishes. Nevertheless, thrombi retain some capacity to interact with platelets and this has a small (but not statistically significant) effect on platelet survival.

3. The injured vessel wall contributes significantly to shortened platelet survival. Unlike the thrombus, the repeatedly injured vessel wall retains its ability to interact with fresh platelets in the circulation. During the time when thrombus reactivity has decreased, the extent of reduction in mean platelet survival time is directly related to the extent of repeated vessel wall injury.

Therefore, both thrombus formation and platelet interaction on a repeatedly injured vessel wall influence mean platelet survival. The

thrombus effect occurs primarily during the acute phase of injury. The vessel wall most likely contributes to shortened platelet survival during the acute phase, but it is the major mechanism of shortened platelet survival during the chronic phase of injury.

4.1 Contribution of Thrombus Formation to Shortened Platelet Survival in Rabbits with Indwelling Aortic Catheters

Since platelet turnover in the circulation was significantly increased during the time when thrombus growth was at its maximum and circulating platelet numbers were decreased during the same time, it suggested that thrombus formation may contribute significantly to shortened platelet survival time. The thrombus effect may be mediated through continued platelet incorporation into thrombi where some of the platelets are lysed or phagocytosed. As well, platelet exchange in thrombi could result in platelet membrane glycoprotein (GP) alterations such that some of the platelets are rapidly cleared upon their return to the circulation while others continue to circulate.

Therefore, we examined:

a) the rate and extent of platelet incorporation into thrombi as they formed in vivo

b) the reactivity of thrombi toward fresh platelets in the circulation

c) whether whole platelets exchanged, lysed, or were phagocytosed in thrombi.

4.1.1 Platelet Incorporation into Thrombi formed on Injured Vessel Walls

Previous studies in humans had demonstrated that platelet survival was shortened in conditions such as disseminated intravascular coagulation where extensive in vivo thrombus formation occurs (McKay, 1965; Harker and Finch, 1969). These studies suggested that platelets are prematurely removed from the circulation simply by their irreversible accumulation into intravascular thrombi.

In rabbits with aortic catheters in which thrombi formed, it was also possible that one of the mechanisms contributing to shortened platelet survival was irreversible platelet accumulation into enlarging thrombi which subsequently organized and became incorporated into the vessel wall.

To investigate this possibility, the extent to which radiolabelled platelets incorporated into thrombi was examined at various times after catheters were inserted into rabbit aortae.

Results from these studies demonstrated that ^{51}Cr-labelled platelets incorporated into developing thrombi for about 24 hours. During the same time, the thrombus achieved its maximum weight. After this time ^{51}Cr was lost from the thrombus although the weight of the thrombus remained constant for as long as 14 days after the catheter had been inserted.

When platelet survival studies were started at various times after thrombi had formed in situ, the greatest reduction in platelet lifespan was observed when the studies included the first 24 hours of thrombus formation when platelet incorporation into thrombi and thrombus growth were at their maximum.

These observations demonstrated that incorporation of platelets into

thrombi during the acute phase of formation, removes large numbers of platelets from the circulation. This loss contributes, in part, to shortened platelet survival time.

Platelet survival time continued to be significantly shortened when survival studies were started 3 days, 6 days or 2 weeks after maximum thrombus formation had occurred although the extent of reduction gradually returned towards control values. During the same time periods, the thrombus weight remained unchanged from the maximum measured at 24 hours. As well, studies of thrombus reactivity demonstrated that during these times the ability of the thrombus to incorporate fresh platelets from the circulation was significantly reduced compared to a newly formed (24 hour) thrombus.

It was apparent that platelet survival time continued to be shortened after maximum thrombus growth and after the period when maximum platelet incorporation into the thrombus had occurred. Thus, it was unlikely that continued, irreversible platelet incorporation into thrombi was the sole mechanism responsible for shortened platelet survival time.

4.1.2 Platelet Turnover in Thrombi

From the first descriptions in the literature of platelet participation in hemostasis and thrombosis, it was established that thrombi are dynamic structures which continually undergo dissolution and reformation (Bizzozero, 1882).

As has been previously discussed (see Section 4.1.1), some of the platelets participating in thrombus formation are lost from the circulation when they incorporate into thrombi that subsequently organize

and incorporate into the vessel wall.

Other platelets which have participated in thrombus formation are probably returned to the circulation as thrombi undergo dissolution and platelet aggregates break up. These platelets are likely to have been exposed to thrombin generated at sites of vessel injury by activated extrinsic and intrinsic coagulation systems, to ADP released from aggregated platelets, TXA_2 formed by activated platelets and to plasmin.

Thrombin treatment of platelets *in vitro* does not alter major glycoproteins of the platelet surface membranes which are important to their normal lifespan (Phillips, 1974; Mosher *et al.*, 1979; Greenberg *et al.*, 1979) . Thus, thrombin has no effect on platelet survival when treated platelets are infused into animals (Reimers *et al.*, 1973, 1976). In addition, direct infusion of thrombin into animals does not shorten platelet survival time (Mustard *et al.*, 1966).

Similarly, exposure of platelets to ADP *in vitro* does not alter platelet surface membrane glycoproteins, nor, membrane sialic acid content, thus, ADP also has no effect on platelet survival time (Packham *et al.*, 1980). Therefore, platelets which had been exposed to thrombin or ADP in thrombi would survive normally upon their return to the circulation.

Some platelets which have participated in thrombus formation are almost certainly exposed to proteolytic enzymes such as plasmin generated at sites of injury and concentrated in areas of fibrin formation (Collen, 1980). Plasmin degrades the fibrin in thrombi thereby destabilizing the mass and facilitating thrombus dissolution. In addition, Greenberg *et al.* (1979) have shown that when platelets are exposed to plasmin *in*

vitro, glycoprotein I and lesser amounts of glycoproteins II and III are cleaved from the surface membranes. These platelets have a significantly shortened survival time.

Studies by Niewiarowski et al. (1972,) provide in vivo confirmation of Greenberg's findings that proteolytic enzymes are important mediators of shortened platelet survival. These workers injected streptokinase into rabbits to activate the fibrinolytic system and observed a transient decrease in platelet count and shortened platelet survival time. However, it should be noted that in these experiments, the rabbits experienced bleeding episodes which may have removed sufficient platelets during plug formation to contribute to shortened platelet survival time. Furthermore, the high concentrations of streptokinase used in these experiments could have played a major role in reducing platelet survival.

In contrast, activation of the fibrinolytic system in humans by urokinase therapy (Harker and Slichter, 1972) had no effect on platelet survival time although the rate of platelet turnover in the circulation was increased. The reasons for these opposing results may be due to the the bleeding problems encountered by Niewiarowski as well as the high concentrations of streptokinase used in the latter experiments. It was also probable that the discrepancy was a result of methods used to analyse the survival curves. Harker (1972) fitted the data to the appropriate curve visually as well as with computer assistance. Based on the pattern of the resultant curve (linear, curvilinear, exponential) the appropriate mathematical calculations were then applied to determine mean platelet survival time (see Section 1.9). We demonstrated in the present study (see results Table 3.2) that the use of different mathematical

approaches to analyse the same survival data can result in very different answers. Had Harker analyzed all his data similarly, the results may have been compatible with those of Niewiarowski and associates.

It was also possible that proteolytic enzymes such as leucocyte elastase and neutral proteases released from lysosomal granules of activated leucocytes modify membrane glycoproteins (Ohlsson and Olsson, 1974). Leucocytes were consistently observed within and around the thrombi in these studies; their presence in experimentally formed thrombi has also been described frequently by other investigators (Jorgensen et al., 1967; Moore, 1973). However, the contribution of the leucocyte to shortened platelet survival time has not been examined.

The action of proteases released from platelets themselves as they incorporate into thrombi (Phillips and Jakabova, 1977; Solum et al., 1980; Sakon et al., 1981) may similarly affect platelet membrane glycoproteins and influence the rate of clearance of these platelets upon their return to the circulation following thrombus dissolution.

In the present study, platelet exchange in thrombi could expose platelets to proteolytic enzymes that are known to modify their surface membrane. This could be an important mechanism of shortened platelet survival in rabbits with indwelling aortic catheters.

We therefore determined:

a) whether thrombi remained reactive and continued to accumulate fresh platelets from the circulation

b) whether whole platelets exchanged in thrombi

c) whether platelets which had participated in thrombus formation returned to the circulation where they could be detected

4.1.2.1 Reactivity of Thrombi to Fresh Platelets in the Circulation

If proteolytic modification by enzymes in thrombi contributes to shortened platelet survival, many platelets must continually exchange in thrombi during the two week period when platelet survival is observed to be shortened. We therefore examined the extent to which thrombi remained reactive to fresh ^{51}Cr-labelled platelets in the circulation at various times after the catheters had been inserted.

The data from these experiments demonstrated that during the early period following insertion of the catheter (first 24 hours), the thrombus was extremely reactive toward labelled platelets in the circulation. Morphological evidence from transmission electron microscopic (TEM) studies of thrombi at 24 hours provided similar evidence. Many platelets that had been stimulated to change shape and release the contents of their granules were observed on the luminal surface of thrombi at this time.

The reactivity toward circulating platelets decreased significantly between 24 hours and 3 days after the thrombus had formed. TEM studies of the surface of 3 day old thrombi also showed fewer platelets on the luminal surface. However, thrombi which had formed *in vivo* for 3, 6 or 14 days continued to accumulate platelets from the circulation although the amount of radioactivity present at these times was about 50% of that observed to accumulate within the first 24 hours.

These results were consistent with those from other investigators

demonstrating a reactive surface on thrombi preformed *in vivo* for various times. For example, Stratton and associates (1981) have shown that ventricular thrombi which had formed *in vivo* for as long as 2 to 72 months retained the ability to accumulate sufficient numbers of ^{111}In-labelled platelets to be detectable by scintigraphic imaging. Similar studies by Thakur *et al.* (1976) have shown that ^{111}In-labelled platelets readily accumulate on preformed venous thrombi. In addition Davis and co-workers (1980) have demonstrated that atherosclerotic lesions in carotid arteries remain reactive toward ^{111}In-labelled platelets in the circulation.

Taken together, the work of Stratton, Thakur and Davis as well as the results from the present study, demonstrate that freshly formed thrombi are highly active toward platelets in the circulation. A significant portion of this reactivity is lost as the thrombus ages *in vivo* but thrombi retain some of their ability to incorporate fresh platelets. The observation that thrombi retain some ability to interact with platelets in the circulation contrasts with results observed after a single balloon catheter injury in which the injured surface of the rabbit aorta quickly becomes less reactive to further platelet accumulation (Groves *et al.*, 1979) and platelet survival is unaffected.

4.1.2.2 Exchange of Whole Platelets in Thrombi

Thrombi which formed in the presence of ^{51}Cr-labelled platelets lost significant amounts of the ^{51}Cr which had incorporated during the time of maximal thrombus growth. This loss of radioactivity was not accompanied by changes in thrombus weight. Moreover, the rate of ^{51}Cr loss was

similar to the rate of removal of ^{51}Cr-labelled platelets from the circulation suggesting that whole platelets are continually escaping from thrombi to be replaced by platelets from the circulation with a lower specific activity. It was also possible that the loss of ^{51}Cr from the thrombus was simply a result of platelet lysis within thrombi. A third possibility was that ^{51}Cr was lost from the thrombus through a combination of: a) platelet lysis, b) phagocytosis by leucocytes and c) loss of whole platelets.

To determine the relative contribution of platelet lysis and platelet escape to the loss of radiolabel from the thrombus, the fate of platelets doubly labelled with diazotized ^{125}I-iodosulphanilic acid ($D^{125}ISA$, a membrane glycoprotein label, George et al., 1976a; Hanson and Harker, 1981) and ^{51}Cr (a cytoplasmic constituent label, Steiner and Baldini, 1970) was examined in rabbits with aortic catheters.

If platelet lysis occurred in thrombi formed from doubly-labelled platelets, selective loss of ^{51}Cr would occur as cytoplasmic constituents were lost. The ^{125}I would be retained in the thrombi with entrapped membranes. Escape of whole platelets from thrombi would result in the simultaneous loss of both ^{125}I and ^{51}Cr from thrombi.

By measuring the ratio of $^{125}I/^{51}Cr$ of doubly labelled platelets before their infusion into the circulation of rabbits, before and after insertion of aortic catheters, and the ratio of $^{125}I/^{51}Cr$ in thrombi formed for various times in vivo, it was possible to determine if platelet lysis occurred and if intact platelets were continually incorporated into and lost from thrombi. Furthermore, during these experiments, it was possible to examine the survival profile of

doubly-labelled platelets based on the membrane ^{125}I label or the cytosolic ^{51}Cr label to determine if whole platelets were removed from the circulation and if membrane modifications were occurring more rapidly in catheter bearing animals.

In these studies, when doubly-labelled platelets were infused into the circulation of rabbits, initially, there was a rapid decrease in the ratio of $^{125}I/^{51}Cr$. The recovery values at 2 hours of 26 to 28% for ^{125}I was much less than the recovery values of 85 to 92% for ^{51}Cr in the same sample. This was most likely due to rapid early loss of ^{125}I from the platelet surface through elution or through enzymatic effects on membrane glycoproteins.

George and his associates (1976a, b), in their original work with doubly-labelled platelets, also observed a rapid loss of ^{125}I from the surface of infused rabbit platelets which had been doubly-labelled with ^{51}Cr and the D^{125}ISA analogue diazotized di^{125}I-iodosulphanilic acid (DD^{125}ISA). However, when these doubly-labelled platelets were harvested from rabbits after 3 hours in the circulation, the preferential loss of ^{125}I could not be demonstrated *in vitro*. George concluded that once the initial loss of loosely bound ^{125}I had occurred immediately upon infusion into the circulation, subsequent loss of ^{125}I from the platelet surface was no longer a consequence of elution.

Rand (1982) has also demonstrated that 60% of the ^{125}I that was initially associated with platelets doubly-labelled with D^{125}ISA and ^{51}Cr appeared in the suspension supernatant, *in vitro* within the first 2.5 hours after labelling. During the same time only 6% of the ^{51}Cr appeared in the suppernatant. Between 2.5 and 5 hours after the double labelling

procedure, further selective loss of ^{125}I was no longer apparent.

Rand suggested that the appearance of the ^{51}Cr in the suspension supernatant probably represents the lysis of a few platelets and that proteases released from lysed platelets may have cleaved ^{125}I-labelled glycoproteins from the surface membranes of remaining platelets.

In the present studies, in agreement with the experimental findings of George et al. (1976a, b, 1978a, b), Hanson and Harker (1981) and Rand (1982), the survival values of doubly-labelled platelets in the circulation of rabbits based upon calculations using the amount of ^{125}I in the circulation, was significanty shorter than survival calculated on the basis of ^{51}Cr in the same platelets.

Since comparison of the ^{125}I and ^{51}Cr survival patterns was complicated by rapid early ^{125}I elution, platelet survival was calculated in two ways:

a) in the normal manner, taking the platelet-associated radioactivity circulating at 2 hours as the initial 100% value

b) taking the platelet associated radioactivity in the circulation at 6 hours as the initial 100% value since the rate of elution at this time was minimal.

In both instances, ^{125}I was lost more rapidly from circulating platelets than ^{51}Cr resulting in a decreasing ratio of $^{125}I/^{51}Cr$ as platelets continued to circulate.

In addition, platelet survival based on either circulating ^{125}I or

^{51}Cr concentration of doubly-labelled platelets was significantly shorter in animals with aortic catheters. Since there was no significant difference in the ratio of $^{125}I/^{51}Cr$ of platelets in the circulation of catheter bearing animals and animals with sham operations, it was apparent the loss of ^{125}I and ^{51}Cr was proportionally the same in both groups and that whole platelets were being removed from the circulation of rabbits with aortic catheters.

George and associates (1978a, b) demonstrated that the selective loss of DD^{125}ISA from the platelet surface *in vivo* could be retarded by treating rabbits with drugs such as a combination of aspirin and dipyridamole (these drugs are known to inhibit different aspects of platelet function). Under these conditions ^{125}I and ^{51}Cr disappeared from the circulation of rabbits at the same rate. Conversely, when platelet aggregation and *in vivo* coagulation was activated by infusion of thrombin into rabbits, the rate of ^{125}I loss from doubly labelled platelets was accelerated in relation to the loss of ^{51}Cr. This is not surprising since thrombin treatment cleaves a minor glycoprotein (GPV) from the platelet surface and this protein was probably also labelled with DD^{125}ISA.

In similar studies, Hanson and Harker (1981) tested a variety of drugs that are known to alter platelet function but could not demonstrate changes in the rate of ^{125}I removal from doubly-labelled (D^{125}ISA and ^{51}Cr) platelets in the circulation of baboons. However, in accord with our results these investigators did observe an accelerated rate of ^{125}I loss from the surface of circulating doubly-labelled platelets. This selective loss of ^{125}I could not be reproduced *in vitro* indicating that

the preferential loss of ^{125}I was a consequence of *in vivo* platelet-vessel wall interactions.

In view of the experimental data from George *et al.* (1976a, b; 1978a, b) showing loss of platelet membrane label as a result of events within the circulation and similar observations by Hanson and Harker (1981), it was most likely in our work, that ^{125}I was continually lost through platelet interactions in processes that cause loss of platelet membrane [illegible] but do not lead to immediate loss of platelets from the [illegible]

These [illegible]tal observations support the concept proposed by Mustard and Murp[illegible] (1963a) that platelets are continually subjected to insults ('hits') as they circulate. The intensity of some 'hits' results in immediate clearance of platelets from the circulation. Others are sublethal and the platelets continue to circulate until they accumulate sufficient damage to be cleared from the circulation. It is probable that the preferential loss of ^{125}I observed in this study and in the work of others represents slow modification of membrane glycoproteins or sublethal 'hits' which eventually leads to platelet clearance.

The ratio of $^{125}I/^{51}Cr$ in thrombi which formed *in vivo* was the same as that of the platelets in the circulation at the time the catheter was inserted. There was no change in the ratio in thrombi that had formed *in vivo* for various times although the total amount of ^{125}I and ^{51}Cr which had incorporated into the thrombus during the first 24 hours of its formation decreased significantly with time.

Based on the assumption that the ^{125}I which initially incorporated into the thrombus remained there with trapped platelet membranes, the

constant $^{125}I/^{51}Cr$ ratio maintained in the thrombus despite a decreasing ratio in circulating platelets was compatible with the following interpretations:

a) all the platelets which initially became incorporated into thrombi remained there unchanged such that loss of ^{51}Cr or ^{125}I did not occur and the ratio of $^{125}I/^{51}Cr$ remained constant

b) whole platelets were removed form the thrombus (simultaneous loss of ^{125}I and ^{51}Cr) and these platelets were not replaced by fresh platelets from the circulation which had a lower $^{125}I/^{51}Cr$ ratio

c) a proportion of platelets in the thrombi turned over (simultaneous loss of ^{125}I and ^{51}Cr) and were replaced by new platelets from the circulation which have less ^{125}I associated with them. Simultaneously, some of the platelets in the thrombus lyse selectively losing ^{51}Cr. The loss of ^{51}Cr through lysis and removal of whole platelets, balanced by influx of platelets with a lower $^{125}I/^{51}Cr$ ratio, would maintain a constant ratio in the thrombus.

The first possibility could be ruled out since we had demonstrated that the total radioactivity of thrombi formed in the presence of radiolabelled platelets decreased significantly as the thrombus aged in vivo. Furthermore, the rate of ^{51}Cr loss from the thrombus paralleled the loss of labelled platelets from the circulation suggesting whole

platelets were exchanging in thrombi.

The second possibility also seemed unlikely since the thrombus weight was maintained despite the loss of radioactivity. Morphological observations indicated that the maintenance of the constant thrombus weight could not be explained solely on the basis of increasing fibrin formation. As well, thrombi formed for various times *in situ* continued to accumulate some fresh platelets from the circulation in sufficient numbers that the $^{125}I/^{51}Cr$ ratio would have changed.

The third possibility was most compatible with all the experimental evidence. The fact that the total amount of ^{125}I and ^{51}Cr in the thrombus decreased simultaneously and the observation that the ratio of $^{125}I/^{51}Cr$ did not increase suggested that whole platelets were lost from the thrombus. Since platelets entering the thrombus from the circulation had a lower $^{125}I/^{51}Cr$ ratio, there must have been some selective ^{51}Cr loss from the thrombus through lysis and retention of membrane bound ^{125}I to maintain the ratio of $^{125}I/^{51}Cr$ constant.

Morphological evidence from transmission electron micrographs (TEM) of thrombi formed *in vivo* for 24 hours or 3 days (see results Figures 3.13, 3.14) support our conclusion that some platelets undergo lysis and some platelets exchanged in thrombi. These figures showed fresh platelets associating with the luminal surface of thrombi indicative of thrombus reactivity and cellular debris suggestive of platelet lysis. Furthermore, the increased proportion of least dense platelets in the circulation of rabbits with indwelling aortic catheters (see next Section 4.1.2.3) provided evidence that platelets which had participated in in thrombus formation returned to the circulation.

We concluded from these studies that some of the ^{51}Cr was lost from the thrombus through lysis; some of the ^{51}Cr was lost as whole platelets. Based on the data obtained from these experiments, it was not possible to calculate the extent of ^{51}Cr loss that occurred as a result of lysis.

It was also recognized in these studies that whole platelets may have been removed from thrombi by phagocytic leucocytes since TEM studies consistently demonstrated leucocytes within and on the surface of thrombi (refer to Figures 3.13, 3.14, 3.15).

In the present study, the extent to which fibrinogen and/or fibrin could accumulate into thrombi and contribute to the maintenance of the thrombus weight was not examined by quantitive methods. The possibility existed that the loss of platelets from thrombi might have been balanced by a simultaneous increase in fibrin content of the thrombus thereby keeping the weight constant.

Other investigators had demonstrated that older venous and arterial thrombi retain some capacity to accumulate fibrinogen from the circulation; however, the amount of fibrinogen incorporated into these thrombi was not significant (Coleman et al., 1975; Salimi et al., 1977; Zimmermann et al, 1979). Morphological evidence from our TEM studies supported this premise. Thrombi which had formed in vivo for 24 hours, 3 days and 4 weeks were primarily composed of platelets and debris trapped between strands or layers of fibrin. Some erythrocytes and leucocytes were also present. The fibrin content of these thrombi appeared to remain constant during this time. Although our TEM studies were not conclusive, they did suggest that as thrombi aged in vivo, the total

fibrin content did not appear to change.

Our observations are supported by the studies of Jorgensen et al. (1967) since these investigators have also shown that there is little change in fibrin content of experimental thrombi between 24 hours and 4 days after their formation in carotid arteries of pigs. In fact, Jorgensen noted that between 2 to 4 days after the thrombi had formed, the fibrin content appeared to decrease. Similar results were reported by Woolf (1981). However, the thrombi formed in these experiments were all the result of a single injury, not a multiple or continuous injury as would result from an indwelling catheter.

4.1.2.3 Identification of Circulating Platelets which had Previously Participated in Thrombus Formation

Since it was probable that whole platelets turned over in thrombi, it was important to determine whether some of the platelets that had participated in thrombus formation returned to and continued to circulate. The approach we used to identify these platelets was to examine the bouyant density of platelets obtained from the circulation of rabbits with indwelling aortic catheters.

Prior to the present study, numerous investigators (Karpatkin, 1969a, Karpatkin and Charmatz 1969b; Penington et al., 1976a, b; Corash et al., 1978, 1982; Rand et al., 1981, 1982) had examined circulating platelets from man and experimental animals and noted that circulating platelets were a heterogeneous population with respect to size, function and bouyant density. The origin of this heterogeneity has remained a controversial subject.

Karpatkin and his group (1969a, b) performed some of the first density studies on human and rabbit platelets. They demonstrated that the large, heavy platelet subpopulation was enriched in younger platelets that had increased metabolic and functional capacities. Karpatkin concluded that a relationship existed between platelet age and platelet bouyant density and proposed that as platelets aged in the circulation, their density decreased.

Extensive studies of human and animal platelets by Corash and co-workers (1978) and Rand (1982) support Karpatkin's findings.

Conversely, Penington et al. (1976a, b) concluded from their studies with rats that the density heterogeneity of circulating platelets was a function of platelet production from different ploidy classes of megakaryocytes which differ in size and concentration of their cytoplasmic constituents.

Busch and Olson (1973) and Boneu et al. (1982) were unable to show a relationship between platelet density and platelet age whereas Mezzano and associates (1981) concluded that platelets actually increase in density as they age in the circulation.

The major source of variation from all these investigators probably arises from diffefences in the techniques used to isolate the different platelet subpopulations. These include:

a) differences in techniques used for platelet isolation from whole blood

b) differences in methodology used for platelet survival studies

c) differences in the type of density gradient used (Stractan, Oil, Albumin, Ficoll-Hypaque)

d) the use of separation techniques in which density alone was not the major basis for platelet separation.

Rand and associates (1979, 1982) extensively studied density subpopulations of circulating rabbit platelets under carefully controlled conditions. They clearly demonstrated that the most dense subpopulation of rabbit platelets was enriched in larger, younger platelets containing significantly higher amounts of protein and sialic acid per platelet than the least dense platelets.

Platelet cohort labelling experiments in which megakaryocyte mucopolysaccharides were labelled with ^{35}S sulfate (Rand, 1982) showed that the label first appeared in the circulation primarily in the most dense platelet subpopulation although a small number of platelets in the least dense subpopulation were also labelled. At subsequent times, the relative specific radioactivity of the most dense platelet population decreased. Simultaneously, the radioactivity in the least dense population slowly increased indicating that platelets become less dense as they age in the circulation.

When platelets (rabbit) are exposed to agonists such as ADP, thrombin or plasmin *in vitro* they become less dense (Packham *et al.*, 1979). Since it is likely that ADP, thrombin and/or plasmin are present in high local concentrations around sites of thrombus formation *in vivo*, some of the platelets which had participated in thrombus formation and

then freed upon thrombus dissolution would be identifiable in the circulation by decreased bouyant density.

Therefore, the bouyant density profile of circulating platelets was examined in rabbits before and at various times after insertion of aortic catheters for evidence of those platelets which had participated in thrombus formation.

In these experiments, the techniques used for platelet isolation were modified since the number of platelets obtained in the plasma (PRP) by conventional methods of isolation was between 70 to 90% of the number expected based upon whole blood platelet counts. It has been argued that the usual isolation methods produce an unrepresentative platelet population as the larger most dense platelets would be more likely to remain with the packed red cells during the separation procedure. However, recent evidence by Mezzano et al. (1981) have shown that platelets isolated in PRP are representative of the whole platelet population.

Nevertheless, the multiple wash procedure developed by Rand (1982) was used in the present study and allowed us to isolate virtually 100% of the platelets from small samples of whole blood collected from rabbits.

The gradients used for the separation of different density subpopulations of rabbit platelets were prepared from a high molecular weight arabinogalactan (Stractan II) obtained from the Western Larch.

This material was easily prepared. It is non toxic; platelets do not react with it; it does not cause the release reaction and platelets which have been exposed to this material aggregate normally upon stimulation with release inducing agents (Corash et al., 1974; Rand,

1982). Platelets separated on this medium also consistently reband to the same density; cross contamination between most dense platelet and least dense platelets in rebanding experiments is negligible (Rand, 1982). Furthermore, platelets which have been separated into density subpopulations on Stractan survive normally when recombined and infused into the circulation of rabbits (Rand, 1982).

In the present studies, the density distribution of rabbit platelets was determined before aortic catheters were introduced, or before animals received sham operations, and, at several times after these surgical procedures. Thus each animal served as its own control thereby minimizing the variation in density profiles that is often observed among normal animals (Cieslar et al., 1979; Rand et al., 1981). As well, the same batch of Stractan II was used throughout all these studies to eliminate another potential source of variation.

Results from these experiments demonstrated that during the first three days after the surgical procedure, there is a significant decrease in the proportion of least dense platelets in the circulation of animals with sham operations and in the circulation of animals with indwelling catheters (refer to Results Table 3.14). Platelets from normal rabbits that separate to the least dense fraction on Stractan density gradients are enriched in older platelets (Rand, 1982) that have undergone some degree of damage during their lifetime in the circulation (multiple hit hypothesis; Mustard et al., 1963a). The decrease in the proportion of least dense platelets during the early period after the surgery is consistent with the observation that these older platelets have a reduced capacity to withstand the additional insults induced by the surgical

procedure. The least dense subpopulation is therefore being cleared more rapidly from the circulation of both groups of rabbits.

The decrease in proportion of least dense platelets is also compatible with an increase in the number of young (dense) platelets entering into the circulation. This selective input into the dense fraction would result in an apparent decrease of the least dense fraction.

However, the increase in proportion of most dense platelets in the circulation was evident only in animals with sham operations. This group also had a significant increase in the number of circulating platelets by 24 hours following the surgical procedure. Thus, the increase in proportion of most dense platelets probably represents an increased rate of platelet production as well as release from sequestration sites in response to surgical trauma. These observations are in agreement with findings of other investigators demonstrating an increase in circulating platelet numbers and rate of platelet production in animals and humans following surgical procedures.

When platelets were harvested from rabbits 3 hours after they had received a sham operation and reinfused into normal animals, the platelets had a prolonged mean platelet survival time. These findings support the conclusion that the surgical procedure induced the release of dense, young platelets in the circulation.

In animals with aortic catheters, a similar increase in the proportion of dense platelets in the circulation does not occur during the first three days after the surgery. During this time, the circulating platelet counts are significantly decreased compared to

animals with sham operations. Extensive thrombus formation and platelet interaction on the freshly injured vessel wall during the first 24 hours after the insertion of the catheter, probably consumed a large proportion of the young dense platelets. This is not unexpected since several investigators (Hirsh et al., 1968; Blajchman et al., 1981) have demonstrated that younger platelets are functionally more active than older platelets.

It was recognized from the increased proportion of young platelets in the circulation of animals with sham operations, that the interpretation of studies of platelet density during the early period after the surgical procedure was complicated by platelet utilization, decreasing platelet counts and thrombus formation as a result of the surgical procedure.

Therefore, platelet density studies were performed at later times following the surgical procedure when platelet incorporation into thrombi had stabilized and an apparent 'steady-state' (in terms of platelet count and thrombus growth) was expected.

The density profiles of circulating rabbit platelets under these conditions demonstrated that there was an increased proportion of least dense platelets in the circulation by 7 days after the insertion of aortic catheters. Similar changes were not evident in the circulating platelets of rabbits with sham operations.

Two conclusions can be drawn from these results:

a) young platelets are preferentially consumed in rabbits with

aortic catheters leaving a population of older less dense platelets in the circulation.

or

b) young platelets which had exchanged in thrombi had their density altered by exposure to ADP, thrombin and plasmin and subsequently returned to the circulation as less dense platelets.

The first possibility was unlikely because platelets, harvested from animals 6 days after aortic catheters were inserted, survived significantly longer in normal rabbits than platelets from animals which had received sham operations 6 days previously.

Based on survival studies, the circulating platelets in rabbits with indwelling aortic catheters were enriched in young platelets. However, this young platelet population did not have the characteristic increased density; rather, these platelets were less dense. This evidence was compatible with the conclusion that some of the platelets turned over in thrombi. These platelets continued to circulate although their density was modified as a result of participation in thrombus formation.

Experimental work by Cieslar et al. (1979) supports our conclusion. Cieslar and his colleagues examined the effect of thrombin treatment on the bouyant density of rabbit platelets separated on Stractan density gradients. In their experiments, 5.6% of control, untreated rabbit platelets separated to the least dense fraction while 62% of the platelets were recovered in the most dense fraction. Following thrombin

treatment, 45.9% of the platelets were in the least dense fraction; 13.7% separated to the most dense fraction.

In our experiments, 40-46% of the total platelet population in animals with aortic catheters were recovered in the least dense fraction between 6 to 9 days after the aortic catheter was inserted. During the same time, the most dense fraction contained 10-13% of the total platelet population. It is apparent that the density profiles of platelets from rabbits with aortic catheters were almost identical to the density profiles of rabbit platelets after thrombin treatment.

The observations from the present study also demonstrated that the relationship between platelet bouyant density and platelet age becomes very complex when intra-vascular thrombosis is present.

In contrast to our observations in rabbits, Winocour et al. (1982) have shown that indwelling aortic catheters in rats can shorten platelet survival time without macroscopic thrombus formation. When platelets from rats with aortic catheters were examined for changes in density, it was observed that an increased number of platelets were present in the most dense fraction 4 days after the catheters has been inserted. As expected, platelets harvested from rats during the time when there was an increased proportion of most dense platelets, survived significantly longer in normal animals than platelets from animals which had received sham operations.

In rats, it was likely that platelets which had interacted with the injured vessel wall (where their density may have been altered by plasmin) were so extensively modified that they were rapidly cleared upon their return to the circulation. Because thrombi were not present in the

circulation of rats and platelets could not exchange with thrombi and return to the circulation, the young newly formed platelets were not exposed to high concentrations of agents which could alter their density.

In rabbits, when consumption of platelets increases at sites of continuous vessel injury and thrombus formation, platelet production increases to maintain equilibrium conditions. A population of young, dense platelets then enters into the circulation. Based on results from rat studies, those platelets which have turned over on the the vessel wall are rapidly cleared from the circulation. The presence of thrombi in the circulation of rabbits with aortic catheters affects the remaining young platelets by modifying their density.

Similar increases in the proportion of least dense platelets have been described in humans with thromboembolic vascular disease (van Oost et al., 1982a). Moreover, it has been suggested that density studies may identify those individuals with active intra-vascular thrombotic disease (Van Oost et al., 1982a, b).

Results from our studies in which we examined the survival of platelets harvested from rabbits with aortic catheters were in agreement with survival results in rats reported by Winocour and co-workers (1982). However, our findings were inconsistent with results from similar studies by Clagett and co-workers in dogs (1981) and Hanson et al. (1982) in baboons. Whereas we observed that circulating platelets from rabbits with aortic catheter in situ were enriched in younger platelets, Clagett demonstrated that shortened platelet survival persisted when platelets from dogs with arterial dacron grafts were cross

transfused into normal animals.

Hanson et al. (1982) reported that baboons with thrombogenic cannular shunts had increased platelet consumption suggesting the presence of a population of younger platelets in the circulation. When platelets were harvested from baboons with cannular shunts and reinfused into normal animals, the platelets survived normally.

The reasons for these conflicting results may be attributed to species differences and to differences in methodology. It was noted in Clagett's experiments that the recovery of labelled platelets from the circulation of recipient dogs was only 49%. Low recovery values are most often indicative of platelet damage during the isolation and labelling procedure and these platelets would be rapidly removed from the circulation of recipient animals. On the other hand, since dacron grafts and thrombogenic shunts are non-biological surfaces, the nature of platelet/graft/shunt interaction may be completely different and considerably more damaging to dog and baboon platelets than the platelet/vessel wall interaction occurring in our study.

In another study, Callow and associates (1980) showed that Dacron arterial prostheses in baboons accumulated ^{111}In-labelled platelets during the first three days after implantation. These data suggested that the interaction between platelet and dacron vascular grafts occur to a greater extent and for longer times than the platelet-vessel wall or platelet-thrombus interaction in our study. This may keep the platelets in close proximity to activated proteolytic enzymes generated on the foreign surface resulting in extensive modification of platelet membrane glycoproteins.

In Hanson's experiments (1982), consumptive shunts positioned in the renal arteries of baboons resulted in progressive occlusion of the renal microcirculation by platelet emboli. It may be that prolonged platelet survival could not be demonstrated in cross transfusion experiments since the young platelets may have been preferentially consumed during the continuous process of embolization on the shunts.

4.1.3 Platelet Consumption on the Surface of the Catheter

It was possible that the indwelling catheter itself affected platelet survival by initiating platelet adhesion and thrombus formation on the foreign surface. This may result in selective consumption of platelets in thrombi which subsequently embolized to the microcirculation as a result of arterial shear forces (Baumgartner, 1973; Harker et al., 1977a, b). Similar consumptive processes have been described by Hanson and his associates (1980) as a probable mechanism for shortened platelet survival in baboons with thrombogenic shunts in renal arteries.

Within the microcirculation anoxic conditions induced by the platelet embolus may reduce blood flow sufficiently to damage or stimulate endothelial cells. Plasminogen activator is released when endothelium is damaged (see reviews by Collen, 1980; Robbins, 1982) and in combination with reduced flow conditions, high local concentrations of proteolytic enzymes are likely to be achieved at sites where emboli undergo dissolution. As described previously, this may result in modification of platelet membranes resulting in premature clearance upon their return to the circulation.

Prior to the present study, other investigators had examined the

possibility that foreign materials initiate thrombus formation.

Jacobsson (1969) studied the problem of platelet consumption by various catheter materials including polyethylene tubing of the type used in this study. He measured the platelet count, erythrocyte count and hematocrit of citrated whole blood before and after a 30 minute in vitro incubation in different catheter materials to determine their relative thrombogenicity. Jacobsson noted that with all of the catheter materials studied, the platelet count was decreased significantly as a result of platelet adhesion to the surface. He also suggested that the observed increase in erythrocyte count and hematocrit of the incubated blood were a result of fluid absorption by the catheter material.

The conclusions drawn by Jacobsson may not be directly applicable to the present work since trace thrombin generation during in vitro conditions may have contributed to the observed thrombogenicity.

In a more recent study, Hanson and his associates (1980) evaluated the effects of numerous artificial surfaces on mean platelet survival time in an in vivo cannular shunt model in baboons. They found that non-polar surfaces with minimal degrees of hydration such as silastic or polyethylene (the catheter material used in the present study) were almost inert to circulating platelets. Short arterio-venous shunts composed of these materials had no significant effect on platelet consumption in baboons while identical shunts of hydrated polymers significantly shortened platelet survival time.

Meuleman et al. (1980) also examined the possibility that direct catheter-platelet contact may have been solely responsible for the shortened platelet survival times observed in rats with aortic

catheters. These investigators demonstrated that platelet survival and platelet count remained unaltered in rats which had their arterial blood passed through an extracorporeal polyethylene cannula of the same composition and similar surface area as that of the indwelling catheters. Meuleman concluded that catheter-platelet interaction was not responsible for the premature clearance of platelets from the circulation of rats.

Collaborative studies performed in our laboratory with Winocour et al. (1982), demonstrated that greater than 90% of radiolabelled platelets that had associated with the vessel wall containing the indwelling catheter, was present on the vessel wall; less than 10% became associated with the catheter.

In the present study, 80-95% of the ^{51}Cr-labelled platelets that accumulated on the vessel containing thrombus and catheter were actually associated with the thrombus. The remainder of the radioactivity became associated with the vessel wall and less than 5% of the total was found on the catheter. Moreover, upon removal from the fixed vessel, macroscopic thrombus formation was never observed on the surface of the catheter suggesting little platelet-catheter interaction.

The possibility of minimal platelet turnover on the catheter was not eliminated in this study. In view of the results from Hanson et al. (1980) and Meuleman and co-workers (1980) demonstrating polyethylene to be relatively inert to circulating platelets and confirming results from our laboratory, it is unlikely that significant numbers of platelets were removed from the circulation of rabbits as a consequence of platelet interaction with and loss from the polyethylene catheter.

4.2 Contribution of the Repeatedly Activated Vessel Wall to Shortened Platelet Survival

The concept proposed by Mustard and Murphy (1963a) that normal platelet destruction is a consequence of 'multiple hits' from their environment raised the possibility that platelet interaction with the vessel wall results in platelet damage which contributes to their eventual removal from the circulation.

Results from studies by George et al. (1976a, b; 1978a, b), Hanson and Harker (1981) and Rand (1982) as well as results from the present study demonstrating that ^{125}I-labelled glycoproteins were lost from the surface of platelets as they circulate, are compatible with the 'multiple hit' hypothesis. Additional evidence which support this concept was provided by George and his associates (1982) who demonstrated that normal human plasma contains microparticles composed of membrane glycoproteins characteristically found in platelets.

Baumgartner (1973) and Groves et al. (1979) have shown that after a single injury to the rabbit aorta by passage of a balloon catheter, an acute, significant platelet-vessel wall interaction takes place as platelets adhere and aggregate on the exposed subendothelial tissue. Baumgartner (1973) has shown that small mural thrombi form on the injured surface but these rapidly embolize to the microcirculation leaving a monolayer of adherent platelets on the vessel wall. Both Groves et al. (1979) and Baumgartner (1973) observed that the injured vessel wall quickly becomes relatively non-reactive to further platelet accumulation.

Platelets which initially adhered to the de-endothelialized surface

were gradually lost from the vessel wall without accumulation of significant numbers of new platelets so that by 4 days, the injured surface had few platelets associated with it (Groves et al. 1979).

It has not been established how platelets are removed from the vessel wall. As indicated previously, it may be that proteolytic enzymes such as plasmin, leucocyte elastase and enzymes released from activated platelets at sites of injury free adherent platelets by hydrolyzing major membrane glycoproteins (GP) that are involved in platelet adherence. Since GP-I is involved in the adherence of platelets to surfaces and plasmin has been shown to cleave GP-I and reduce platelet survival time, these platelets would be rapidly cleared from the circulation.

Since de-endothelialized rabbit aortae accumulated only a small proportion of the total platelets in the circulation during the short time the vessel remained reactive, it was not surprising that Groves and her colleagues (1979) were unable to demonstrate shortened platelet survival after a single balloon catheter injury.

At variance with Groves' findings, Harker (1978) reported that platelet survival was shortened in monkeys which had their aortae de-endothelialized by passage of a balloon catheter and that the extent of de-endothelialization correlated directly with the extent to which survival was shortened. Although the reasons for these discordant findings have not been resolved, it is possible that species differences and variation in methodology were responsible. Similarly, in an abstract, Kohler and Schaffer (1979) reported platelet survival was shortened to the same extent in rabbits with indwelling aortic catheters as in rabbits after a single balloon catheter injury. In the same study,

Kohler reported a value of 33.9 hours for the mean platelet survival time in normal rabbits. This value is less than half of that observed in our study and in studies by many other investigators (Groves et al., 1979; Buchanan et al., 1979; Blajchman et al., 1981; Rand, 1982). It appears likely that platelets prepared by Kohler were damaged during the isolation and labelling procedure and that their subsequent removal from the circulation of rabbits was a consequence of in vitro manipulation and not a result of the type of vessel injury induced in the animal.

In contrast to the findings that a single injury to the aortic endothelium may not affect platelet survival, repeated or continuous vessel injury is associated with shortened platelet survival. For example, Harker and his colleagues (1979) have shown that a continuous chemical injury to the vessel endothelium as a result of homocysteine infusion shortens platelet survival in baboons. However, platelet survival studies in humans with homocysteinemia have remained controversial.

Harker and his co-workers (1974), and Roulaud et al. (1982) have reported that platelet survival is shortened in humans with homocystinemia. Two other groups were unable to confirm these results and it was suggested by Hill-Zobel et al. (1982) that differences in methods used to analyse the survival data was the most reasonable explanation for the variable observations.

Ross and Harker (1976) observed that hypercholesterolemic diets could induce continuous endothelial cell injury in monkeys. They also noted that platelet survival was shortened in these animals. Similar experiments in monkeys by Armstrong et al. (1980) also inferred that

hypercholesterolemia injured or stimulated the endothelium and contributed to shortened platelet survival time.

In man, diets rich in animal fat are associated with reduced platelet survival and when these products are eliminated from the diet, platelet survival returns toward normal (Mustard and Murphy, 1962). Furthermore, diets enriched in eicosapentanoic acid prolongs shortened platelet survival time (Hay et al., 1982).

In most of these studies it was concluded or suggested that platelet survival was shortened as a result of selective consumption of platelets in microthrombi which formed on the continuously injured surface and subsequently embolized to the microcirculation.

Collaborative studies performed concurrently in our laboratory. (Winocour et al., 1982) have shown that repeated injury to the aortic endothelium of rats by indwelling catheters, can result in shortened platelet survival without macroscopic thrombus formation. We observed that the extent to which platelet survival was decreased was proportional to the length of the catheter inserted into the vessel; longer catheters caused a greater reduction in platelet survival than shorter catheters. Furthermore, the injured vessel wall remained active to circulating platelets for as long as 42 days after the catheter had been inserted.

In view of the fact that the catheter in rat aortae accumulated few radiolabelled platelets, it was concluded that platelet turnover on the repeatedly injured vessel wall resulting in proteolytic modification of platelet membranes was the major mechanism contributing to shortened platelet survival in rats.

The suggestion that proteolytic enzymes are important mediators of

platelet clearance is supported by several lines of evidence.

Winocour and associates (1983) pretreated rats with epsilon aminocaproic acid (EACA), an inhibitor of the fibrinolytic system, before insertion of aortic catheters. EACA treated rats had reduced whole blood fibrinolytic activity and extensive fibrin deposits on the vessel wall. Similar findings were not evident in untreated control animals with aortic catheters. Shortened platelet survival in animals with aortic catheters was significantly prolonged if the rats were pretreated with EACA. In addition, EACA pretreatment induced a significant increase in the number of ^{51}Cr-labelled platelets associated with the injured vessel wall suggesting a decreased rate of platelet loss from the surface.

Therefore, the available evidence indicates that an accelerated rate of platelet-vessel wall interaction through repeated or continuous stimulation or injury, may result in increased rates of platelet modification thereby contributing to shortened platelet survival time.

In the present study, it was possible that platelets continually interacted with the repeatedly injured vessel wall and that this could be a major mechanism of shortened platelet survival in rabbits with aortic catheters.

4.2.1 Reactivity of the Injured Vessel Wall Toward Platelets in the Circulation

Morphological evidence had shown that the vessel wall could accumulate platelets for a prolonged period. Furthermore, since platelet turnover on a repeatedly injured surface could contribute to shortened survival, it was important to determine to what extent the vessel wall

remained reactive to fresh platelets in the circulation.

Two similar experimental approaches were used to examine this question.

The first approach measured the association during a 24 hour period of ^{51}Cr-labelled platelets with the vessel wall that had catheters *in situ* for varying times. Results from these experiments demonstrated that in contrast to the loss of thrombus reactivity *in vivo*, the vessel wall which had been injured for 6 days to 14 days accumulated as many platelets from the circulation as a freshly (3 hour) injured surface.

Similar results were observed in collaborative studies in our laboratory (Winocour *et al*, 1982). In those studies, we reported that the vessel wall of rats with aortic catheters *in situ* for 6 days was as reactive to circulating platelets as a freshly injured vessel wall.

In the second approach, the association of ^{111}In-labelled platelets with the vessel wall during a 2 hour period, was determined in animals which had catheters *in situ* for various times.

^{111}Indium was used because of its high efficiency of labelling and high specific activity which would enable accurate quantitation of small numbers of platelets which may accumulate on the vessel wall during this short time period.

Prior to this study, the procedure for labelling platelets with ^{111}In had been well established. It had been demonstrated that ^{111}In does not readily elute from platelets nor was it reutilized.

In one control experiment in this study, the survival pattern of ^{111}In-labelled rabbit platelets was identical to the survival profile obtained with ^{51}Cr-labelled platelets.

These results were compatible with numerous other studies of human, dog and rabbit platelets which had demonstrated essentially identical platelet survival values using either isotope although higher recovery values were reported for ^{111}In-labelled platelets (Joist et al., 1983).

When we examined the reactivity of the vessel wall with ^{111}In-labelled platelets, the results were similar to experiments using ^{51}Cr-labelled platelets. At 3 days or 6 days after the insertion of the catheter, the vessel wall was as reactive to infused ^{111}In-labelled platelets as a vessel wall that had been injured 3 hours previously.

Since these experiments were performed, several reports have appeared in the literature which showed that rabbit platelets may incorporate ^{111}In into their dense bodies (Baker et al., 1982; Joist et al., 1983). Thrombin and collagen induced stimulation caused these platelets to release significant amounts of the label under experimental conditions of minimal lysis. This added a complicating factor to the interpretation of our results since the release of radiolabel from platelets adherent to the vessel wall would underestimate the extent of vessel wall reactivity. However, comparison of the vessel wall reactivity at different times after insertion of aortic catheters would still be valid as there is no evidence to suggest that adherent platelets undergo different extents of release in response to exposed subendothelial tissue.

Furthermore, Rand (1982) has demonstrated that when rabbit platelets are labelled with ^{111}In in the same media as that used in the present study, they released negligible amounts of ^{111}In in response to concentrations of thrombin which caused greater than 90% release of their

amine storage granule content.

Thus our observations confirmed that the vessel wall of rabbits with aortic catheters maintained the capacity to interact with circulating platelets.

4.2.2 Platelet Survival in Rabbits with Varying Extents of Vessel Injury

In our studies of the contribution of the thrombus to shortened platelet survival in rabbits with aortic catheters, we had established that platelet survival continued to be shortened past the time when the growth and reactivity of the thrombus had decreased significantly. During the same time the vessel wall continued to remain reactive.

If shortened platelet survival at these times was a consequence of platelet turnover on the continuously active vessel wall, then the degree in reduction of the platelet survival time should relate to the extent of vessel injury.

To examine this possibility, catheters of varying length (20 cm, 16 cm, 10 cm) were inserted into rabbit aortae. We had previously observed that thrombus formation occurred in rabbits with any length of indwelling catheter and we hypothesized that differences in catheter length would result in varying extents of injury with similar patterns of thrombus formation. This would allow us to measure and correlate the effect of vessel injury on platelet survival independent of thrombus effects.

Morphological evidence from Evans blue dye studies confirmed that the longest catheter (20 cm) which extended about 12 cm into the aorta, did injure a larger area of the vessel than either the 16 cm or the 10 cm catheters which extended 8 cm and 2 cm respectively, into the vessel.

These studies also established that all three lengths of catheter induced thrombus formation at the aortic trifurcation and in the area of the catheter tip. However, an unexpected finding was that the 10 cm catheter consistently resulted in formation of a thrombus that was about three times larger than thrombi which formed with the longer catheter.

When mean platelet survival time was measured in rabbits which had received an infusion of ^{51}Cr-labelled platelets followed 2 hours later by insertion of long or short catheters, platelet survival was shortened to the same extent in all animals irrespective of the extent of vessel injury.

Thus, if platelet survival measurements included the acute phase of thrombus formation, the incorporation of large numbers of platelets into developing thrombi had a significant effect on platelet survival during this time.

These results are at variance with earlier studies performed in our laboratory (Winocour et al., 1982) in which we reported that platelet survival in rabbits was unaffected by 10 cm aortic catheters whereas the longer (20 cm) catheters significantly shortened platelet survival time.

The reasons for these conflicting results may have been due to differences in animal size. The animals used in the original experiments were approximately twice as large as those in the present study. It is possible that differences in the relation between aortic diameter and catheter diameter may have influenced the extent of injury in large and small animals.

In addition, in the earlier study, due to differences in the region of catheter insertion, the 10 cm catheter extended about 4 cm into the

rabbit aorta and the thrombus that formed was similar in size with either length of catheter.

In the present work, the tip of the 10 cm catheter was located about 2.5 cm above the trifurcation. It was likely that the cumulative effects of catheter induced injury as well as disturbed flow close to the aortic trifurction contributed to the formation of significantly larger thrombi which incorporated sufficient numbers of platelets to shorten platelet survival time.

Since we had demonstrated that platelet incorporation into thrombi did influence platelet lifespan, it was surprising to find that significantly larger thrombi formed in animals with short catheters did not reduce platelet survival to a greater extent than the smaller thrombi which formed in animals with long aortic catheter. These results were compatible with any of the following conclusions:

a) in animals with short catheters (10 cm) platelet survival was shortened primarily through the effects of thrombus formation on platelet loss from the circulation

b) in animals with longer catheters (20 cm), the shortened platelet survival was the net balance between irreversible platelet accumulation into smaller thrombi and extensive platelet accumulation on an increased area of injured vessel wall.

It was apparent from these experiments that during the early period following the insertion of the catheter, the influence of the forming thrombus could have masked the effects of varying extents of

vessel injury.

Therefore, platelet survival time was measured in rabbits which had long (20 cm) or short (10 cm) catheters *in situ* for 3 days before survival studies were started. We had previously demonstrated that at this time, platelet incorporation into thrombi was significantly reduced compared to earlier time periods. Results from these studies demonstrated that once thrombus growth had ceased, platelet survival was not significantly affected by the presence of short (10 cm) catheters. In contrast, the longer catheters continued to shorten platelet survival time significantly.

Other experiments excluded platelet turnover on thrombi as a major contributing factor since thrombi formed with either short or long catheters retained similar capacities to interact with platelets; but, survival was shortened only in animals with long catheters. Thus the contribution of the thrombus to shortened platelet survival at this time is probably less than that of the vessel wall.

However, platelet survival time in rabbits with short catheters tended to be shorter (60.5 ± 4.4 hours, n=5) than that observed in control animals (69.3 ± 2.5 hours, n=5). These results were in agreement with our previous conclusion that platelet turnover in thrombi does contribute to shortened platelet survival time.

Thus, evidence from the latter studies showed that shortened platelet survival in rabbits with aortic catheters was primarily related to the extent of vessel injury and not to thrombus reactivity. Similar conclusions were reached by Winocour *et al.* (1982) in which they demonstrated shortened platelet survival in rats with indwelling aortic

catheters under experimental conditions where thrombus formation did not occur.

Therefore, it was reasonable to conclude that platelet turnover on a continuously activated vessel wall can be a major mechanism causing shortened platelet survival.

4.3 Summary and Conclusions

Platelet survival is shortened under experimental conditions in which the vessel wall is repeatedly or continuously injured and thrombus formation occurs.

If thrombus formation is extensive, sufficient numbers of platelets are removed from the circulation during the acute phase of thrombus growth to influence platelet survival time significantly. When maximum thrombus formation has been completed, platelet incorporation into thrombi decreases significantly, nevertheless, the thrombus remains reactive to circulating platelets for at least 2 weeks after being formed.

Some of the platelets that initially become incorporated into thrombi undergo lysis. Others escape as whole platelets as the thrombus undergoes dissolution and reformation to be replaced by fresh platelets from the circulation thereby contributing to the maintenance of the constant thrombus weight.

A proportion of the platelets which had participated in thrombus formation are likely to be modified sufficiently such that they are cleared from the circulation thus contributing to shortened platelet

survival. Some of the platelets which are freed from the thrombus continue to circulate in a modified form.

In contrast to the loss of thrombus reactivity, the repeatedly injured vessel wall does not lose its ability to interact with platelets in the circulation. Once the acute phase of thrombus formation has ceased, shortened platelet survival is directly related to the extent of vessel injury. Thus, the experimental findings support the conclusion that repeated vessel injury can itself shorten platelet survival irrespective of thrombus formation.

These conclusions are compatible with the observations from numerous drug studies (see review by Genton, 1976) demonstrating that shortened platelet survival can be normalized by compounds such as dipyridamole which inhibit platelet vessel wall interactions. In contrast, drugs such as aspirin which inhibit platelet aggregation but do not inhibit platelet adhesion to the vessel wall, appear to have little effect on shortened platelet survival. These observations are consistent with the present study that demonstrates platelet interaction with a continuously activated vessel wall contributes to a much greater extent to shortened platelet survival than platelet participation in thrombus formation particularly after initial thrombus growth.

These conclusions are also in agreement with the observations from many human studies showing platelet survival is shortened in conditions such as hypertension, hypercholesterolemia and diabetes which may be characterized by continuous injury to the endothelium.

It is apparent from the present work in which shortened platelet survival was primarily related to the extent of vessel injury that

platelet survival studies may identify those individuals at risk for developing thromboembolic vascular disease.

4.4 Unresolved Questions

Throughout the course of this study, a number of interesting questions arose which should be resolved to enhance our understanding of the mechanisms of shortened platelet survival in thromboembolic disease.

The extent of fibrinogen/fibrin incorporation into thrombi and its contribution to the weight maintenance of the thrombus *in vivo* has not been investigated thoroughly. Indirect evidence from our morphological studies and from the work of others (Jørgensen *et al.*, 1967) suggest that fibrin accumulation into thrombi does not change significantly during the first four weeks after thrombus formation. It is not clear whether this is primarily a consequence of fibrin turnover in the thrombus. Studies designed to examine the incorporation of radiolabelled rabbit fibrinogen into forming thrombi and into pre-existing thrombi under experimental conditions described in this study may provide definitive answers to this question.

Another area of particular interest to the present study is that of examining the surface characteristics of the platelet membranes obtained from animals with aortic catheters. Other studies have established that the removal of sialic acid and sialopeptides from platelet membranes by proteolytic enzymes, shorten their survival *in vivo* (Greenberg *et al.*, 1979). Studies of membrane glycoproteins and sialic acid content of platelets which have interacted *in vivo* with the thrombus or the injured

vessel wall would establish the importance of platelet membrane modification as a means of platelet clearance in thromboembolic disease.

The factors in the vessel wall which influence platelet survival have not been identified. Since the vessel wall must be continuously stimulated to shorten platelet survival, the available evidence suggests that proteolytic enzymes are responsible for the platelet modifications which result in their clearance from the circulation. Thus, there is a need for studies designed to examine the effects of proteolytic inhibitors on platelet survival under experimental conditions similar to those of the present study.

It has also been suggested that platelets are removed from the circulation as a result of IgG binding to exposed cryptic antigens which are normally masked by sialic acid. Studies designed to examine the role of immunoglobulin association with platelet membranes under conditions of repeated vessel injury and thrombus formation would provide important answers to these questions.

Finally, the importance of activated leucocytes in platelet survival studies has never been determined. Morphological evidence from this study demonstrating large numbers of leucocytes at sites of vessel injury and thoughout thrombi suggest that leucocytes may play a significant role in platelet modification and subsequent platelet clearance from the circulation.

CHAPTER 5

REFERENCES

5.0 References

Aas, K.A. and Gardner, F.H. Survival of blood platelets labeled with chromium (1958). J. Clin. Invest. 37: 1257.

Abilgaard, C.F. and Simone, J.V. (1967). Thrombopoiesis. Semin. Hematol. 4:424.

Abrahamsen, A.F. (1970). Survival of ^{51}Cr-labelled autologous and isologous platelets as differential diagnostic aid in thrombocytopenic states. Scand. J. Haematol. 7:525.

Abrahamsen, A.F. (1968). Platelet survival studies in man. Scand. J. Haematol. (Suppl. 3): 7.

Abramowitz, J., Stracher, A. and Detwiler, T.C. (1974). Proteolysis of myosin during platelet storage. J. Clin. Invest. 53: 1493.

Adelson, E., Kaufman, R.M., Berdeguez, C., Lear, A.A. and Rheingold, J.J. (1965). Platelet tagging with tritium labeled diisopropylfluorophosphate. Blood 26: 744.

Adelson, E., Rheingold, J.J., Parker, O., Buenaventura, A. and Crosby, W.H. (1961). Platelet and fibrinogen survival in normal and abnormal states of coagulation. J. Haematol. XVII: 267.

Akkerman, J.W.N., Van Brederode, W., Gorter, G., Zegus, B.J.M, and Kuis, W. The Wiskott-Aldrich syndrome: studies on a possible defect in mitochondrial ATP resynthesis in platelets. Br. J. Haematol. 51: 561.

Aminoff, D., Vorder Bruegge, W.F., Bell, W.C., Sarpolis, K. and Williams, R. (1977). Role of sialic acid in survival of erythrocytes in the circulation: interaction of neuraminidase-treated and untreated erythrocytes with spleen and liver at the cellular level. Proc. Natl. Acad. Sci. 74: 1521.

Amorosi, E., Garg, S.K. and Karpatkin, S. (1971). Heterogeneity of human platelets. IV. Identification of a young platelet population with [^{75}Se]selenomethionine. Br. J. Haematol. 21: 227.

Ardlie, N.G., Packham, M.A. and Mustard, J.F. (1970). Adenosine diphosphate-induced platelet aggregation in suspensions of washed rabbit platelets. Br. J. Haematol. 19: 7.

Armstrong, M.L., Peterson, R.E., Hoak, J.C., Megan, M.B., Cheng, F.H. and Clarke, W.R. (1980). Arterial platelet accumulation in experimental Hypercholesterolemia. Atherosclerosis 36: 89.

Aster, R.H. (1972). Platelet sequestration studies in man. Br. J. Haematol. 22: 259.

Aster, R.H. and Jandl, J.H. (1964a). Platelet sequestration in man. I. Methods. J. Clin. Invest. 41: 843.

Aster, R.H. and Jandl, J.H. (1964b). Platelet sequestration in man. II. Immunological and clinical studies. J. Clin. Invest. 43: 856.

Baker, J.R.J., Butter, K.D., Eakins, M.N., Pay, G.F. and White, M.A. (1982). Subcellular localization of ^{111}In in human and rabbit platelets. Blood 59: 351.

Baker, J.R.J., Bullock, G.R., Crawford, N. and Taylor, D.G. (1977). Localization in platelets of sodium ^{51}Cr-chromate, ^{125}I-antibody to whole membrane, and ^{3}H-diisopropylfluorophosphate using electron microscope autoradiography. Am. J. Pathol. 88: 277.

Baldini, M.G. and Myers, T.J. (1980). Uses and limitations of platelet survival time measurements. Haematologica 65: 689.

Baldini, M. (1968). Platelet circulation and survival. Bibl. Haematol. 29(1); 107 (Karger, Basel/New York).

Barnes, M.J. (1981). Collagen polymorphism in relation to the role of collagen induced platelet aggregation in haemostasis and thrombosis. Connective Tissue Research: Chemistry, Biology and Physiology (Alan R. Liss, Inc. New York). p. 163.

Baumgartner, H.R., Tschopp, T.B., Meyer, D. (1980). Shear rated dependent inhibition of platelet adhesion and aggregation on collagenous surfaces by antibodies to human factor VIII/von Willebrand factor. Br. J. Haematol. 44: 127.

Baumgartner, H.R. (1973). The role of blood flow in platelet adhesion, fibrin deposition and formation of mural thrombi. Microvasc. Res. 5: 167.

Becker, C.G., Dubin, T., Wiedemann, H.P. (1976). Hypersensitivity to tobacco antigen. Proc. Natl. Acad. Sci. U.S.A. 73: 1712.

Benditt, E.P. (1977). Implications of the monoclonal character of human atherosclerotic lesions. Am. J. Pathol. 86: 693.

Bensoussan, D., Levy-Toledano, S., Passa, S., Caen, J., and Canivet, J. (1975). Platelet hyperaggregation and increased plasma level of von Willebrand factor in diabetics with retinopathy. Diabetologia 11: 307.

Bergstrom, K., Blomback, B. and Kleen, G. (1960). Studies on the plasma fibrinolytic activity in a case of liver cirrhosis. Acta Med. Scand. 168: 291.

Bern, M.B. (1978). Platelet functions in diabetes mellitus. Diabetes 27: 342.

Bessler, H., Notti, E. and Djaldetti, M. (1981). The effect of partial splenectomy on platelet production in mice. Thromb. Haemost. 46: 602.

Bevers, E.M., Comfurius, P., Van Rijn, J.L.M.L., Hemker, H.C. and Zwaal, R.F.A. (1982). Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. Eur. J. Biochem. 122: 429.

Bird, G.W.G., Shinton, N.K. and Wingham, J. (1971). Persistent mixed-field polyagglutination. Br. J. Haematol. 21: 443.

Bithell, T.C., Athens, J.W., Cartwright, G.E. and Wintrobe, M.M. (1967). Radioactive diisopropyl fluorophosphate as a platelet label: an evaluation of *in vitro* and *in vivo* technics. Blood 29: 354.

Bizzozero, J. (1882). On a new blood particle and its role in thrombosis and blood coagulation (translated by Beck, E.A., 1982). Virchow's Arch. Pathol. Anat. Physiol. Klin. Med. 90: 261.

Bjornson, J. and Aursnes, I. (1977). The haemostatic effect of ^{51}Cr-labelled blood platelets. An experimental study in the rabbit. Scand. J. Haematol. 18: 326.

Bjornson, J. (1974). The effect of the ^{51}Cr-labelling procedure on platelet aggregability. Scand. J. Haematol. 13: 252.

Blajchman, M.A., Senyi, A.F., Hirsh, J. and Genton, E. (1981). Hemostatic function, survival, and membrane glycoprotein changes in young versus old rabbit platelets. J. Clin. Invest. 68: 1289.

Bolin, R.B. (1981). Changes in platelet membranes and bouyant density with storage of platelet concentrates at 22°C. Vox. Sang. 40 (Suppl. 1): 117.

Bolin, R.B., Medina, F. and Chesney, B.A. (1981). Glycoprotein changes in fresh vs room temperature stored platelets and their bouyant density cohorts. Lab. Clin. Med. 98: 500.

Boneu, B., Vigoni, F., Boneu, A., Caranobe, C. and Sie, P. (1982). Further studies on the relationship between platelet buoyant density and platelet age. Am. J. Haematol. 13: 239.

Booyse, F.M., Osikowicz, G. and Quarfoot, A.J. (1981). Effects of chronic oral consumption of nicotine on the rabbit aortic endothelium. Am. J. Pathol. 2: 229.

Branehog, I., Kutti, J. and Weinfeld, A. (1974). Platelet survival and platelet production in idiopathic thrombocytopenic purpura. Br. J. Haematol. 27: 127.

Branehog, I., Weinfeld, A. and Roos, B. (1973). The exchangeable splenic platelet pool studied with epinephrine infusion in idiopathic thrombocytopenic purpura and in patients with splenomegaly. Br. J. Haematol. 25: 239.

Bretscher, M.S. and Raff, M.C. (1975). Mammalian plasma membranes. Nature 258: 43.

Brinkhous, K.M., Sultzer, D.L., Reddick, R.L. and Griggs, T.R. (1980). Elevated plasma von Willebrand factor (vWF) levels as an index of acute endothelial injury: use of hypotonic injury model in rats. Fed. Proc. 39: 630.

Bull, B.S., Schneiderman, M.A. and Brecher, G. (1965). Platelet counts with the coulter counter. Am. J. Clin. Path. 44: 678.

Buchanan, M.R., Carter, C.J. and Hirsh, J. (1979). Decreased platelet thrombogenecity in association with increased platelet turnover and vascular damage. Blood 54: 1369.

Busch, C_{51} and Olson, P.S. (1973). Density distribution of ^{51}Cr-labelled platelets within the circulating dog platelet population. Thromb. Res. 3: 1.

Caen, J.P., Nurden, A.T., Jeanneau, C., Michel, H., Tobelem, G. and Levy-Toledano, S. (1976). Bernard-Soulier syndrome: a new platelet glycoprotein abnormality. Its relationship with platelet adhesion to subendothelium and with the Factor VIII von Willebrand protein. J. Lab. Clin. Med. 87: 586.

Callow, A.D., Ledig, C.B., O'donnell T.F., Kelly, J.J., Rosenthal, D., Korwin, S., Hotte, C., Kahn, P.C., Vecchione, J.J., and Valeri, C.R. (1980). A primate model for the study of the interaction of "In-labeled baboon platelets with dacron arterial prostheses. Ann. Surg. 191: 362.

Carroll, R.C., Butler, R.G., Morris, P.A. and Gerrard, J.M. (1982). Separable assembly of platelet pseudopodal and contractile cytoskeletons. Cell 30: 385.

Catalano, P., Murphy, S. and Smith, J.B. (1979). Does recovery from aspirin measure platelet survival? Thromb. Haemost. 42: 329.

Charmatz, A. and Karpatkin, S. (1974). Heterogeneity of rabbit platelets. I. Employment of an albumin density gradient for separation of a young platelet population identified with Se^{75}-Selenomethionine. Thromb. Diath. Haemorrh. 31: 485.

Cheng, C.M. and Hawiger, J. (1979). Affinity isolation and characterization of immunoglobulin G Fc fragment-binding glycoprotein from human blood platelets. J. Biol. Chem. 254: 2165.

Chesney, C.M., Harper, E. and Colman, R.W. (1974). Human platelet collagenase. J. Clin. Invest. 53: 1647.

Chiang, T.M., Mainardi, C.L., Seyer, J.M. and Kang, A.H. (1980). Collagen-platelet interaction. Type V (A-B) collagen induces platelet aggregation. J. Lab. Clin. Med. 95: 99.

Cieslar, P., Greenberg, J.P., Rand, M.L., Packham, M.A., Kinlough-Rathbone, R.L. and Mustard, J.F. (1979). Separation of thrombin-treated platelets from normal platelets by density-gradient centrifugation. Blood 53: 867.

Clagett, G.P., Russo, M. and Hufnagel, H. (1981). Platelet changes after placement of aortic prosthesis in dogs. I. Biochemical and functional alterations. J. Lab. Clin. Med. 97: 345.

Clemetson, K.J., Capitanio, A. and Luscher, E.F. (1979). High resolution two-dimensional gel electrophoresis of the proteins and glycoproteins of human blood platelets and platelet membranes. Biochim. Biophys. Acta 553: 11.

Cohen, P., Cooley, M.H. and Gardner, F.H. (1965). The use of selenomethionine (Se^{75}) as a label for canine and human platelets. J. Clin. Invest. 44: 1036.

Coleman, R.E., Harwig, S.S.L., Harwig, J.F., Siegel, B.A. and Welch, M.J. (1975). Fibrinogen uptake by thrombi: effect of thrombus age. J. Nucl. Med. 16: 370.

Collen, D. (1980). On the regulation and control of fibinolysis. Thromb. Haemost. 43: 77.

Cooney, D.P., Smith, B.A. and Fowley, D.E. (1968). The use of ^{32}Di-isopropylfluorophosphate (^{32}DFP) as a platelet label: evidence for reutilization of this isotope in man. Blood 31: 791.

Cooper, G.W. (1970). The regulation of thrombopoiesis. Regulation of Hematopoiesis, Vol. 11. White cell and platelet production Ed.: Gordon, A.S. (Appleton-Century-Crofts, New York). p. 1611.

Corash, L. and Shafer, B. (1982). Use of asplenic rabbits to demonstrate that platelet age and density are related. Blood 60: 166.

Corash, L., Shafer, B. and Perlow, M. (1978). Heterogeneity of human whole blood platelet subpopulations. II. Use of a subhuman primate model to analyze the relationship between density and platelet age. Blood 52: 726.

Corash, L.M., Piomelli, S., Chen, H.C., Seaman, C. and Gross, E. (1974). Separation of erythrocytes according to age on a simplified density gradient. J. Lab. Clin. Med. 84: 147.

Daimon, T. and Gotoh, Y. (1982). Cytochemical evidence of the origin of the dense tubular system in the mouse platelet. Histochem. 76: 189.

D'Amore, P. (1978). Platelet-endothelial interaction and the maintenance of the microvasculature. Microvasc. Res. 15: 137.

Danielli, J.F. (1940). Capillary permeability and oedema in the perfused frog. J. Physiol. 98: 109.

da Prada, M., Lorez, H.P., and Richards, J.G. (1982). Platelet granules. Eds.: Poisner and Trifaro. The secretory granule. (Elsevier Biomed. Press). p. 279.

da Prada, M., Richards, J.G.and Kettler, R. (1981). Amine storage organelles in platelets. In: Platelets in Biology and Pathology - 2. Ed.: Gordon, J.L. (Elsevier/North-Holland Biomedical Press). p. 108.

Dassin, E. and Najean, Y. (1979). The use of ^{75}Se-methionine as a tracer of thrombocytopoiesis. 1. In vivo incorporation of the tracer into platelet proteins: a biochemical study. Acta Haematol. 61: 61.

Dassin, E., Balitrand, N. and Najean, Y. (1976). An analysis of the effect of random and ageing mechanisms on the survival of platelets. Biomed. 25: 23.

Davey, M.G. and Lander, H. (1964). The behaviour of infused human platelets during the first twenty-four hours after infusion. Br. J. Haematol. 10: 94.

Davis, H.H., Siegel, B.A., Sherman, L.A., Heaton, W.A., Naidich, T.P., Joist, H.J. and Welch, M.J. (1980). Scintigraphic detection of carotid atherosclerosis with indium-111-labeled autologous platelets. Circulation. 61: 982.

Davis, H.H., Siegel, B.A., Joist, J.H., Heaton, W.A., Mathias, C.V., Sherman, L.A. and Welch, M.V. (1978). Scintigraphic detection of atherosclerotic lesions and venous thrombi in man by indium-111-labelled autologous platelets. Lancet 1: 1185.

de Gabriele, G. and Penington, D.G. (1967a). Physiology of the regulation of platelet production. Br. J. Haematol. 13: 202.

de Gabriele, G. and Penington, D.G. (1967b). Regulation of platelet production: thrombopoietin. Br. J. Haematol. 13: 210.

Deuel, T.F., Senior, R.M., Chang, D., Griffin, G.L., Heinrikson, R.L. and Kaiser, E.T. (1981). Platelet factor 4 is chemotactic for neutrophils and monocytes. Proc. Natl. Acad. Sci. U.S.A. 78: 4584.

Dixon, R.H. and Rosse, W.F. (1975). Platelet antibody in autoimmune thrombocytopenia. Br. J. Haematol. 31: 129.

Donne, A. (1842). De L'origine des globules du sang, de leur mode de formation et de leur fin. Acad. Sci. Paris 14: 336.

Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Devare, S.G., Robbins, K.C., Aaronson, S.A. and Antoniades, H.N. (1983). Simian sarcoma onc Gene, v-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science 221: 275.

Drury, R.A.B. and Wallington, E.A. (1967). Carleton's Histological Technique. Oxford University Press, Toronto.

Duke, W.W. (1911). Rate of regeneration of blood platelets. J. Exp. Med. 14: 265.

Ebbe, S. (1970). Megakaryopoiesis. Regulation of Haematopoiesis. Vol. II. White cell and platelet production. Ed.: Gordon, A.S. (Appleton-Century-Crofts, New York). p. 1587.

Ebbe, S., Baldini, M. and Donovan, J. (1965). Comparative studies of platelet survival by different methods in the rabbit. Blood 25: 548.

Fedorko, M.E. and Lichtman, M.A. (1982). Megakaryocyte structure, maturation and ecology. In: Hemostasis and Thrombosis: Basic Principles and Clinical Practice. Ed.: Coleman, R.W., Hirsh, J., Marder, V.J. and Salzman, E.W. (J.B. Lippencott Co., Philadelphia, Toronto). p. 210.

Firket, J. (1922). Recherches sur la regeneration des plaquettes dans l'intoxication par la saponine et apres defibrination du sang. Arch. de biol. Liege & Paris. 32: 539.

Friedman, R.J., Stemerman, M.B., Wenz, B., Moore, S., Gauldie, J., Gent, M., Tiell, M.L. and Spaet, T.H. (1977). The effect of thrombocytopenia on experimental arteriosclerotic lesion formation in rabbits. J. Clin. Invest. 60: 1191.

Friedman, R.J., Moore, S. and Singal, D.P. (1975). Repeated endothelial injury and induction of atherosclerosis in normolipemic rabbits by human serum. Lab. Invest. 30: 404.

Fuster, V., Chesebro, J.H., Frye, R.L. and Elveback, L.R. (1981). Platelet survival and the development of coronary artery disease in the young adult: effects of cigarette smoking, strong family history and medical therapy. Circulation 63: 546.

Gardner, F.H. and Cohen, P. (1966). Platelet life span. Transfusion 6: 23.

Gear, A.R.L. (1981). Preaggregation reactions of platelets. Blood 58: 477.

Geissinger, H.D., Mustard, J.F., Riddell, P.E., Robinson, G.A. and Rowsell, H.C. (1963). The fate of di-isopropylfluorophosphonate-^{32}P and sulphur-^{35}S labels on platelets during blood coagulation. Br. J. Haematol. 9: 77.

Genton, E. (1976). Platelet Survival Measurement - Clinical Results. In: Platelet Function Testing. Eds.: Day, H.J., Holmsen, H., Zucker, M.B. DHEW Publication No. (NIH) 78-1087. U.S. Dept. Health, Education, Welfare Public Health Service.

George, J.N., Thoi, L.L., McManus, L.M. and Reimann, T.A. (1982). Isolation of human platelet membrane microparticles from plasma and serum. Blood 60: 834.

George, J.N., Thoi, L.L. and Morgan, R.K. (1981). Quantitative analysis of platelet membrane glycoproteins: effect of platelet washing procedures and isolation of platelet density subpopulations. Thromb. Res. 23: 69.

George, J.N., Lewis, P.C. and Morgan, R.K. (1978a). Studies on platelet plasma membranes. III. Membrane glycoprotein loss from circulating platelets in rabbits: inhibition by aspirin - dipyridamole and acceleration by thrombin. J. Lab. Clin. Med. 91: 301.

George, J.N., Mogan, R.K. and Lewis, P.C. (1978b). Studies on platelet plasma membranes. IV. Quantitative analysis of platelet membrane glycoproteins by (^{125}I)-diazotized diiodosulfanilic acid labeling and SDS-polyacrylamide gel electrophoresis. J. Lab. Clin. Med. 430: 1978b.

George, J.N. (1976c). Platelet membrane glycoproteins: alterations during storage of human platelet concentrates. Thromb. Res. 8: 719.

George, J.N., Potterf, R.D., Lewis, P.C. and Sears, D.A. (1976a). Studies on platelet plasma membranes. I. Characterization of surface proteins of human platelets labeled with diazotized (^{125}I)-diiodosulfanilic acid. J. Lab. Clin. Med. 88: 232.

George, J.N., Lewis, P.C. and Sears, D.A. (1976b). Studies on platelet plasma membranes. II. Characterization of surface proteins of rabbit platelets in vitro and during circulation in vivo using diazotized (^{125}I)-diiodosulfanilic acid as a label. J. Lab. Clin. Med. 88: 247.

Gerrard, J.M., Peterson, D.A. and White, J.G. (1981). Calcium mobilization. In: Platelets in biology and pathology. 2. Ed. Gordon, J.L. (Elsevier/North-Holland Biomedical Press, New York). p. 407.

Gibson, J.B., Carson, N.A.J. and Neill, D.W. (1964). Pathological findings in homocystinuria. J. Clin. Pathol. 17: 427.

Gimbrone, M.A., Aster, R.H., Cotran, R.S., Corkery, J., Jandl, J.H. and Folkman, J. (1969). Preservation of vascular integrity in organs perfused in vitro with a platelet-rich medium. Nature 222: 33.

Ginsburg, A.D. and Aster, R.H. (1972). Changes associated with platelet aging. Thromb. Diath. Haemorrh. 27: 407.

Glöckner, W.M., Kaulen, H.D. and Uhlenbruck, G. (1978). Immunochemical detection of the Thomsen-Friedenreich antigen (T-antigen) on platelet plasma membranes. Thromb. Haemost 39: 186.

Greenberg, J.P., Packham, M.A., Guccione, M.A., Rand, M.L., Reimers, H.-J. and Mustard, J.F. (1979). Survival of rabbit platelets treated in vitro with chymotrypsin, plasmin, trypsin or

neuraminidase. Blood 53: 916.

Greenberg, J.P., Rand, M.L. and Packham, M.A. (1977). Relation of platelet age to platelet sialic acid. Fed. Proc. 36: 453.

Greenberg, J.P., Packham, M.A., Cazenave, J.-P., Reimers, H.-J. and Mustard, J.F. (1975). Effects on platelet function of removal of platelet sialic acid by neuraminidase. Lab. Invest. 32: 476.

Grottum, K.A. and Solum, N.O. (1969). Congenital thrombocytopenia with giant platelets: a defect in the platelet membrane. Br. J. Haematol. 16: 277.

Groves, H.M., Kinlough-Rathbone, R.L., Richardson, M., Moore, S. and Mustard, J.F. (1979). Platelet interaction with damaged rabbit aorta. Lab. Invest. 40: 194.

Hagen, I. and Solum, N.O. (1978). Further studies on the protein composition and surface structure of normal platelets and platelets from patients with Glanzmann's thrombasthenia and Bernard-Soulier Syndrome. Thromb. Res. 13: 845.

Handin, R.I. and Stossel, T.P. (1974). Phagocytosis of antibody-coated platelets by human granulocytes. N. Eng. J. Med. 290: 989.

Handin, R.I., Pressens, W.F. and Moloney, W.C. (1973). Stimulation of nonimmunized lymphocytes by platelet-antibody complexes in idiopathic thrombocytopenic purpura. N. Eng. J. Med. 289: 714.

Hanson, S.R. and Harker, L.A. (1981). Survival of baboon platelets labeled with diazotized (125-I)-Iodosulfanilic acid: no effect of drugs that modify platelet behaviour. Thromb. Res. 23: 133.

Hanson, S.R., Harker, L.A., Ratner, B.D. and Hoffman, A.S. (1980). In vivo evaluation of artificial surfaces with a nonhuman primate model of arterial thrombosis. J. Lab. Clin. Med. 95: 289.

Harker, L.A. and Ritchie, J.L. (1980). The role of platelets in acute vascular events. Circulation 62 (Suppl. V): 13.

Harker, L.A. and Hanson, S.R. (1979a). Experimental arterial thromboembolism in baboons. Mechanism, quantitation and pharmacologic prevention. J. Clin. Invest. 64: 559.

Harker, L.A. and Hazzard, W. (1979b). Platelet kinetic studies in patients with hyperlipoproteinemia: effects of clofibrate therapy. Circulation 60: 492.

Harker, L.A., Wall, R.T., Haslam, J.M. and Ross, R. (1978a). Sulfinpyrazone prevention of homocysteine-induced endothelial cell injury and arteriosclerosis. Clin. Res. 26: 554.

Harker, L.A. (1978b). Platelet survival time: its measurement and use. In: Progress in Hemostasis and Thrombosis. Vol. 4,. Ed.: Spaet, T.H. (Grune and Stratton, New York). p. 321.

Harker, L.A., Slichter, S.J. and Sauvage, L.R. (1977a). Platelet consumption by arterial prostheses: the effects of endothelialization and pharmacologic inhibition of platelet function. Ann. Surgery 186: 594.

Harker, L.A., Hanson, S.R. and Hoffman, A.S. (1977b). Platelet kinetic evaluation of prosthetic material in vivo. Ann. N.Y. Acad. Sci. 283: 317.

Harker, L.A., Ross, R., Slichter, S.J. and Scott, C.R. (1976). Homocystine-induced arteriosclerosis. The role of endothelial cell injury and platelet response in its genesis. J. Clin. Invest. 58: 731.

Harker, L.A., Slichter, S.J., Scott, R.S. and Ross, R. (1974). Homocystinemia. Vascular injury and arterial thrombosis. N. Eng. J. Med. 291: 537.

Harker, L.A. and Slichter, S.J. (1972). Platelet and fibrinogen consumption in man. N. Eng. J. Med. 287: 999.

Harker, L.A. and Slichter, S.J. (1970). Studies of platelet and fibrinogen kinetics in patients with prosthetic heart valves. N. Eng. J. Med. 283: 1302.

Harker, L.A. and Finch, C.A. (1969). Thrombokinetics in man. J. Clin. Invest. 48: 963.

Harker, L.A. (1968). Kinetics of thrombopoiesis. J. Clin. Invest. 47: 458.

Harrington, W.J., Minnick, V., Hollingsworth, J.W. and Moore, C.V. (1951). Demonstration of a thrombocytopenic factor in the blood of patients with thrombocytopenic purpura. J. Lab. Clin. Med. 38: 1.

Hauch, T.W. and Roose, W.F. (1977). Platelet-bound complement (C3) in immune thrombocytopenia. Blood 50: 1129.

Haut, M.J., Cowan, D.H. and Harris, J.W. (1973). Platelet function and survival in sickle cell disease. J. Lab. Clin. Med. 82: 44.

Hay, C.R.M., Durber, A.P. and Saynor, R. (1982). Effect of fish oil on platelet kinetics in patients with ischaemic heart disease. Lancet, June 5, 1982: 1259.

Heaton, W.A., Davis, H.H., Welch, M.J., Mathias, C.J., Joist, J.H., Sherman, L.A. and Siegel, B.A. (1979). Indium-III: A new radionuclide label for studying human platelet kinetics. Brit. J. Haematol. 42: 613.

Hessler, J.R., Robertson, A.L. and Chisolm, G.M. (1979). LDL-induced cytotoxicity and its inhibition by LDL in human vascular smooth muscle and endothelial cells in culture. Atherosclerosis 32: 213.

Heynos, A., du, P., Lotter, M.G., Badenhorst, P.W., de Kock, F., Pieters, H., Herbst, C., van Reenen, O.R., Kotze, H., Minnaar, P.C. (1982). Kinetics and sites of destruction of ^{111}indium-oxine-labeled platelets in idiopathic thrombocytopenic purpura. Am. J. Haemat. 12: 167.

Heyns, A., du, P., Lotter, M.G., Badenhorst, P.N., van Reenen, O.R., Pieters, H., Minnaar, P.C. and Retief, F.P. (1980). Kinetics, destruction and sites of distribution of ^{111}indium-labelled human platelets. Br. J. Haematol. 44: 269.

Heyns, A., du, P., Badenhorst, P.N., Pieters, H., Lotter, M.G., Minnaar, P.C., Duyvene de Wit, L.J., van Reenen, O.R. and Retief, F.P. (1979). Preparation of a viable population of indium-111-labelled human blood platelets. Thromb. Haemost. 42: 1473.

Hill-Zobel, R.L., Pyeritz, R.E., Scheffel, U., Malpica, O., Engin, S., Camargo, E.E., Abbott, M., Guilarte, T.R., Hill, J., McIntyre, P.A., Murphy, E.A. and Tsan, M.F. (1982). Kinetics and distribution of ^{111}indium-labeled platelets in patients with homocystinuria. N. Eng. J. Med. 307: 781.

Hirsh, J., Glynn, M.F. and Mustard, J.F. (1968). The effect of platelet age on platelet adherence to collagen. J. Clin. Invest. 47: 466.

Holmsen, H. (1982). Platelet secretion. In: Hemostasis and Thrombosis: basic principles and clinical practice. Eds.: Colman, R.W., Hirsh, J., Marder, V.J. and Salzman, E.W. (J.B. Lippincott Co., Toronto). p. 390.

Holmsen, N. and Day, H.J. (1970). The selectivity of the thrombin-induced platelet release reaction: subcellular localization of released and retained constituents. J. Lab. Clin. Med. 75: 840.

Hudson, E.M., Ramsey, R.B. and Evatt, B.L. (1981). Subcellular localization of Indium^{111}-labeled platelets. J. Lab. Clin. Med. 97: 577.

Humphry, J.H. (1955). Origin of blood platelets. Nature 176: 38.

Humphrey, W.A. (1983). An assay for rabbit FVIIIvWF: Detecting vessel wall damage. M.Sc. Thesis, McMaster University.

International Committee for Standardization in Hematology (1977). Recommended methods for radioisotope platelet survival studies. Blood 50: 1137.

Jacobsson, B. (1969). Platelet adhesion and aggregation following contact between blood and vascular catheters in vitro. Scand. J. Haematol. 6: 216.

Jaffe, E.A., Leung, L.K., Nachman, R.L., Levin, R. and Mosher, D.F. (1982). Thrombospondin is the endogenous lectin of human platelets. Nature 295: 246.

Jaffe, E.A., Hoyer, L.W. and Nachman, R.L. (1973). Synthesis of antihemophilic factor antigen by cultured endothelial cells. J. Clin. Invest. 52: 2757.

Jamieson, G.A., Okumura, T., Fishback, B., Johnson, M.M., Egan, J.J. and Weiss, H.J. (1979). Platelet membrane glycoproteins in thrombasthenia, Bernard-Soulier Syndrome, and storage pool disease. J. Lab. Clin. Med. 93: 652.

Jamieson, G.A. (1978). Surface glycoproteins of normal and abnormal platelets. In: Cell Surface Carbohydrate Chemistry. Ed. Hornor, R.E. (Academic Press, Inc.). p. 225.

Jamieson, G.A. and Okumura, T. (1978). Reduced thrombin binding and aggregation in Bernard-Soulier platelets. J. Clin. Invest. 61: 861.

Jancik, J., Schauer, R. and Streicher, H.J. (1975). Influence of membrane-bound N-acetylneuraminic acid on the survival of erythrocytes in man. Hoppe-Seyler's Z. Physiol. Chem. 356: 1329.

Jenkins, C.S.P., Ali-Briggs, E.F., Zonneveld, G.T.E., Stark, R. and Clemetson, K.J. (1979). Human blood platelet membrane glycoproteins. Resolution in different polyacrylamide gel electrophoretic systems. Thromb. Haemost. 42: 1490.

Jenkins, C.S.P. and Clemetson, K.J. (1977). The role of platelet membrane glycoproteins. In: Sereno Symposium on Platelets and

Thrombosis. Ed.: Mills, D.C.B. and Pareti, F.I. (Academic Press, N.Y.). p. 9.

Jirillo, E., Miragliotta, G. and Fumarola, D. (1982). Effects of bacterial lipopolysaccharides (LPS) on leukocytes and platelets. Boll. Ist Sieroter Milan: 285.

Joist, J.H., Baker, R.K. and Welch, M.J. (1983). Methodologic and basic aspects of indium-111 platelets. Sem. Thromb. Hemost. 9: 86.

Joist, J.H., Baker, R.K., Thakur, M.L. and Welch, M.J. (1978). Indium-111-labelled human platelets: uptake and loss of label and in vitro function of labeled platelets. J. Lab. Clin. Med. 92: 829.

Jørgensen, L., Hovig, T., Rowsell, H.C. and Mustard, J.F. (1970). Adenosine diphosphate-induced platelet aggregation and vascular injury in swine and rabbits. Am. J. Pathol. 61: 161.

Jørgensen, L., Rowsell, H.C., Hovig, T. and Mustard, J.F. (1967). Resolution and organization of platelet-rich mural thrombi in carotid arteries of swine. Am. J. Pathol. 51: 681.

Julliard, J., Maupin, B., Chary, R., Theilleux, R., Nau, P. and Loverdo, A. (1952). Transfusion au lapin de leucocytes et de plaquettes du sang humain apres marquage par le radio-phosphore. C.R. Soc. Biol. (Paris) 146: 211.

Kane, W.H. and Majerus, P.W. (1982). The interaction of human coagulation factor Va with platelets. J. Biol. Chem. 257: 3963.

Kaplan, J.E. and Saba, T.M. (1978). Platelet removal from the circulation by the liver and spleen. Am. J. Physiol. 235: H314.

Kaplan, K.L., Broekman, M.J., Chernoff, A., Lesznik, G.R. and Drillings, M. (1979). Platelet -granule proteins: studies on release and subcellular localization. Blood 53: 604.

Karas, S.P., Rosse, W.F. and Kurlander, R.J. (1982). Characterization of the IgG-Fc receptor on human platelets. Blood 60: 1277.

Karpatkin, S., Schur, P.H., Strick, N. and Siskind, G.W. (1973). Heavy chain subclass of human anti-platelet antibodies. Clin. Immunol. Immunopath. 2: 1.

Karpatkin, S., Garg, S.K. and Siskind, G.W. (1971). Autoimmune thrombocytopenic purpura and the compensated thrombocytolytic state. Am. J. Med. 51: 1.

Karpatkin, S. and Charmatz, A. (1969a). Heterogeneity of human platelets. 1. Metabolic and kinetic evidence suggestive of young and old platelets. J. Clin. Invest. 48: 1073.

Karpatkin, S. (1969b). Heterogeneity of human platelets. II. Functional evidence suggestive of young and old platelets. J. Clin. Invest. 48: 1083.

Kattlove, H.E. and Spaet, T.H. (1970). The effect of chromium on platelet function in vitro. Blood 35: 659.

Kaufman, R.M., Airo, R., Pollock, S., Crosby, W.H. and Doberneck, R. (1965). Origin of pulmonary megakaryocytes. Blood 25: 767.

Kelemen, E., Lehoczky, D., Cserhati, I., Kriza, F. and Rak, K. (1963). Demonstrability of a serum factor inducing thrombocytosis prior to acute rises of platelets in mice and man. Acta Haematol. 29: 16.

Kelemen, E., Cserhati, I. and Tanos, B. (1958). Demonstration and some properties of human thrombopoietin in thrombocythaemic sera. Acta Haematol. 20: 350.

Kelton, J.G. (1983). The measurement of platelet-bound immunoglobulins: An overview of the methods and the biological relevance of platelet-associated IgG. In: Progress in Hematology, Vol. XIII. Ed.: Brown, E.G. (Grune and Stratton, Inc.).

Kelton, J.G. and Denomme, G. (1982). The quantitation of platelet-associated IgG on cohorts of platelets separated from healthy individuals by buoyant density centrifugation. Blood 60: 136.

Kelton, J.G., Neame, P.B., Bishop, J., Ali, M., Gauldie, J. and Hirsh, J. (1979). The direct assay for platelet-associated IgG (PA IgG); lack of association between antibody level and platelet size. Blood 53: 73.

Kernoff, L.M., Blake, K.C.H. and Shackleton, D. (1980). Influence of the amount of platelet-bound IgG on platelet survival and site of sequestration in autoimmune thrombocytopenia. Blood 55: 30.

Kinlough-Rathbone, R.L., Packham, M.A. and Mustard, J.F. (1983). Vessel injury, platelet adherence and platelet survival. Arteriosclerosis 3: 529.

Kinlough, R.L., Lander, H., Ardlie, N.G. and Schwartz, C.J. (1968). Platelet behaviour in patients with Starr-Edwards prostheses. S. Aust. Clin. Dec; 263.

Kinlough, R.L., Davey, M.G. and Lander, H. (1966). An evaluation of anticoagulant solutions used in the preparation of platelet concentrates. Transfusion 6: 213.

Kinosita, R., and Ohno, S. (1960, 1961). Biodynamics of Thrombopoiesis. In: Blood Platelets. Henry Ford Hospital Int. Symposium 1960. Little, Brown & Company, Boston, 1961. p. 611.

Kohler, C.A. and Schaffer, S.A. (1979). Platelet survival as an index of vascular damage. Abstract. V. International Symposium on Atherosclerosis. Houston, Texas. 1979.

Kutti, J., Zaroulis, C.G., Kutti, S.S., Dinsmore, R.E., Day, N.K. and Good, R.A. (1981). Evidence that circulating immune complexes remove transfused platelets from the circulation. Am. J. Haematol. 11: 255.

Kutti, J. and Weinfeld, A. (1979). Platelet survival and platelet production in acute myocardial infarction. Acta Med. Scand. 205: 501.

Kutti, J. and Weinfeld, A. (1971). Platelet survival in man. Scand. J. Haematol. 8: 336.

Kwaan, H.C. (1982). Fibrinolytic Proteins. In: Hemostasis and Thrombosis: basic principles and clinical practice. Eds.: Colman, R., Hirsh, J., Marder, V.J., Salzman, E.W. (J.B. Lippincott Co., Toronto). p. 748.

Kwaan, H.C., McFadzean, A.J.S. and Cook, J. (1956). Plasma fibrinolytic activity in cirrhosis of the liver. Lancet 270: 132.

Larke, R.P.B., Turpie, A.G.G., Scott, S. and Chernesky, M.A. (1977). Paramyxovirus-induced platelet aggregation. Requirement for neuraminic acid receptors on platelets. Lab. Invest. 37: 150.

Legrand, Y., Pignaud, G. and Caen, J.P. (1977). Human blood platelet and proelastase. Activation of proelastase and release of elastase after adhesion of platelets to collagen. Haemostasis 6: 180.

Leeksma, C.H.W. and Cohen, J.A. (1956). Determination of the life span of human blood platelets using labelled diisopropylfluorophosphate. J. Clin. Invest. 35: 964.

Ljungqvist, U. and Bergentz, S.E. (1971). Platelet adhesiveness and aggregation studied with chromium^{51}-labelled platelets. Acta Chir. Scand. 137: 15.

Lotter, M.G., Badenhorst, P.N., Heyns A. du, P., van Reenen, O.R., Pieters, H. and Minnaar, P.C. (1980). Kinetics, distribution, and sites of destruction of canine blood platelets with In-111. J. Nucl. Med. 21: 36.

Lowenhaupt, R.W., Silverstein, E.B., Sperling, M.I. and Mayfield, G. (1982). A quantitative method to measure human platelet chemotaxis using indium-111-oxine-labeled gel-filtered platelets. Blood 60: 1345.

Luscher, J.M. and Barnhart, M.I. (1977). Congenital disorders affecting platelets. Semin. Thromb. Hemost. 4: 123.

Luskotoff, D.J., Levine, E. and Mussoni, L. (1982). Fibrinolytic components of cultured endothelial cells. In: Pathobiology of the endothelial cell. Eds.: Nossel, H.L., Vogel, H.J. (Academic Press, Toronto). p. 167.

Lutz, H.U. and Kay, M.M.B. (1981). An age specific cell antigen is present on senescent human red blood cell membranes. A brief note. Mech. Ageing and Develop. 15: 65.

Maca, R.D., Gry, G.L., Hoak, J.C. and Loh, P.T. (1977). The effects of intact platelets on cultured human endothelial cells. Thromb. Res. 11: 715.

Marcus, A.J., Broekman, M.J., Weksler, B.B., Jaffe, E.A., Safier, L.B., Ullman, H.L. and Tack-Goldman, K. (1982). Platelets, endothelial cells, and the thrombotic process. In: Cardiovascular Pharmacology of the Prostaglandins. Eds.: Herna, A.G., Vanhoutte, P.M., Denolin, H., Goosens, A. (Raven Press, N.Y.). p. 12.

Martin, J.F. and Trowbridge, E.A. (1982). Theoretical requirements for the density separation of platelets with comparison of continuous and discontinuous gradients. Thromb. Res. 27: 513.

Meuleman, D.G., Vogel, G.M.T., Slutemeyer, S.M. and Moelker, H.C.T. (1980a). Analysis of platelet survival curves in an arterial thrombosis model in rats. Thromb. Res. 20: 31.

Meuleman, D.G., Vogel, G.M.T. and Van Delft, A.M.L. (1980b). Effects of intra-arterial cannulation on blood platelet consumption in rats. Thromb. Res. 20: 45.

Meuleman, D.G. (1975). Reduction of platelet survival: development of a thrombosis model. Thromb. Diath. Haemorrh. 34: 586.

Mezzano, D., Hwang, K-I., Catalano, P. and Aster, R.H. (1981). Evidence that platelet buoyant density, but not size,

correlates with platelet age in man. Am. J. Hematol. 11: 61.

Moncada, S. and Vane, J.R. (1982). The role of prostaglandins in platelet vessel wall interactions. In: Pathobiology of the Endothelial Cell. Eds.: Nossel, H.L., Vogel, H.J. (Academic Press, Toronto). p. 253.

Moore, A. and Nachman, R.L. (1981). Platelet Fc receptor. J. Clin. Invest. 67: 1064.

Moore, S. (1973). Thromboatherosclerosis in normolipemic rabbits. A result of continued endothelial damage. Lab. Invest. 29: 478.

Moschos, C.B., Oldewurtel, H.A., Haider, B. and Regan, T.U. (1976). Effect of coronary thrombus age on fibrinogen uptake. Circulation 54: 653.

Mosher, D.F., Vaheri, A., Choate, J.J. and Gahmberg, C.G. (1979). Action of thrombin on surface glycoproteins of human platelets. Blood 53: 437.

Mueller, J.F. (1953). Pathologic physiology of mammalian blood platelet utilized P^{32} tagged rabbit platelets. Proc. Soc. Exp. Biol. (N.Y.) 83: 557.

Murphy, E.A., Francis, M.E. and Bolling, D.R. (1973). Estimation of blood platelet survival. V. A method for the analysis of population data. J. Chron. Dis. 26: 797.

Murphy, S., Oski, F.A., Naiman, J.L., Lusch, C.J., Goldberg, S. and Gardner, F.H. (1972). Platelet size and kinetics in hereditary and acquired thrombocytopenia. N. Eng. J. Med. 286: 499.

Murphy, E.A. and Francis, M.E. (1971a). The estimation of blood platelet survival. II. The multiple hit model. Thromb. Diath. Haemorrh. 25: 53.

Murphy, E.A. (1971b). The estimation of blood platelet survival. III. The robustness of the basic models. Thromb. Diath. Haemorrh. 26: 431.

Murphy, E.A. and Francis, M.E. (1969a). The estimation of blood platelet survival. I. General principles of the study of cell survival. Thromb. Diath. Haemorrh. 22: 281.

Murphy, S. and Gardner, F.H. (1969b). Platelet preservation. Effect of storage temperature on maintenance of platelet viability - deleterious effect of refrigerated storage. N. Eng. J. Med. 280: 1094.

Murphy, E.A. and Mustard, J.F. (1962). Coagulation tests and platelet economy in atherosclerotic and control subjects. Circulation 25: 114.

Mustard, J.F. and Packham, M.A. (1979). The reaction of blood to injury. In: Inflammation, Immunity and Hypersensitivity. Ed.: Movat, H.Z. (Harper & Row, N.Y.). p. 557.

Mustard, J.F. (1978). Platelet survival. Thromb. Haemost. 40: 154.

Mustard, J.F., Packham, M.A. and Kinlough-Rathbone, R.L. (1978). Platelet survival. In: Platelet Function Testing. Eds.: Day, H.J., Holmsen, H., Zucker, M.B. (U.S. Government Printing Office, Washington, D.C., DHEW Publ. No. (NIH) 78-1089), p. 545.

Mustard, J.F. and Packham, M.A. (1977). Platelets and diabetes mellitus. N. Eng. J. Med. 297: 1345.

Mustard, J.F., Rowsell, H.C. and Murphy, E.A. (1966). Platelet Economy (Platelet Survival and Turnover). Brit. J. Haemat. 12: 1.

Mustard, J.F., Murphy, E.A., Robinson, G.A., Rowsell, H.C., Ozge, A. and Crookston, J.H. (1963a). Blood platelet survival. In: Fibrinogen and Fibrin: Turnover of Clotting Factors. Eds.: Koller, F., Beck, E. (F.K Schattauer-Verlag, Stuttgart). p. 246.

Mustard, J.F., Murphy, E.A., Ogryzlo, M.A. and Smythe, H.A. (1963b). Blood coagulation and platelet economy in subjects with primary gout. Can. Med. Assoc. J. 89: 1207.

Mustard, J.F. and Murphy, E.A. (1963c). Effect of smoking on blood coagulation and platelet survival in man. Br. Med. J. 1: 846.

Mustard, J.F. and Murphy, E.A. (1962). Effect of different dietary fats on blood coagulation, platelet economy and blood lipids. Br. Med. J. 1: 1651.

McDonald, T.P. (1980). Effects of thrombopoietin on platelet size of mice. Exp. Hematol. 8: 527.

McDonald, T.P., Clift, R. and Lange, R.D. (1975). Thrombopoietin production by human embryonic kidney cells in culture. J. Lab. Clin. Med. 85: 59.

McKay, D.G. (1965). Disseminated intravascular coagulation: an intermediary mechanism of disease. (Hoeber Medical Division, Harper and Row, New York, 1965).

Nachman, R.L. and Ferris, B. (1972). Studies on the proteins of human platelet membranes. J. Biol. Chem. 247: 4468.

Najean, Y., Dassin, E., Vigneron, N. and Wacquet, M. (1979). Platelet survival studies in patients with vascular diseases. Eur. J. Clin. Invest. 9: 461.

Najean, Y., Ardaillou, N. and Dresch, C. (1969a). Platelet lifespan. Ann. Rev. Med. 20: 47.

Najean, Y. and Ardaillou, N. (1969b). The use of ^{75}Se-methionine for the *in vivo* study of platlelet kinetics. Scand. J. Haematol. 6: 395.

Nel, J.D., Stevens, K., Mouton, A. and Pretorius, F.J. (1983). Platelet-bound IgM in autoimmune thrombocytopenia. Blood 61: 119.

Nel, J.D. and Stevens, K. (1980). A new method for the simultaneous quantitation of platelet-bound immunoglobulin (IgG) and complement (C_3) employing an enzyme-linked immunosorbent assay (ELISA) procedure. Br. J. Haematol. 44: 281.

Niewiarowski, S. and Kodungallore, G.V. (1982). Biochemistry and Physiology of Secreted Platelet Proteins. In: Hemostasis and Thrombosis: basic principles and clinical practice. Eds.: Colman, R.W., Hirsh, J., Marder, V.J. and Salzman, E.W. (J.B. Lippincott Co., Toronto). p. 421.

Niewiarowski, S., Senyi, A.F. and Gillies, P. (1973). Plasmin induced platelet aggregation and platelet release reaction. Effects of hemostasis. J. Clin. Invest. 52: 1647.

Norcott, H.C., Goldman, M., Hawker, R.J., Rafiqi, E.I., Drolc, Z., and McCollum, C.N. (1982). Platelet inhibitory drugs: an *in vivo* method of evaluation in patients. Thromb. Haemost. 48: 307.

Nurden, A.T. and Caen, J.P. (1979). The different glycoprotein abnormalities in thrombasthenic and Bernard-Soulier platelets. Semin. Hematol. 16: 234.

Nurden, A.T. and Caen, J.P. (1978). Membrane glycoproteins and human platelet function. Br. J. Haematol. 38: 155.

Nurden, A.T., Butcher, P.D. and Hawkey, C.M. (1977). Comparative studies on the glycoprotein composition of mammalian platelets. Comp. Biochem. Physiol. 56B: 407.

Nurden, A.T. and Caen, J.P. (1975). Specific roles of platelet surface glycoproteins in platelet function. Nature 255: 720.

Odell, T.T., McDonald, T.P. and Detwiler, T.C. (1961). Stimulation of platelet production by serum of platelet depleted rats. Proc. Soc. Exp. Biol. Med. 108: 428.

Odell, T.T., Tausche, F.G. and Gude, W.D. (1955a). Uptake of radioactive sulfate by elements of the blood and the bone marrow of rats. Am. J. Physiol. 180: 491.

Odell, T.T., Tausche, F.G. and Furth, J. (1955b). Platelet life span as measured by transfusion of isotopically labeled platelets into rats. Acta Haematol. 13: 45.

Ohlsson, K. and Olsson, I. (1974). The neutral proteases of human granulocytes. Isolation and partial characterization of granulocyte elastases. Eur. J. Biochem. 42: 519.

Packham, M.A., Guccione, M.A., Kinlough-Rathbone, R.L. and Mustard, J.F. (1980). Platelet sialic acid and platelet survival after agregation by ADP. Blood 56: 876.

Packham, M.A., Mustard, J.F., Guccione, M.A., Winocour, P.D., Groves, H.M., Rand, M.L., Kinlough-Rathbone, R.L., Greenberg, J.P. and Cieslar, P. (1979). Investigation of in vivo factors that may influence platelet survival and density. Thromb. Haemost. 42: 328.

Packham, M.A., Rowsell, H.C., Jorgensen, L. and Mustard, J.F. (1967). Localized protein accumulation in the wall of the aorta. Exp. Mol. Pathol. 7: 214.

Parker, R.C. (1962). In: Methods of Tissue Culture. 3rd Edition. (Hoeber Medical Division, Harper & Row, New York).

Parkman, R., Kenney, D.M., Remold-O'Donnell, E., Perrine, S., Rosen, F.S. (1981). Surface protein abnormalities in lymphocytes and platelets from patients with Wiskott-Aldrich Syndrome. Lancet, Dec. 19; 1387.

Patterson, J.C. (1969). The pathology of venous thrombi. In: Thrombosis. Eds.: Sherry, S., Brinkhous, K.M. and Genton, E. (National Academy of Science). p. 321.

Pearson, T.A., Solez, K., Dillman, J. and Heptinstall, R.H. (1979). Monoclonal characteristics of organizing arterial thrombi: significance in the origin and growth of human atherosclerotic plaques. Lancet, Jan. 6: 7.

Penington, D.G. (1979). The cellular biology of megakaryocytes. Blood Cells 5: 5.

Penington, D.G., Lee, N.L.Y., Roxburgh, A.E. and McGready, J.R. (1976a). Platelet density and size: the interpretation of heterogeneity. Br. J. Haematol. 34: 365.

Penington, D.G., Streatfield, K. and Roxburgh, A.E. (1976b). Megakaryocytes and the heterogeneity of circulating platelets. Br. J. Haematol. 34: 639.

Penny, R., Rozenberg, M.C. and Firkin, B.G. (1966). The splenic platelet pool. Blood 27: 1.

Peters, A.M., Rozkovec, A., Bell, K.N., Hallidie-Smith, K.A., Goodwin, J.F. and Lavender, J.P. (1982). Platelet kinetics in congenital heart disease. Cardiovasc. Res. 16: 391.

Podolsak, B., Mingers, A.-M., Engelke, A. (1980). The influence of platelet homogenates on thrombopoiesis in rabbits. Thromb. Res. 20: 191.

Phillips, D.R. (1980). An evaluation of membrane glycoproteins in platelet adhesion and aggregation. In: Progress in Hemostasis and Thrombosis, Vol. 5. Ed.: Spaet, T.H. (Grune and Stratton, Inc.). p. 81.

Phillips, D.R. and Jakabova, M. (1977). Ca^{2+}-dependent protease in human platelets. Specific cleavage of platelet polypeptides in the presence of added Ca^{2+}. J. Biol. Chem. 252: 5602.

Phillips, D.R., Jenkins, C.S.P., Luscher, E.F. and Larrieu, M.-J. (1975). Molecular differences of exposed surface proteins on thrombasthenic platelet plasma membranes. Nature 257: 599.

Phillips, D.R. and Agin, P.P. (1974). Thrombin substrates and the proteolytic sites of thrombin action on human platelet plasma membranes. Biochim. Biophys. Acta 352: 218.

Phillips, D.R. (1972). Effect of trypsin on the exposed polypeptides and glycoproteins in the human platelet membrane. Biochem. 11: 4582.

Pittilo, R.M., Mackie, I.J., Rowles, P.M., Machin, S.J. and Woolf, N. (1982). Effects of cigarette smoking on the ultrastructure of rat thoracic aorta and its ability to produce prostacyclin. Thromb. Haemost. 48: 173.

Pumphrey, C.W., Chesebro, J.H., Dewanjee, M.K., Wahner, H.W., Hollier, L.H., Pairolero, P.C. and Fuster, V. (1983). In vivo quantitation of platelet deposition on human peripheral arterial bypass grafts using indium-111-labeled platelets. Effect of dipyridamole and aspirin. Am. J. Cardiol. 51: 796.

Radley, J.M. and Scurfield, G. (1980). The mechanism of platelet release. Blood 56: 996.

Rand, M.L. (1982). Title: Studies of changes in rabbit platelets as they age in vivo. Ph.D. Thesis, Department of Biochemistry, University of Toronto.

Rand, M.L., Greenberg, J.P., Packham, M.A. and Mustard, J.F. (1981). Density subpopulations of rabbit platelets: size, protein and sialic acid content, and specific radioactivity changes following labeling with ^{35}S-sulfate in vivo. Blood 57: 741.

Reimers, H.-J., Kinlough-Rathbone, R.L., Cazenave, J.-P., Senyi, A.F., Hirsh, J., Packham, M.A. and Mustard, J.F. (1976). In vitro and in vivo functions of thrombin-treated platelets. Thromb. Haemost. 35: 151.

Reimers, H.-J., Buchanan, M.R. and Mustard, J.F. (1973). Survival of washed rabbit platelets in vivo. Proc. Soc. Exp. Biol. Med. 142: 1222.

Reynolds, E.S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17: 208.

Robbins, K.C. (1982). The plasminogen-plasmin enzyme system. In: Hemostasis and Thrombosis: basic principles and clinical practice. Eds.: Coleman, R.W., Hirsh, J., Marder, V.J., Salzman, E.W. (J.B. Lippencott Co., Philadelphia). p. 623.

Robert, B., Robert, L., Legrand, Y., Pignaud, G. and Caen, J. (1971). Elastolytic protease in blood platelets. Ser. Haematol. 4: 175.

Ross, R. (1981). Atherosclerosis: A problem of the biology of arterial wall cells and their interactions with blood components. Arteriosclerosis 1: 293.

Ross, R. and Harker, L. (1976). Hyperlipidemia and atherosclerosis. Science 19: 1094.

Rouland, F., Boisseau, M.R., Collignon, G., Hourdille, P. and Vezon, G. (1982). Homocysteinuric congenitale et antiagregants plaquettaires. Interct du Flurlipofene dans la prevention des thromboses XXII Reunion du groupe d'etude sur l'hemostase et la thrombose. French Society of Haematology, Strasbourg. p. 23.

Saba, T.M. (1970). Physiology and physiopathology of the reticuloendothelial system. Arch. Intern. Med. 126: 1031.

Saba, T.M. and DiLuzio, N.R. (1969). Reticuloendothelial blockade and recovery as a function of opsonic activity. Am. J. Physiol. 216: 197.

Sakon, M., Kambayashi, J.-I., Ohno, H. and Kosaki, G (1981). Two forms of Ca^{++} activated neutral proteases in platelets. Thromb. Res. 24: 207.

Salimi, A., Oliver, G.C., Lee, J. and Sherman, L.A. (1977). Continued incorporation of circulating radiolabeled fibrinogen into preformed coronary artery thrombi. Circulation 56: 213.

Scheffel, U., Tsan, M.-F., and McIntyre, P.A. (1979). Labeling of human platelets with [^{111}In]8-hydroxyquinolone. J. Nucl. Med. 20: 524.

Scheffel, U., McIntyre, P.A., Evatt, B., Dvornicky, J.A., Natarajan, T.K., Bolling, D.R. and Murphy, E.A. (1977). Evaluation of indium-111 as a new high photon yield gamma-emitting "physiological" platelet label. Johns Hopkins Med. J. 140: 285.

Schmaier, A.H., Zuckerberg, A., Silverman, C., Kuchibhotta, J., Tuszynski, G.P. and Colman, R.W. (1983). High-molecular weight kininogen a secreted platelet protein. J. Clin. Invest. 71: 1477.

Scott, S., Reimers, H.-J., Chernesky, M.A., Greenberg, J.P., Kinlough-Rathbone, R.L., Packham, M.A. and Mustard, J.F. (1978). Effect of viruses on platelet aggregation and platelet survival in rabbits. Blood 52: 47.

Sedar, A.W., Silver, M.J., Kocsis, J.J. and Smith, J.B. (1978). Fatty acids and the initial events of endothelial damage seen by scanning and transmission of electron microscopy. Atherosclerosis 30: 273.

Senior, R.M., Griffin, G.L., Huang, J.S., Walz, D.A. and Deuel, T.F. (1983). Chemotactic activity of platelet alpha granule proteins for fibroblasts. J. Cell Biol. 96: 382.

Seppa, H., Grotendorst, G., Seppa, S., Schiffmann, E. and Martin, G.R. (1982). Platelet-derived growth factor is chemotactic for fibroblasts. J. Cell Biol. 92: 584.

Sevitt, S. (1974). The structure and growth of valve-pocket thrombi in femoral veins. J. Clin. Pathol. 26: 517.

Sharefkin, J.B., Latker, C., Smith, M., Cruess, D., Clagett, G.P. and Rich, N.M. (1982). Early normalization of platelet survival by endothelial seeding of dacron arterial prostheses in dogs. Surgery 92: 385.

Shouse, S.S., Warren, S.L. and Whipple, G.H. (1931). Aplasia of marrow and fatal intoxication in dogs produced by reontgen radiation of all bones. J. Exp. Med. 53: 421.

Sixma, J.J., Sakariassen, K.S. and Bolhuis, P.A. (1982). The role of factor VIII-von Willebrand factor in adhesion of platelets to the vessel wall. In: Pathobiology of the endothelial cell. (Academic Press Inc., Toronto). p. 139.

Slichter, S.J. and Harker, L.A. (1976). Preparation and storage of platelet concentrates. 1. Factors influencing the harvest of viable platelets from whole blood. Br. J. Haematol. 34: 395.

Slichter, S.J. and Harker, L.A. (1976). Preparation and storage of platelet concentrates. 2. Storage variables influencing platelet viability and function. Br. J. Haematol. 34: 403.

Solum, N.A., Haggen, I., Filion-Myklebust, C. and Stabaek, T. (1980). Platelet glycocalicin: its membrane association and solubilization in aqueous media. Biochim. Biophys. Acta 597: 235.

Solum, N.A., Hagen, I., Peterka, M. and Gjemdal, T. (1979). Absence of the 145,000 molecular weight, soluble platelet membrane glycoprotein-lack of platelet agglutination. Thromb. Haemost. 42: 1626.

Spurr, A.R. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. Ultrastruct. Res. 26: 31.

Steele, P., Rainwater, J. and Vogel, R. (1979). Platelet suppressant therapy in patients with prosthetic cardiovascular valves. Relationship of clinical effectiveness to alteration of platelet survival time. Circulation 60: 910.

Steele, P., Ellis, J. and Genton, E. (1978a). Effects of platelet suppressant, anticoagulant and fibrinolytic therapy in patients with recurrent venous thrombosis. Am. J. Med. 64: 441.

Steele, P. and Rainwater, J. (1978b). Effects of dietary and pharmacologic alteration of serum lipids on platelet survival time. Circulation 58: 365.

Steele, P., Rainwater, J. and Vogel, R. (1978c). Abnormal platelet survival time in men with myocardial infarction and normal coronary arteriogram. Am. J. Cardiol. 41: 60.

Steele, P., Ellis, J.H., Weily, H.S. and Genton, E. (1977). Platelet survival time in patients with hypoxemia and pulmonary hypertension. Circulation 55: 660.

Steele, P., Weily, H., Davies, H., Pappas, G. and Genton, E. (1975a). Platelet survival time following aortic valve replacement. Circulation 51: 358.

Steele, P., Battock, D. and Genton, E. (1975b). Effects of clofibrate and sulfinpyrazone on platelet survival time in coronary artery disease. Circulation 52: 473.

Steele, P.P., Weily, H.S. and Genton, E. (1973). Platelet survival in recurrent venous thrombosis. N. Eng. J. Med. 288: 1148.

Stefanini, M. and Chatterjea, J.B. (1951). Rate of platelet survival in thrombocytopenia. Clin. Invest. 30: 676.

Stein, S.F. and Harker, L.A. (1982). Kinetic and functional studies of platelets, fibrinogen, and plasminogen in patients with hepatic cirrhosis. J. Lab. Clin. Med. 99: 217.

Steiner, M. and Baldini, M. (1970). Subcellular distribution of ^{51}Cr and characterization of binding sites in human platelets. Blood 35: 787.

Stemerman, M.B. (1974). Vascular intimal components: precursors of thrombosis. In: Progress in Hemostasis and Thrombosis. Vol. 2. Ed.: Spaet, T.H. (Grune and Stratton, New York). p. 1.

Stoltz, J.F. and Voisin, P.H. (1979). Evaluation of a biochemical method for measuring platelet lifespan. Cell Biol. Int. Rep. 3: 441.

Stratton, J.R., Ritchie, J.L., Hamilton, G.W., Hammermeister, K.E. and Harker, L.A. (1981). Left ventricular thrombi: in vivo detection by Indium-111 platelet imaging and two dimensional echocardiography. Am. J. Cardiol. 47: 874.

Stuart, M.J., Murphy, S. and Oski, F.A. (1975). A simple nonradioisotope technique for the determination of platelet lifespan. N. Eng. J. Med. 292: 1310.

Taylor, F.B. and Muller-Eberhard, H.J. (1970). Qualitative description of factors involved in the retraction and lysis of dilute whole blood clots and in the aggregation and retraction of platelets. J. Clin. Invest. 49: 2068.

Tessier, C., Steiner, M. and Baldini, M.G. (1974). Measurement of platelet kinetics using ^{51}Cr. In: Platelets: Production, Function, Transfusion and Storage. Eds.: Baldini, M.G., Ebbe, S. (Grune and Stratton, Inc.). p. 327.

Thakur, M.L. (1983). Radioisotopic labeling of platelets. A historical perspective. Semin. Thromb. Hemost. 9: 79.

Thakur, M.L., Welch, M.J., Joist, H. and Coleman, R.E. (1976). Indium-111 labeled platelets: studies on preparation and evaluation of in vitro and in vivo functions. Thromb. Res. 9: 345.

Thomas, D.P. (1982). Pathogenesis of venous thrombosis. In: Hemostasis and Thrombosis: basic principles and clinical practice. Eds.: Colman, R.W., Hirsh, J., Marder, V.J. and Salzman, E.W. (J.B. Lippincott Co., Toronto). p. 820.

Thompson, M.E., Lewis, J.H., Porkolab, F.L., Hasiba, U., Spero, J.A., Wilson, J. and Snyder, M. (1983). Indexes of intravascular hemolysis, quantification of coagulation factors and platelet survival in patients with porcine heterograft valves. Am. J. Cardiol. 51: 489.

Thomsen, H.K. (1974). Carbon monoxide induced atherosclerosis in primates. An electron-microscopic study on the coronary arteries on Macaca Irus monkeys. Atherosclerosis 20: 233.

Tschopp, T.B. and Baumgartner, H.R. (1981). Platelet adhesion and mural platelet thrombus formation on aortic subendothelium of rats, rabbits and guinea pigs correlate negatively with the vascular PGI_2 production. J. Lab. Clin. Med. 98: 402.

Tsukada, T., Steiner, M. and Baldini, M. (1971). Mechanism and kinetics of chromate transport in human platelets. Am. J. Physiol. 221: 1697.

Turitto, V.T. and Weiss, H.J. (1980). Red blood cells: their dual role in thrombus formation. Science 207: 541.

Uhlemann, E.R., TenPas, J.H., Lucky, A.W., Schulman, J.D., Mudd, S.H. and Shulman, N.R. (1976). Platelet survival and morphology in homocystinuria due to cystathionine synthase deficiency. N. Eng. J. Med. 295: 1283.

von Oost, B.A., van Hien-Hagg, I.H., Valdhuyzen, B.F.E., Timmermans, A.P.M. and Sixma, J.J. (1982a). Determination of the density distribution of human platelets-methodological aspects and comparison with other tests for platelet activation. Thromb. Haemost. 47: 239.

von Oost, B.A., Veldhuyzen, B.F.E., von Houwelingen, H.C., Timmermans, A.P.M. and Sixma, J.J. (1982b). Tests for platelet changes, acute phase reactants and serum lipids in diabetes mellitus and

peripheral vascular disease. Thromb. Haemost. 48: 289.

Wakisaka, G., Yasunaga, K., Karumoto, A., Furukawa, H. and Okuma, H. (1963). Survival study of ^{51}Cr-labeled human platelets. Proc. 2nd Ann. Meeting. J. Assoc. Nucl. Med. Nov. 25, 2: 33.

Walsh, P.N. (1982). Platelet-coagulant protein interactions. In: Hemostasis and Thrombosis: basic principles and clinical practice. Eds.: Coleman, R.W., Hirsh, J., Marder, V.J., Salzman, E.W. (J.B. Lippincott Co., Philadelphia, Toronto). p. 404.

White, J.G. and Gerrard, J.M. (1982). Anatomy and structural organization of the platelet. In: Hemostasis and Thrombosis: basic principles and clinical practice. Eds.: Coleman, R.W., Hirsh, J., Marder, V.J., Salzman, E.W. (J.B. Lippincott Co., Philaelphia, Toronto). p. 343.

White, J.G. and Gerrard, J.M. (1976). Ultrastructural features of abnormal blood platelets. Am. J. Pathol. 83: 590.

White, J.G. and Krivit, W. (1967). An ultrastructural basis for the shape changes induced in platelets by chilling. Blood 30: 625.

Wilkinson, A.R., Hawker, R.J. and Hawker, L. (1979). The influence of antiplatelet drugs on platelet survival after aortic damage or implantation of a dacron arterial prosthesis. Thromb. Res. 15: 181.

Winocour, P.D., Kinlough-Rathbone, R.L., Perry, D.W., Rand, M.L., Packham, M.A. and Mustard, J.F. (1983). Changes in the properties of platelets from rats with experimentally induced shortened platelet survival. J. Lab. Clin. Med. 101: 175.

Winocour, P.D., Kinlough-Rathbone, R.L., Richardson, M. and Mustard, J.F. (1983). Reversal of shortened platelet survival in rats by antifibrinolytic agent epsilon aminocaproic acid. J. Clin. Invest. 71: 159.

Winocour, P.D., Cattaneo, M., Somers, D.A., Richardson, M., Kinlough-Rathbone, R.L. and Mustard, J.F. (1982). Platelet survival and thrombosis. Arteriosclerosis 2: 458.

Witte, L.D., Kaplan, K.L., Nossel, H.L., Lages, B.A., Weiss, H.J. and Goodman, D. (1978). Studies of the release from human platelets of the growth factor for cultured human arterial smooth muscle cells. Circ. Res. 42: 402.

Woods, M.C., Gamble, F.N., Furth, J. and Bigelow, R.R. (1953). Control of post irradiation hemorrhagic state by platelet transfusion. Blood 8: 545.

Woolf, N. (1981). Thrombosis and atherosclerosis. In: Hemostasis and Thrombosis. Eds.: Bloom, A.L., Thomas, D.P. (Churchill Livingstone, New York). p. 527.

Wright, J.H. (1906). Origin and nature of blood platelets. Bos. Med. Surg. J. (New Eng. J. Med.) 154: 643.

Zajicek, J. (1957). Studies on the histiogenesis of blood platelets and megakaryocytes. Acta Physiol. Scand. 40 (Suppl. 138): 1.

Zar, J.H. (1974). Biostatistical analysis. (Prentice Hall, Englewood Cliffs, N.J.).

Zimmermann, R., Zeltsch, C. and Lange, D. (1979). Estimation of thrombus formation by labeling of platelets, red cells and fibrinogen in experimental thrombosis. Thromb. Res. 16: 147.

Zucker-Franklin, D. (1981). Endocytosis by human platelets: metabolic and freeze-fracture studies. J. Cell Biol. 91: 706.

Zucker-Franklin, D. (1970). The ultrastructure of megakaryocytes and platelets. Regulation of Hematopoiesis, Vol. II. White cell and platelet production. Ed.: Gordon, A.S. (Appleton-Century-Crofts, New York). p. 1553.

Zucker, M., Heleman, L. and Zumoff, B. (1964). Rapid disappearance of C^{14}-labelled serotonin from platelets in patients with carcinoid syndrome. J. Lab. Clin. Med. 63: 137.