Distribution and orientation of blood platelets flowing in small arterioles

DISTRIBUTION AND ORIENTATION OF BLOOD PLATELETS FLOWING IN SMALL ARTERIOLES

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1. BLOOD PLATELETS: A SHORT SURVEY OF LITERATURE AND INTRODUCTION TO THE PRESENT STUDY

1.1. HISTORY

Blood platelets are the smallest cellular elements in the blood* and their discovery needed the great improvements in the compound microscope attained in the first half of the nineteenth century (Robb-Smith, 1967; James, 1976). The earliest descriptions of small elements in the blood distinct from red and white blood cells appeared in the literature between 1841-1846 (Tocantins, 1948; Robb-Smith, 1967; Maupin, 1969; Rebuck, 1971). Subsequently the strong tendency of these small elements to form granular aggregates and the relation between these aggregates and fibrin was noted (Tocantins, 1948; Rebuck, 1971; Brinkhous and Shermer, 1971). Osler (1874) observed that in venules of freshly killed rats these small bodies appeared in the circulation as single units with no tendency to stick together, but that they formed aggregates when the blood was shed. At that time it became evident that these particles were no artifacts or by-products of the other blood cells, but that they had to be considered as normal constituents of the circulating blood. Hayem (1878) counted them and found in human blood average numbers between 220.000-320.000/mm^3. Similar values are found today (Maupin, 1969).

Bizzozero (1882) was one of the first who observed these particles circulating in the blood of a living animal, among others in the intact vessels of the wing of an unanesthetized bat. He called them "blood platelets", which has become their name ever since, and described them as: "very pale, colorless and disk-shaped plaques with parallel surfaces or, more seldom, with an oval or round lens shape, and a diameter equal to a third or a half of that of the red blood cells". Moreover, he showed that platelets adhere and accumulate at the wall at a site of injury and demonstrated that the resulting white thrombus consists mainly of platelets. In the same year, Hayem (1882) observed that puncture wounds in veins were sealed by a "hemostatic plug". In the following years a number of investigators extended these observations and the essential role of the blood platelets in hemostatis and thrombosis was

*In mammals these cells lack a nucleus; comparative cells in non-mammalian vertebrates do contain a nucleus and are called thrombocytes.

established (Tocantins, 1948; Bergqvist, 1973; Sixma and Wester 1977).

In the same period a relation was found between a decrease in platelet number and purpura haemorrhagica. The poor clot retraction in these patients was correctly attributed to the paucity of platelets. Furthermore the procoagulant activity of the platelets was noted. The role of platelets in spontaneous hemostasis and in disorders of hemostasis was extended by Duke (1910), who described a method for determining the skin bleeding time. He and later investigators (Hirsh et al, 1976; Burnstein and Harker, 1981) found a correlation between the number of platelets in the blood and the bleeding time.

The problem of the origin of the blood platelets was solved by Wright (1906, 1910), who convincingly described their formation from the mature megakaryocytes in the bone marrow by a process of cytoplasmic fragmentation. Employing the newer dyes coming available at that time Wright also found granules within the platelet. He confirmed earlier findings that platelets were capable of forming protoplasmic processes and he saw some of these pseudopods contracting.

Thus, although not generally accepted at that time, all major aspects about platelets were recognized at the beginning of this century.

During the following 30 years progress made in obtaining knowledge of platelets was slow, partially because of the lack of adequate instruments, partially because most attention was paid to the identification of the different coagulation factors. Worth mentioning, however, with respect to the present study is an increasing interest in vital dyes and their application to biological research at that time (Barbosa and Peters, 1971). In this way the presence of mitochondria and two types of granules in platelets could be demonstrated (Tocantins, 1937).

In 1939 the first electron microscopic study on platelets was performed, confirming the earlier findings about pseudopods (Rebuck, 1971). At that time interest in hemostasis was renewed and direct observation of transected microvessels in transparant animal tissue (Apitz, 1941; Zucker, 1947; Hugues, 1953) further established the fundamental role of platelets in the formation of the hemostatic plug. The electron microscope provided final evidence that the freshly formed hemostatic plug was composed of densely packed platelets (Kjaerheim and Hovig, 1962). Furthermore, it revealed a much more complicated

ultrastructure of the platelet than their appearance under the light microscope suggested (White, 1971a,b).

Around 1960 new isotope techniques and the improvements in platelet preservation and transfusion, the latter kind of research being stimulated by the fear of atomic warfare in the early 1950's (Johnson, 1971a), allowed accurate determination of platelet lifespan: 8-11 days. Nowadays this value is generally accepted for human platelets (Maupin, 1969; Ebbe, 1971; Penington, 1981). Moreover, in 1961 transfusion of radiolabeled platelets and autoradiography showed apparent labeling of the capillary beds in many organs of thrombocytopenic animals, whereas normal animals showed no evidence of deposition of radioactive material. This provided the first experimental evidence for an interaction between platelets and endothelial cells (Johnson, 1971b). At present substantial evidence exists that platelets deliver supportive agents to the endothelium (D'Amore, 1978), thereby maintaining vascular integrety apart from hemostasis.

Grette (1962) used the term "blood platelet release reaction" to describe the secretion of biochemical substances from the platelet in response to specific stimuli. Subsequent electron microscopic studies showed that this was caused by the discharge of the contents of the granules, while the platelet itself remained intact (Kjaerheim and Hovig, 1962; Day and Holmsen, 1971; White, 1973, 1974; Holmsen, 1975). In the same year a simple photometric method was introduced to study platelet aggregation in vitro (Born, 1962; O'Brien, 1962). This "aggregometer" became a standard technique and has since been used extensively (Ludlam, 1981), among others in the search for anti-platelet drugs (Born and Hardisty, 1976).

With this and other techniques it was found that in vitro platelets could be stimulated by a variety of chemical agents , among others: adenosine diphosphate (ADP), thrombin, collagen, endoperoxides, thromboxane A_2, serotonin, adrenalin, immune complexes and free fatty acids (Mustard and Packham, 1970a; Zucker, 1974; Walsh, 1975; Luscher and Massini, 1975; Samuelsson et al, 1976; Holmsen et al, 1977; Frojmovic, 1978; Frojmovic and Milton, 1982). In addition, many physical agents that could activate platelets were discovered, such as: temperature, centrifugation, pH, osmotic pressure and shear stress (for definition of shear stress see note on page 16; Massini and Luscher, 1971; Dickson et al, 1971; Mason, 1972; Nossel, 1975; Goldsmith et al, 1975; Karino and Goldsmith, 1979; Stein and Sabbah, 1980; Frojmovic and Milton, 1982).

In the following years the interest in platelets increased steadily with a consequent increase in the number of publications on these cells. Beginning in 1954 Maupin set himself the task to read all publications related to blood platelets. However, in 1969 Crosby wrote in the preface to Maupin's book (Maupin, 1969): "Rather, the swelling tide of publications has finally swamped Colonel Maupin's resources and sadly he informs me it is no longer possible, even with the best intention and devotion of all his leisure to find and read everything about the platelet". One reason for this interest and the swelling tide of publications was that platelets became recognized as multifunctional cells, despite their lack of a nucleus. The capability of platelets to exhibit a variety of cellular reactions, such as: receptor binding, active transport and uptake of monoamines, adhesion, aggregation, unmasking membrane phospholipids, contraction, relaxation, secretion from storage granules, prostaglandin synthesis and phagocytosis as well as the fact that a sufficient number of them can be obtained intact, repeatedly and relatively easily even from humans, made them a suitable model for studying certain basic cellular reactions (Gordon, 1976, 1981; Holmsen et al, 1977; De Gaetano and Garattini, 1978).

Another reason for the rising interest was that it became clear that, beside their primary task in hemostasis - including their role in thrombosis (Maupin, 1969), blood coagulation (Walsh, 1977) and fibrinolysis (Kwaan, 1971; Crawford, 1977) -, platelets are or might be involved in many other physiological processes: for instance scavenging, inflammation, tissue repair and wound healing (Gordon, 1976; De Gaetano and Garattini, 1978); as well as pathological processes, like atherosclerosis (Mustard and Packham, 1975; Friedman and Burns, 1978), immunological diseases (Kincaid-Smith, 1970; Gordon, 1981), and migraine (Gawel et al, 1979; Diamond and Medina, 1980; Malmgren et al, 1980; Masel et al, 1980).

1.2. STRUCTURE AND BIOCHEMISTRY

In freshly drawn whole blood 65-90% of the platelets are disk-shaped (Allen et al, 1979; Frojmovic and Milton, 1982). Ultrastructural studies show the resting platelet as a lenticular cell without a nucleus (see fig. 1.1), containing several organelles, membrane systems and fiber systems (White, 1971a,b; White et al, 1981).

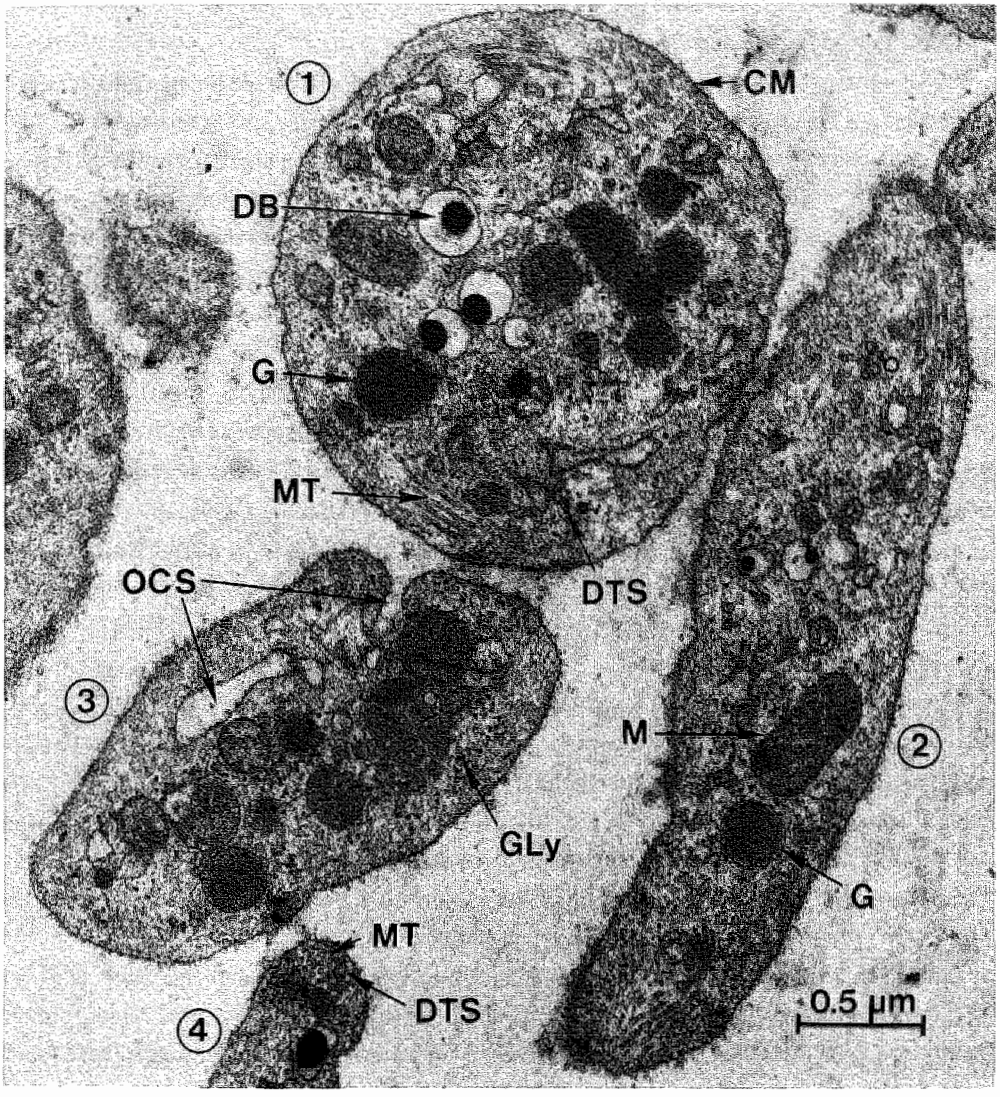

Fig. 1.1
Electron microscopic picture of a thin section of rabbit blood platelets. The cell at the top (No.1) is cut face-on and the cell at the right (No. 2) side-on, showing the disk-shape of the platelets. A nucleus is not present in blood platelets. The cell membrane (CM) invaginates at different sites (see No.3), forming a system of tortuous channels in the interior of the platelet: the open canalicular system (OCS). Just below the CM a band of microtubules (MT) is present in the equatorial plane of the cell. In No.1 the MT band is cut longitudinally, and is seen in cross-section in No.4. Throughout the cytoplasm a second membrane system is found: the dense-tubular system (DTS). In platelet No.4 part of the DTS can be seen associated with the MT. Dense bodies (DB), alpha- and lysosome-like granules (G), mitochrondia (M) and glycogen particles (Gly) are shown. For preparation see chapter 2.2.4.

1.2.1. Cell membrane

On the surface of the cell, which appears smooth at first sight, small indentations and elevations can be discovered on closer examination. The small elevations are thought to result from the outward projection of large vesicles in the cytoplasm (Allen et al, 1979; Werner and Morgenstern, 1980; Frojmovic and Milton, 1982). The indentations are the openings of the surface connected open canalicular system (OCS), a system of tortuous channels tunneling through the cytoplasm (Behnke, 1970; White et al, 1981). The OCS greatly increases the total surface area of the platelet and consequently the possibilities for contact and exchange with the environment. The walls of the OCS are continuous with the cell surface. The barrier separating the platelet cytoplasm from the environment consists of a lipid bilayer, which is coated with an extensive exterior coat or glycocalix (Behnke, 1970; White, 1971b; Holmsen, 1972). Important constituents of this glycocalix are the sialic acid and carbohydrate containing moieties of sialoglycoproteins, which appear to be the majority, if not all, of the membrane proteins exposed on the outer surface (Jamieson and Smith, 1976; Phillips, 1980; Berndt and Phillips, 1981). Protrusions of glycolipids are also present in the glycocalix (Jamieson and Smith, 1976; Schick, 1979). It is assumed that the membrane receptors for stimuli and antagonists, as well as the binding sites for plasma proteins, aggregation and adhesion are sialoglycoproteins (Jamieson and Smith, 1976; Phillips, 1980; Berndt and Phillips, 1981) or glycolipids (Jamieson and Smith 1976; Schick, 1979; Zwaal and Hemker, 1982).

The lipid bilayer consists of different phospholipids with an asymmetric distribution. The two negatively charged phospholipids, phosphatidylinositol and phosphatidylserine, are almost completely located in the inner layer (Schick, 1979; Zwaal and Hemker, 1982). Negatively charged phospholipid surfaces, especially when containing phosphatidylserine, accelerate the coagulation steps in which the vitamin-K dependent proteins are involved (Jackson and Nemerson, 1980). Therefore, it was postulated that the procoagulant activity of stimulated platelets was caused by the release or unmasking at their surface of negatively charged phospholipids (Jackson and Nemerson, 1980). However, the presence of phosphatidylserine on the surface of thrombin-activated platelets could not be demonstrated (Schick, 1979). Moreover, part of the procoagulant activity of platelets can be explained by the fact that factor V is secreted during the release reaction, that small amounts of thrombin can convert it into its activated form, factor V_a, and that this

factor bound to the platelet surface greatly accelerates the conversion of prothrombin into thrombin (Jackson and Nemerson, 1980; Zwaal and Hemker, 1982). Nevertheless, it has been shown recently that when platelets are stimulated by a mixture of collagen and thrombin, the amount of phosphatidylserine at the exterior of the plasma membrane increases considerably (Zwaal and Hemker, 1982). This results in a high prothrombin converting activity of these platelets as compared to that of platelets stimulated by either thrombin or collagen alone (Zwaal and Hemker, 1982).

1.2.2. Fiber systems and contractile proteins

In the equatorial plane of the disk shaped platelet a circumferential band of microtubules is prominent, subjacent to but always separated from the cell membrane (Sixma and Molenaar, 1966; White, 1971a; White et al, 1981). It probably consists of a single coiled tubule serving as a cytoskeleton to support the lenticular cell shape (White, 1971a; Harris, 1981). Between the ring of microtubules and the cell membrane a system of fine filaments in close association with the membrane was discovered (Sixma and Molenaar, 1966; White, 1971a). It appeared that the submembrane filaments are also present outside these areas of the cell membrane (White, 1971a; White et al, 1981). Subsequently numerous microfilaments were discovered throughout the platelet cytoplasm forming an irregular network (White, 1971a; White et al, 1981).

Upon stimulation and subsequent shape change of the platelet, i.e. from the disk shape to a sphere with pseudopods, reorganisation of microtubules and microfilaments occurs. In each pseudopod, which can reach a length of 10 μm, bundles of parallel microfilaments and sometimes microtubules are found (Sixma and Molenaar, 1966; White, 1971a; Crawford, 1976; Harris, 1981). The microtubule-ring is reduced in circumference or disappears (White, 1971a; Crawford, 1976; Harris, 1981; White et al, 1981). Simultaneously the organelles are displaced toward the central region of the cell, packed within a web of microfilaments and microtubules (White, 1971a; White et al, 1981). This contractile wave may be reversed with return of the microtubule ring to the position just below the cell membrane and recovery of the disk-shape of the cell (White, 1971a; White et al, 1981). These motile events must be subject to delicate and local regulation as illustrated by the fact that a platelet may extend a pseudopod at one site, while simultaneously retracting one at another site (Harris, 1981). In addition, platelets are able to extend a single pseudopod while maintaining their disk-shape (White et al, 1981).

Platelets are the first nonmuscular cells in which the presence of actin and myosin was demonstrated (Adelstein and Pollard, 1978). Actin is the most abundant protein in platelets, comprising 20-30% of the total cellular protein content (Harris, 1981). It is the major constituent of the submembrane and cytoplasmic microfilaments. Beside the actin polymerized in the microfilaments, a large pool of actin monomers is present. The concentration of myosin is only 1% of that of actin. In addition, a number of "actin-organizing proteins" has been found as well as proteins regulating the interaction of actin with myosin, an interaction which provides a contractile system. One of the proteins crosslinks actin filaments and plays a role in reversible gel-sol transformations of the cytoplasm, which determines the consistency of the cytoplasm. Proteins regulating the equilibrium between monomeric and polymeric actin have been found and it is assumed that monomeric actin can be triggered to polymerize rapidly into ordered microfilaments, leading to the formation of pseudopods. In all these regulations calcium seems to play a central role. A transmembrane sialoglycoprotein has been found with an internal portion resembling alpha-actinin from the Z-line in skeletal muscle. Such a protein or family of proteins may serve as anchorage points on the membrane for actin filaments and provide a link between cytoplasmic fiber systems and the external environment.

The main protein in platelet microtubules, tubulin, closely resembles the tubulin from microtubules in nerve tissue (Crawford, 1976). In the resting platelet tubulin seems to exist principally in the polymerized form, i.e. in microtubules (White et al, 1981). The microtubules appear to be labile structures which can be rapidly assembled or disassembled. Again calcium seems to be involved in this process (Crawford, 1976).

1.2.3. Granules and secreted substances

At present three types of secretion granules can be distinguished within the platelet: dense bodies, alpha-granules and lysosome-like granules (Kaplan 1981; Niewiarowski, 1981; Skaer, 1981). Constituents released from dense bodies, so-called because of their electron-dense content, are serotonin, adenosine triphosphate (ATP), ADP and calcium (Pletscher and Da Prada, 1975; Niewiarowski, 1981; Skaer, 1981). Alpha-granules contain a variety of proteins which can be classified as follows: 1) proteins similar or identical to plasma proteins and 2) proteins immunologically or functionally specific to platelets (Niewiarowski, 1981). Examples from the first group are fibrinogen,

clotting factor V, von Willebrand factor, proteolytic inhibitors and fibronectin, a protein involved in the adherence of cells to the extracellular matrix (Kaplan, 1981; Niewiarowski, 1981; Skaer, 1981). The second group includes growth factors, stimulating fibroblasts, arterial smooth muscle cells and glial cells; a vascular permeability factor that causes mast cells to release histamine; a factor that has chemotactic activity and that activates the 5th component of complement; and proteins binding to heparin or other glycosaminoglycans such as heparan sulphate, which occurs at the surface of many cells including endothelial cells (Lindahl and Hook, 1978; Kaplan, 1981; Niewiarowski, 1981; Skaer, 1981). Beta-thromboglobulin is a weak heparin binding protein for which radio-immunoassays have been developed to measure plasma levels in patients with various disorders as an indicator of platelet release in vivo (Kaplan, 1980). The lysosome-like granules contain a number of hydrolases which are generally found in the lysosomes of various cells (Kaplan, 1981; Niewiarowski, 1981; Skaer, 1981).

The term "platelet release reaction" may suggest an overall emptying of the platelet upon stimulation. Actually, the secretion of granule contents from the platelet is much more selective and must be subject to specific control mechanisms (Kaplan, 1981; Skaer, 1981). The different types of granules do not secrete their contents at the same level of stimulation and even within the group of alpha-granules subgroups seem to exist with different contents and susceptibilities to release. At maximal stimulation only part of the content of the granules is secreted. Release from dense bodies, alphagranules and lysosome-like granules can be achieved by thrombin and collagen, while ADP and arachidonic acid derivatives induce release from the first two granule-types, but probably not from lysosome-like granules (Kaplan, 1981; Skaer, 1981). Most inducers act synergistically with each other and close cell contact enhances the release reaction (Massini and Luscher, 1971; Niewiarowski, 1981).

Different mechanisms of secretion may exist. Centripetal movement of the granules accompanying the contractile wave (see section 1.2.2.) and the presence of granule contents in the OCS has been observed (White, 1973, 1974). However, movement of individual or confluent granules to the cell surface with subsequent disappearance during the release reaction has also been observed (Ginsberg et al, 1980; Skaer, 1981). There is some evidence that circulating platelets continuously release proteins from alpha-granules (Niewiarowski, 1981).

1.2.4. Mitochondria and energy metabolism

Platelets contain mitochondria as well as glycogen, which is stored in the cytoplasm as discrete particles or concentrated masses (White, 1971a; White et al, 1981). The platelets contain all the enzymes for glycolysis, glycogenolysis, Krebs cycle, fatty acid oxidation and oxidative phosphorylation (Holmsen, 1972; Holmsen et al, 1977). The resting platelet has a relatively high energy consumption. It is suggested that a large portion of this energy is involved in cycles of polymerization and depolymerization of actin (Mills, 1981). Release, aggregation and shape change are inhibited with a decrease in the adenylate energy charge, which in normal platelets is close to the value found in other cell types (Holmsen et al, 1977; Mills, 1981).

1.2.5. Internal membranes, calcium and prostaglandins

Beside the OCS, another membrane system has been identified in blood platelets, which consists of narrow, membrane limited tubules containing an electron dense substance (Behnke, 1970; White et al, 1981).This dense tubular system (DTS) was found randomly dispersed throughout the cytoplasm. However, some tubules were always seen in close association with the circumferential band of microtubules, suggesting a role in the assembly and disassembly of this system. (Behnke, 1970; White et al, 1981). In addition, in every platelet the DTS forms membrane complexes with channels of the OCS in one or two areas of the cytoplasm.

It has been demonstrated that platelets contain a system for calcium sequestration and the available data suggest that the DTS is the major calcium sequestering site (Gerrard et al, 1978, 1981). It is generally assumed that calcium movement within the cell plays a key role in the regulation of, probably, all platelet functions, initiating cellular activities when calcium is released into the cytoplasm from its storage sites following stimulation of the platelet (Detwiler et al, 1978; Massini et al, 1978; Feinstein, 1981; Gerrard, 1981). The removal of calcium from the cytoplasm by the calcium sequestering pumps is stimulated by cyclic-AMP, which is considered to be the major intracellular modulator opposing the actions of calcium (Haslam, 1978; Feinstein, 1981).

The DTS has been identified as a site of prostaglandin synthesis by cytochemical studies (Gerrard and White, 1978; Gerrard et al, 1978). In platelets as in other cells, arachidonic acid is liberated from membrane phospholipids by one or more calcium-dependent phospholipases (Gerrard and White, 1978;

Gerrard et al, 1978; Burch and Majerus, 1979; Smith, 1981). Approximately one third of the arachidonic acid is metabolized through the lipoxygenase pathway (Burch and Majerus, 1979; Smith, 1981), yielding among others a substance which is chemotactic for polymorphonuclear leukocytes (Gerrard and White,1978 Burch and Majerus, 1979). The rest is converted by the enzyme cyclo-oxygenase to endoperoxides, which induce platelet aggregation when added exogenously (Gerrard and White, 1978; Burch and Majerus, 1979; Smith, 1981). However, most of these endoperoxides are rapidly converted into thromboxane A_2, a strong aggregating agent and vasoconstrictor with a half-life in plasma of about 5 minutes. Aggregating platelets release large quantities of thromboxane A_2. It has been suggested that thromboxane A_2 stimulates platelets by acting as a physiologic ionophore, transporting calcium from the inside of the DTS to the cytoplasm (Gerrard and White, 1978; Gerrard et al, 1978,1981). In contrast to platelets, endothelial cells and vascular smooth muscle cells convert their endogenously produced endoperoxides to prostacyclin, a potent inhibitor of platelet aggregetion and a vasodilator. Prostacyclin increases the levels of cyclic-AMP in the platelet (Gerrard and White, 1978; Burch and Majerus, 1979; Smith, 1981). Aspirin inhibits prostaglandin synthesis by acetylating the active site of cyclo-oxygenase. Since the acetylation is irreversible and platelets only have a marginal protein synthesis, thromboxane A_2 formation is blocked for the rest of their life time. In contrast, the effect of aspirin on prostacyclin synthesis appears to be reversible within hours (Burch and Majerus, 1979; Smith, 1981). This has raised interest in the possibility of shifting the "thromboxane A_2-prostacyclin balance" in favor of the latter substance by small doses of aspirin given infrequently.

1.3. FUNCTION

Although platelets may participate in many physiological and pathological processes, their main and, at present, only firmly established function is keeping the blood in the proper compartment, i.e. within the blood vessels. They do so by contributing to the normal healthy status of the endothelial lining , their so called endothelium supporting function, as well as by playing a key role in the arrest of bleeding at a site of injury. Adequate hemostasis is of vital importance for the individual. Several processes contribute to the arrest of bleeding or may do so: contraction and retraction of the

injured blood vessel, formation of a platelet plug, reinforcement and stabilization of this initial hemostatic plug by the formation of fibrin, a fall in blood pressure, an increase in tissue pressure due to a hematoma, formation of a scab on the wound surface and, mainly in capillaries, endothelial adhesion (Macfarlane, 1976; Born, 1977; Mustard and Packham, 1977). The relative contribution of each of these processes varies depending on the size and location of the vessel and the type of injury. In medium sized arteries or veins the formation of an effective hemostatic plug usually needs the assistance of other mechanisms, e.g. vessel retraction and contraction or a fall in blood pressure, while in smaller vessels the combined action of platelets and the coagulation system alone is usually sufficient for effective hemostasis (Mustard and Packham, 1977). In the older literature the mass sealing an injured vessel has been referred to as "white thrombus" or "hemostatic thrombus", illustrating the similarity between an arterial (or white) thrombus* and a hemostatic plug (Apitz, 1941; Sixma and Wester, 1977). Indeed, thrombosis has been called "hemostasis in the wrong place" (Macfarlane, 1977), and both processes can be regarded as a result of the same reaction: the response of the blood to vessel wall injury (Mustard and Packham, 1970b; Arfors and Bergqvist, 1976; Mustard et al, 1981). Therefore, platelet function will be treated in more detail by describing the formation of the hemostatic plug.

Direct microscopic observation of hemostatic plug formation in small microvessels has been performed in different animal species, but most extensively in rabbits using the mesentery preparation (Apitz, 1941; Zucker, 1947; Hugues, 1953; Kjaerheim and Hovig, 1962; Marr et al, 1965; Hovig et al, 1967, 1968; Sanders, 1970; Bergqvist and Arfors, 1973, 1974, 1976a,b; Bergqvist, 1974; Arfors and Bergqvist, 1974, 1975; Bergqvist et al,1974; Herrmann,1975). Histological and ultrastructural studies in animals (Sixma and Wester, 1977) and in humans, where skin biopsies from bleeding time wounds were investigated (Sixma and Wester, 1977; Wester et al, 1978, 1979), revealed no essential differences (Sixma, 1981). A clear difference between arterioles and venules was observed in the formation time and stability of the hemostatic plug (Hugues, 1953; Sanders, 1970; Bergqvist, 1974; Arfors and Bergqvist,

*A clear distinction has to be made between a (white) thrombus and a blood clot. A thrombus is formed in the living, active circulation and contains mainly platelets, fibrin and leukocytes. A clot is formed outside the body in a static system and consists of (mainly red) blood cells entrapped in a meshwork of fibrin.

1974, 1975; Bergqvist and Arfors, 1974), but not in the ultrastructure (Kjaerheim and Hovig, 1962). However, treatment with aspirin or indomethacin abolished the differences between both vessel types and it was suggested that prostaglandins produced in the handled tissue inhibited platelet function on the venular side of the microcirculation (Arfors et al, 1972, 1976b; Arfors and Bergqvist, 1978).

1.3.1. Adhesion

After transection of a microvessel bleeding starts immediatly from both ends of the transected vessel. Most vessels contract within approximately 30 to 60 s following transection (Hugues, 1953) with a maximum constriction of 18-45% (Hugues, 1953; Arfors and Bergqvist, 1975). In the microcirculation (see section 1.4) no correlation was found between the degree of contraction and hemostatic plug formation time and it was concluded that in microcirculatory hemostasis the role of vascular contraction is limited (Hugues, 1953; Arfors and Bergqvist, 1975). Platelets adhere to the injured vessel and the surrounding connective tissue, and within seconds a growing white mass can be observed due to adhesion and aggregation of more platelets. Although platelets can adhere to different subendothelial structures (Santoro and Cunningham, 1981; Mustard et al, 1981), they seem to attach mainly to collagen fibers (Kjaerheim and Hovig, 1962; Sixma and Wester, 1977). Moreover, collagen fibers are probably the only subendothelial structures that induce the release reaction and the production of endoperoxides and thromboxane A_2 (Santoro and Cunningham, 1981; Mustard et al, 1981).

Normal adhesion to microfibrils needs in a flow situation the presence in the blood of the factor VIII-related von Willebrand factor (Sixma, 1981; Berndt and Phillips, 1981). The von Willebrand activity is associated with the polymeric, high molecular weight forms of a glycoprotein that exists as a series of disulfide-linked multimers in plasma. The factor VIII procoagulant activity is associated with both the high and low molecular weight forms (Sixma, 1981; Berndt and Phillips, 1981). The size of this polymeric molecule suggests that it is suited to serve as a bridge between the platelet membrane and the subendothelial surface. Although the interaction between the von Willebrand factor and the platelet is still poorly understood, the available data support the hypothesis that the receptor on the platelet membrane surface is glycoprotein Ib (Phillips, 1980; Berndt and Phillips, 1981).

1.3.2. Aggregation

With the aggregation of more and more platelets the white mass at the end of the vessel grows extravascularly and narrows the blood stream (Hugues, 1953; Hovig et al, 1967). New channels may open and close in different directions through this platelet mass even opposite to the original direction of flow. The amount of blood lost becomes gradually smaller and finally bleeding stops. Most arterioles in the rabbit mesentery stop within 0.5-2 minutes (see fig. 2.7). The plug appears as a capsule protruding from the vessel (Kjaerheim and Hovig, 1962). This primary hemostatic plug is not always stable and one or more rebleedings may occur through newly opened channels that are subsequently occluded (Hovig et al, 1967; Bergqvist, 1974). Histologic observations on plug formation showed that plugs consisted of platelets that had lost their discoid shape and had formed pseudopods. In the more central parts of the plug the platelets were loosely packed and still retained their organelles and most of their granules. However, the platelets in contact with collagen had lost their organelles and appeared as swollen, empty balloons with many gaps in their cell membrane. At this stage little fibrin was found and only near the vessel margins (Sixma, 1977; Wester et al, 1978). The formation of this primary hemostatic plug depends mainly on platelet aggregation and less on fibrin formation, since induction of fibrinolysis or anti-coagulation did not prolong its formation time (Bergqvist and Arfors, 1974, 1976). A decrease in platelet count progressively prolonged this time (Bergqvist and Arfors, 1973).

The precise mechanism of the initiation and formation of the primary hemostatic plug is still unknown. Many aggregating agents may play a role. It is likely that they act synergistically at concentration levels which by themselves have little effect (Vermijlen et al, 1973; Mustard and Packham, 1977). Platelets in contact with collagen secrete ADP, serotonin and thromboxane A_2. In combination with the activation of clotting factor VII by the damaged tissue they may promote rapid thrombin formation. In addition, ADP may become available from the damaged vessel wall or from red blood cells (Schmid-Schonbein et al, 1976; Born, 1977). Shear stresses in the transected vessel may rise sufficiently high to promote or induce platelet activation (Goldsmith et al, 1975; Schmid-Schonbein et al, 1976; Born, 1977; Stein and Sabbah, 1980). The existence of more than one activation pathway is supported by clinical conditions like storage pool (dense body) deficiency or defects in thromboxane synthesis (for instance caused by aspirin) in which generally

only a mild prolongation of the bleeding time is found (Hardisty and Caen, 1981).

Platelets of patients with Glanzmann's thrombasthenia adhere to collagen and change shape in response to stimuli, but are not able to form a hemostatic plug consisting of aggregated platelets (Mustard and Packham, 1977; Hardisty and Caen, 1981). In these patients two membrane components, glycoprotein IIb and IIIa, are present in reduced concentrations or are missing (Phillips, 1980; Berndt and Phillips, 1981; Hardisty and Caen, 1981). Therefore, the role of the receptor in platelet to platelet interaction has been attributed to these proteins. Fibrinogen and calcium-ions are required for aggregation, and it is suggested that fibrinogen - linked to the receptor with calcium - serves as a bridging molecule between aggregating platelets (Phillips, 1980; Berndt and Phillips, 1981). Evidence exists that the glycoproteins (especially III) interact on the intracellular side of the membrane with actin filaments (Phillips, 1980; Berndt and Phillips, 1981).

1.3.3. Release and fibrin formation

Hemostatic plugs fixed at different time intervals after their formation show that the platelets become more closely packed with firmly interdigitated pseudopods in the minutes following their formation (Hovig et al, 1967; Sixma and Wester, 1977; Wester et al, 1978, 1979; Sixma, 1981). At the same time an increasing number of platelets shows progressively more degranulation, although the cell membranes of the majority of the platelets remain intact. After 10 minutes several fibrin strands are found on the periphery of the plug, but not in the center. In the following two hours more and more fibrin is deposited between the platelets from the periphery toward the center of the plug. Simultaneously the platelets are rounded off, become less densely packed and the interdigitated pseudopods disappear. Platelets seen as empty vesicles with perforated cell membranes appear throughout the plug. Leukocytes accumulate around the plug and infiltrate it. These changes progress and the initial platelet plug is transformed into a dense network of fibrin (Hovig et al, 1968; Sixma and Wester, 1977; Wester et al, 1979).

Although an inhibition of the formation of thrombin and fibrin does not clearly influence the development of the primary hemostatic plug, subsequent stabilization of the labile plug is impaired, resulting in frequent and prolonged rebleeding (Hovig et al, 1967, 1968; Bergqvist and Arfors, 1974). Platelets remain loosely packed with little degranulation (Hovig et al, 1967,

1968; Sixma and Wester, 1977; Sixma, 1981). Therefore, thrombin seems to play a central role in strengthening and stabilizing the initial plug, not only by means of fibrin formation, but also by inducing the release reaction and causing denser packing of the platelets and more interdigitation of the pseudopods. Moreover, the release and subsequent binding to the platelet surface of clotting factors, notably factor V, will accelerate the formation of thrombin. As mentioned, platelets secrete substances that attract leukocytes and stimulate fibroblasts. Secreted lysosomal enzymes may play a role in the lytic changes seen during plug transformation. However, damage of the membrane by the C_{5b-9} complex of the complement system is also possible, since platelets have been shown to secrete a complement activator and to initiate the production of the C_{5b-9} complex at their surface (Bennett and Ogston, 1981). Fibrin formation during transformation of the plug was especially seen between empty vesicles (Wester et al, 1979). Therefore, the lytic changes may promote fibrin formation, for instance, by exposing the cytoplasmic side of the cell membrane which is rich in phosphatidylserine, thereby accelerating clotting reactions (see section 1.2.1.).

1.3.4. Physical aspects of platelet function

In considering platelet function *in vivo* one should realize that the platelets are suspended in a constanly flowing liquid* in which the bigger red blood cells are present in excess.

One consequence of flow is that biochemical substances liberated from a damaged vessel wall or secreted from blood platelets are washed away down stream (Leonard, 1972; Schmid-Schonbein et al, 1976). The displacement toward the vessel center of small molecules originating from a wall injury on the basis of diffusive motion alone is small as compared to the downstream displacement due to flow. Consequently, platelets passing at a distance of

*Blood is a liquid flowing through cylindrical tubes (blood vessels). In most instances this flow is laminar, i.e. stable and with well ordered streamlines, and can be considered as many concentric, cylindrical layers of fluid sliding over each other. It is assumed that the layer of fluid adjacent to the vessel wall is stationary and that toward the center of the tube the velocity of the layers increases. The velocity gradient is called shear rate. Due to the internal friction of the fluid a force must be exerted to "shear" or move one layer of fluid past another. This deforming force is called shear stress. A measure for the internal friction of the fluid is the viscosity, which is defined as the ratio of the applied shear stress to the resulting shear rate (Charm and Kurland, 1972; Feigl, 1974).

approximately 1 μm or more from the wall will be reached by activating substances liberated from a wall injury downstream of the site of injury. Even if the diffusive motion is augmented by lateral and rotational movements of the red blood cells, causing small convection currents as has been proposed by Blackshear (Leonard, 1972), the downstream displacement will still prevail. However, beside radial displacement of activating substances, lateral displacement of the flowing platelets has to be considered (see below), promoting, among other things, contact of the platelets with the activating substances.

Shear stresses in the flowing fluid tend to tear off sticking platelets and to cause embolization of platelet aggregates. Moreover, due to their own velocity the time available for platelet activation and subsequent adhesion or aggregation is limited. In fact, in arterioles the available time must be in the order of milliseconds, which is considerably shorter than the 1-2 seconds "activation time" as determined in vitro in the aggregometer (Arfors and Bergqvist, 1974; Arfors et al, 1976a; Born, 1977).

Flow is essential to transport platelets toward a site of injury. For subsequent adhesion and aggregation platelets must come into contact with the wall and with each other by means of collisions. In this respect the following results obtained in in vitro experiments may be relevant (Goldsmith, 1972, 1978; Yu and Goldsmith, 1975; Goldsmith and Karino, 1977). Disk-shaped particles flowing through a small tube in a dilute suspension show an angular rotation. In addition, the flexible red blood cells tend to migrate away from the wall. It is assumed therefore that the concentration of red blood cells increases toward the center of the tube and that the interactions between the densely packed red blood cells result in continuous radial displacement of individual cells. The radial and rotational movements of the red blood cells are thought to cause considerable lateral displacement of platelets, resulting in frequent collisions with the wall and with each other. Such lateral excursions with wall encounters were observed microscopically in concentrated ghost cell suspensions for red blood cells, small latex particles and platelets, although the experiments were performed in creeping flow. However, in a perfusion chamber model at more physiological shear rates the deposition rate of platelets on subendothelium increased approximatly 57 times in the presence of red blood cells (Turitto and Baumgartner, 1975).

The possibility of activation of platelets by deforming shear stresses in the fluid has already been mentioned. Since these stresses are highest near

the wall, lateral displacement of platelets by red blood cell motions will favor this kind of activation.

1.4. PLATELET BEHAVIOR IN THE MICROCIRCULATION

The picture of the platelet emerging from present day knowledge is that of a highly complex and explosive cell which quickly responds to even slight changes in its environment. In vitro studies may therefore easily introduce unknown and uncontrolled artifacts, especially with regard to their functional behavior. Moreover, in contributing to physiological and pathological processes in the body, platelets always function in close connection and mutual influence with many other cells and functional systems, for instance the clotting system. The discrepancy between the effects of drugs or diets on platelet function in vitro and in vivo is well known (Butler and White, 1977; Hornstra, 1980). However, not only the efficacy of drugs or diets has to be tested in vivo, but also the correctness of other in vitro findings on platelet behavior as well as the hypotheses about the contribution of these findings to platelet-involved processes in the body.

Within the scope of platelet research in vivo, the microcirculation, comprising the smallest blood vessels (in general < 100 μm in diameter), offers a special advantage. It allows direct observation of platelets in their natural environment through the microscope and of their involvement in the reaction of blood to local trauma. However, such observations are difficult to perform in humans and hence have been restricted to experimental animals. Because platelets are small and transparent cells, study of the behavior of individual platelets in vivo has been limited to capillaries containing few red blood cells, and in larger vessels to situations with slowly flowing or stagnant plasma (Bizzozzero, 1882; Silver and Stehbens, 1965; Kwaan et al, 1967). In arterioles or venules with undisturbed flow platelets could only be observed if they were located close to the endothelial wall (Bloch, 1968; Schmid-Schonbein and Zweifach, 1975). Most studies have been engaged in observing the formation of a thrombus mass or hemostatic plug following trauma. Injury causing a break in the vessel wall, for instance puncture or transection of the vessel, results in a hemostatic plug (see section 1.3). To study thrombus formation a variety of less severe stimuli has been used, among others: ADP-iontophoresis (Begent and Born, 1970; Begent et al, 1972),

pinching of the vessel (Henry, 1962; Honour and Ross Russel, 1962; Didisheim, 1972), electrical trauma (Henry, 1962; Didisheim, 1972; Gordon et al, 1973) and laser injury (Wiedeman and Margulies, 1973; Kovacs et al, 1973, 1975; Poliwoda et al, 1973; Wiedeman, 1974; Hovig et al, 1974; Mckenzie et al, 1974; Arfors et al, 1976a). Such studies have yielded important information, many of which seem also relevant to platelet behavior in humans because no essential histological or ultrastructural differences were found between hemostatic plugs in experimental animals and in humans (section 1.3).

In conclusion, the survey shows that in contrast to the rapidly growing knowledge of platelet structure and biochemistry, the information about the rheology of platelets and the more physical aspects of platelet function is limited, while at present this information, which is largely based on models and in vitro studies, has not yet been adequately evaluated on the basis of animal experiments in vivo.

1.5. PRESENT STUDY

The starting point of the present study was the idea of visualizing the individual platelets flowing in a microvessel amidst the excess of red blood cells, since this would open up many new possibilities for the study of platelet behavior in the microcirculation. The search for a fluorescent dye which labels the platelets, but not the red blood cells, directly in vivo is described in chapter 2. This aim was achieved with the dye acridine red.

To study the rheology of platelets flowing in a microvessel, it is necessary to know their position in that vessel, not only perpendicular to the optical axis where the position is well defined, but also along the optical axis where the position is less clear. Therefore, we investigated whether it was possible to localize fluorescent platelets flowing in a microvessel within a finite and thin optical section (chapter 3). Using the intensity distribution of the microscopic image of the platelets an optical section could be defined with a depth of approximately 6μm, which is shallow enough to allow the investigation of their rheological behavior in, for instance, small arterioles. Consequently the distribution or concentration profile (chapter 4) and the orientation (chapter 5) of the platelets flowing in small arterioles was determined. Chapter 6 presents a summary and conclusions.

2. FLUORESCENT LABELING OF BLOOD PLATELETS IN VIVO*

2.1. INTRODUCTION

Processes in which platelets are involved can be studied in vivo in the microcirculation using intravital microscopy. After transection of a small vessel or infliction of a wall injury, the formation of a hemostatic plug (Bergqvist, 1974) and a platelet thrombus (Mckenzie et al, 1974) can be recorded, respectively. However, individual blood platelets flowing in microvessels outside the capillary region cannot be observed by normal brightfield microscopy (Schmid-Schonbein and Zweifach, 1975) unless they are located close to the endothelial wall. The detection of individual platelets flowing more centrally is hampered by the excess of red blood cells (RBC's).

In this chapter of the thesis we describe the investigations as to whether flowing platelets could be visualized individually by labeling them preferentially with a fluorescent dye, so that their rheological behavior could be studied. The aim was to label the platelets directly in vivo to avoid activation of the platelets due to handling in vitro and to obtain a high percentage of labeling of the circulating platelets.

Fluorescent staining or fluorochroming of blood cells, either in fixed smears or supravitally, has been reported for a number of, mostly basic, dyes (Schloshardt and Heilmeyer, 1942; Strugger, 1948; Kosenow, 1952; Contier, 1956; Price and Schwartz, 1956; Rothstein, 1958; Sokal, 1963; Morgenstern et al, 1963; Shively and Gott, 1967; Barbosa and Peters, 1971; Koslov, 1975; Da Prada and Pletscher, 1975; Thaer and Becker, 1975; Lorez et al, 1977). These dyes stain platelets, especially their granules, leukocytes and reticulocytes, but not RBC's. Intravital staining by intravenous injection of the fluorochrome has also been reported (Loeser et al, 1960; Lorez et al, 1977; Rosenblum, 1978). In addition, a number of fluorochromes can stain mast cell granules orange or red (Lillie, 1977). Several of these fluorochromes were investigated in the present study. Secondly, staining by fluorochromasia was investigated, i.e. , staining by intracellular enzymatic conversion of a non-fluorescent substrate into a fluorescent product which is accumulated within

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the cell because of its higher polarity (Rotman and Papermaster, 1966; Rotman 1973). Fluorochromasia with fluorescein-esters has been observed in leukocytes and platelets, but not in RBC's (Rotman and Papermaster, 1966; Rotman, 1973; Sengbusch et al, 1976), and with umbelliferone-glucuronide in swimming sperm cells (Udenfriend, 1969). Fluorogenic substrates were selected on the basis of the enzyme content of the platelet (Maupin, 1969).

In this study compounds that labeled platelets in vitro, were subsequently investigated in vivo. To prevent a smeared microscopic image of the flowing platelets, illumination with a flash of short duration had to be used. At the low light intensity of fluorescent emission under these circumstances, observation of fluorescent platelets in vivo could only be performed adequately with the fluorochrome acridine red (AR). Therefore, this dye and its influence on some platelet functions was studied in more detail.

2.2. MATERIALS AND METHODS

2.2.1. Animal preparation and test procedures

The experiments were performed on rabbits of various breeds and either sex, ranging in weight from 2.5-4.5 kg. The animals were anesthetized with intramuscular injections of 0.2 ml/kg body wt Vetalar (100 mg/ml ketamine hydrocloride, Parke-Davis) and 0.4 ml/kg body wt Rompun (2% solution, Bayer) when the mesentery preparation was used (anesthesia technique and mesentery preparation are described in more detail in chapter 3). In the other experiments, the rabbits were anesthetized with an intramuscular injection of 0.1-1.0 ml/kg body wt Hypnorm (fluanison 10 mg/ml and fentanyl 0.2 mg/ml, Philips Duphar). Blood was collected from the central ear artery, using an 18 G needle, after discarding the first drops. Platelet rich plasma (PRP) was obtained by centrifugation (Labofuge III, Heraeus Christ)- at 140g for 20 min- of blood collected in 0.1 vol trisodium citrate (0.129 M in all experiments, except for the aggregometer experiments where 0.153 M was used). Electronic platelet counts were made in duplicate in blood collected in 0.1 vol disodium EDTA (0.027 M) with a Coulter Counter (model ZF), using the method previously described by De Bruyere et al (1974); the diluting steps were performed with automatic diluters (diluter III, Hook and Tucker). The average value of the two measurements is presented in the result section. The hematocrit was measured in duplicate in an Autocrit II Centrifuge (Clay Adams).

2.2.2. Microscope and recording system

The microscope and recording system are described in more detail in chapter 3. In short, all observations were made with a modified Leitz intravital microscope. Fluorescence microscopy was performed with an incident fluorescence illuminator (Leitz Ploemopak 2.2), using water-immersion objectives with a high numerical aperture. The filter combinations employed with each dye (selected on the basis of literature data about color and spectral properties of the dye: Porro et el, 1963; Udenfriend, 1969; Guilbault, 1973; Lillie, 1977) are listed in table 2.1. In all experiments, a pulsed xenon arc (flash duration < 0.1 ms, 1-200 flashes/s) was used. In the in vitro experiments we also used a high pressure mercury lamp (HBO 200 Watt) with the filter combinations 3, 4, 5 and 6 (table 2.1). Registrations were made on a video-tape recorder (IVC 801 PSM) through a TV camera (RCA TC 1005/tube RCA 4532) coupled to a 3-stage image intensifier (RCA 4550).

2.2.3. Fluorochromes and enzyme substrates

Twenty fluorochromes and eleven fluorogenic enzyme substrates were tested in vitro. They are listed in table 2.1 together with their commercial source. Of each compound a stock solution, containing 3-5 mg/ml, was made in physiological saline (PS; 0.154 M NaCl), ethanol (96%), acetone, or a mixture of acetone or ethanol with PS. Of this solution, 5-80 µl was added to 1 ml PRP. After 1-30 min of incubation with the dye at room temperature, a drop of PRP was put on an object glass and the platelets were examined for fluorescence under the microscope. In case of a too strong background fluorescence, the incubated PRP was centrifuged at 1300g for 20 min. After removing the supernatant, the platelet pellet was washed (1-3x) and resuspended in PS (pH 7.3), containing Tris buffer (10 mM), and the platelets were again examined under the microscope.

The first six fluorochromes in the left column of table 2.1 as well as fluoresceindiacetate (FDA) and 4(5)-carboxyfluoresceindiacetate (CFDA) were also tested in vivo. Of the fluorochromes 2-4 ml stock solution (10-12 mg dye) were injected into a marginal ear vein within 20-30 s. FDA and CFDA (1-2 mg) were injected intravenously within 60-120 s after dilution (1:5) of their stock solution with PS. In these experiments, the mesenteric microcirculation was examined through the microscope during and after injection of the dyes.

TABLE 2.1

Fluorochromes and Fluorogenic Enzyme Substrates, their Commercial Sources, and the Filter Sets Employed.

Dye	Source	Filter	Dye	Source	Filter
Fluorochromes			Fluorescein (F)	P/B	2
			Xylenol O	E/K	1
Acridine Red (AR)	C	1			
Acridine Orange (AO)	C	2			
Pyronin B	Si	1	Enzyme Substrates		
Acriflavine	C	5,(3)			
Mepacrine	P/B	5,(3)	F-diacetate (FDA)	P/B	2
Auramine O	C	4,(2)	F-dibutyrate (FDB)	P/B	2
Safranin O	E/K	1	CF-diacetate (CFDA)	H	2
Euchrysine	P/B	3,(2)	F-dilaurate (FDL)	Se	2
Coriphosphine	C	4,(2)	MUF-N-acetyl-β-D-		
Neutral Red	C	1	galactosamid	Se	6,(3)
Acridine Yellow	C	3,(2)	MUF-N-acetyl-β-D-		
Rhodamine 6G	M	1	glucosamid	Se	6,(3)
Rhodamine B	P/B	1	MUF-sulphaat	Se	6,(3)
Rose Bengal	P/B	1	MUF-α-D-manno-		
Thiazine Red R	Se	1	pyranosid	Se	6,(3)
Alizarin Red S	E/K	1	MUF-β-D-glycuronid	Se	6,(3)
4(5)-carboxyfluo-			MUF-β-D-galacto-		
rescein (CF)	E/K	2	pyranosid	Se	6,(3)
4-methyl-umbelli-			MUF-β-D-gluco-		
ferone (MUF)	Se	6,(3)	pyranosid	Se	6,(3)

Source: C=Chroma, Si=Sigma, P/B=Pfalz and Bauer, E/K=Eastman Kodak, M=Merck, Se=Serva, H=CF converted to CFDA by treatment with acetic acid anhydride and pyridine (Merck) at 60°C (Korytnik, 1967).
Filter sets: 1=Leitz interchangeable filter set (LF) No. N2,1 (excitation filter: BP 515-560, dichroic mirror: RKP 580, barrier filter: LP 580); 2=LF No. I2 (BP 450-490, RKP 510, LP 515); 3=LF No. D (BP 355-425, RKP 455, LP 460); 4,5 and 6: filter combinations with 3 mm excitation filter, dichroic mirror and barrier filter, respectively (all components were obtained from Leitz) ; 4=UV-BG3 (BG12), RKP 455, K 530; 5=UV-BG3 (BG12), RKP 455, K 490; 6=UV-UG1, RKP 400, K 430. Filter sets within parentheses were less suitable.

2.2.4. Acridine Red

Working solution. A working solution of AR (dimethyl[6-(methylamino)--3H-xanthen-3-yliden] ammonium chloride; mol.wt 274.7) was made by dissolving 50 mg of the dye in 1 ml (in the first experiments 2 ml) PS, containing ethanol (50% v/v), and further diluting the solution with PS till a final concentration of 5 mg/ml (18.2 mM) was reached. In most experiments, this solution was subsequently filtered through a 0.22 μm filter (Cathivex).

A fresh solution was made at the day of the experiment. The pH of the working solution was 3.31, as measured with a PHM 62 Standard pH-meter (Radiometer). To get an approximation of the pKa of the dye by simple calculation (Williams and Williams, 1973), a solution of AR (10 mM) in distilled (2x) water was made. The pH of this solution was 3.36, and an apparent pKa of 4.72 was calculated.

Absorption and emission spectrum. The absorption spectrum of AR and of oxygenated whole blood, measured with a Beckman spectrophotometer (model 25), and the emission spectrum of AR, determined with a Zeiss fluorospectrophotometer (PMQ 2/ZFM 4), are shown in fig. 2.1. Both spectra of AR and especially the emission spectrum are favorably positioned with respect to the absorption spectrum of blood.

Labeling in vivo. For labeling in vivo the working solution was injected intravenously through a polyethylene catheter in the marginal ear vein, either by hand or by a syringe infusion pump (Harvard Apparatus, model 940A, or Sage Instruments, model 351). A dose of 0.5-3 ml was injected within 20-40 s. When repeated injections were given the interval between injections was at least 10-15 min.

Plasma concentration. To assess plasma concentrations of the dye during and after injection of 1-3 ml working solution, small blood samples were collected from 10 to 360 s after the start of the injection. The animals were heparinized with 500 IU/kg Thromboliquine (5000 IU/ml, Organon). No further anticoagulation was used. After centrifugation at 1300g for 20 min, the concentration of the dye was determined spectrophotometrically (wavelength 526 nm).

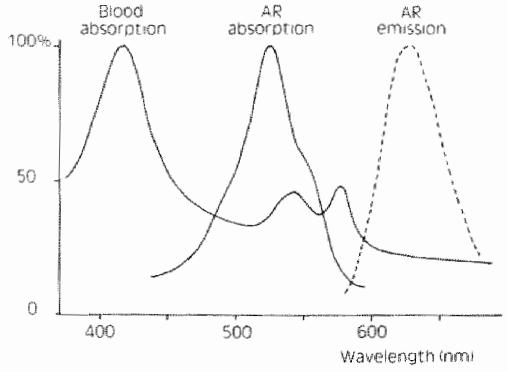


Fig. 2.1
Light absorption spectra of oxygenated whole blood and Acridine Red (AR), and fluorescence emission spectrum of AR.

Percentage of labeled platelets. To obtain an estimate of the percentage of labeled platelets, electronic (see above) and microscopic platelet counts were made simultaneously in the same blood samples by independent observers. The blood was collected in EDTA, 60 s after the end of an injection of 2 ml working solution. Microscopic platelet counts were performed in a Levy Ultra Plane Improved Neubauer Counting Chamber (model 4000, Clay Adams) after 1:100 dilution of the blood in isoton (Coulter). The fluorescent platelets within 5 to 6 scattered groups of 16 squares were counted at a final magnification of 500x. To locate the squares, short periods of weak transillumination were regularly combined with fluorescence microscopy.

Labeling of reticulocytes, if any. Basic dyes and especially Acridine Orange (AO) can stain reticulocytes (Schloshardt and Heilmeyer, 1942; Price and Schwartz, 1956; Thaer and Becker, 1975). Fluorescent reticulocytes might be mistaken for fluorescent platelets. Therefore,labeling of reticulocytes, if any, was assessed by fluorescence microscopy of individual RBC's located with short periods of weak transillumination. After labeling in vivo (2 ml working solution within 20 s) and processing of the blood as described above, 2000-2500 RBC's were studied in each blood sample. Labeling in vitro was done by adding AR (final concentrations 180 and 360 μM) or AO (final concentration 330 μM) to packed cells (for preparation see PRP, section 2.2.1.). In each sample 530-1400 RBC's were studied.

Electron microscopy. Transmission electron microscopy was performed on platelets labeled in vivo as well as in vitro. In the in vivo experiment, 3 ml blood was collected in a mixture of 1 ml EDTA and 6 ml 2.5% glutaraldehyde in phosphate buffer (0.1 M; pH 7.2) before and 1 min after the injection of 2 ml working solution. In the in vitro experiments, 1 ml PRP was incubated with the working solution (final concentrations 10 and 90 μM) or an equal amount of PS during 1 min, and then 2 ml of the 2.5% glutaraldehyde solution was added. After fixation (120 min, $4^{o}C$), platelet pellets were made by centrifugation of PRP at 2000g for 20 min, and postfixed in 1% osmiumtetroxide in phosphate buffer (60 min, $4^{o}C$). The pellets were dehydrated in graded ethanols and embedded in Epon 812. Ultrathin sections (Ultracut, Reichert-Jung) were stained with uranyl acetate and lead citrate, and examined on a Philips 400 electron microscope.

Influence on platelet aggregation in vitro. The influence of AR on platelet aggregation in vitro was studied with an aggregometer (Sixma, 1972). In a Payton Dual Channel Aggregation Module ($37^{o}C$, 1000 rev/min) 450 μl PRP

(blood collected in 0.153 M citrate) was preincubated with 20 µl $CaCl_2$ solution (0.033 M) for 2 min, and then 20 µl reagent solution was added. Reagents were graded dilutions of AR working solution or PS, containing ethanol (5% v/v), with PS. Aggregation was induced 1 min later by adding 10 µl of one of the following solutions (kept at 0°C): ADP (Sigma) 25 µg/ml in PS; bovine thrombine (prepared by the department of Biochemistry, Maastricht; Rosing et al, 1980) 0.25 mg/ml and collagen (Horm) 4 mg/ml. Recording was done on a LKB Biocal 2066 potentiometer recorder.

Influence on platelet behavior in vivo. In vivo the influence of AR on platelet behavior, if any, was evaluated by counting the number of circulating platelets and by testing their ability to form thrombi and hemostatic plugs, before and after the injection of AR.

Electronic platelet counts were made in blood samples taken before and at different intervals after the injection of 1-3 ml working solution.

Their ability to form thrombi was tested by inflicting a 200 Joule laser injury (ruby laser, model 153, TRG, New York) in mesenteric arterioles (20-30 µm diameter), either before or shortly following injection of 1-2 ml working solution. No attempt was made to quantify the reaction.

Hemostatic plug formation was evaluated as follows:

-in each of 6 animals, small blood vessels (< 0.5 mm) on the ears, between the central artery and marginal veins, were cut and the bleeding time (BT) was measured by sucking away the drop of blood every 10 s. After 6-8 cuts within 15 min, 3 ml PS were injected intravenously and again 6-8 transections were made within 15-20 min. Then 3 ml of the AR working solution were injected and another 6-8 vessels were cut within 15-20 min. Each vessel was observed for up to 5 min.

-twelve rabbits were randomly allocated to 2 groups. In one group, 1 ml of the AR working solution was injected intravenously 5 times; the other group served as control. In both groups, mesenteric arterioles (20-50 µm diameter) were transected and the primary hemostatic plug formation time (PHT) of the proximal end was measured (Bergqvist, 1974). The microvessels were observed for maximally 10 min. In the AR group, transections were made from 0.5-10 min after the end of each injection because platelet fluorescence was maximal at this interval.

-in 6 rabbits, 11 mesenteric arterioles (20-50 µm diameter) were transected following 2-7 injections of 2-3 ml AR working solution. The platelet reaction and plug formation in the proximal arteriolar ends were examined.

2.2.5. Statistical methods

To present platelet counts the mean values ($\bar{x}$) and standard deviations (s.d.) were used. To display BT and PHT, schematic plots were used (Tukey, 1977) because they exhibit an excellent visual summary of the data with preservation of the most important characteristics. The schematic plot shows the median, the interquartile range (i.e., the interval between the two values below which 25% and 75% of the population falls, respectively) and the range, minus those values which are straying out and minus indefinite values (BT > 300 s and PHT > 600 s), which are indicated separately. Stray values were calculated according to the definitions of Tukey (1977) for 'outside' and 'far out' values. Because BT and PHT have a skew distribution (Bergqvist, 1974; Healy and Ingram, 1978) and because indefinite values occur, nonparametric statistical tests were used to assess the significance of differences between groups. The Kruskal-Wallis one-way analysis of variance (two-tailed probability) was applied in the case of three groups (Dixon and Brown, 1979). To test a difference between two groups the Mann-Whitney U test (two-tailed probability) was used (Dixon and Brown, 1979).

2.3. RESULTS

2.3.1. Selection of the proper dye

In vitro, strong to good labeling of the platelets was achieved with AR, AO, pyronin B, acriflavine and mepacrine (in this order), and with FDA, fluoresceindibutyrate (FDB) and CFDA. Moderate to very weak labeling was obtained with safranin O, auramine O, euchrysine, coriphosphine and neutral red. No labeling could be achieved with the other dyes. At higher concentrations of the good labeling fluorochromes, the whole platelet was fluorescent. At lower concentrations individual granules could be distinguished, especially with AO which labeled the granules orange and the cytoplasm green. Specific labeling of granules was also easily achieved with auramine O. With pyronin B and safranin O spontaneous platelet aggregates were observed. FDA, FDB and CFDA were converted not only intracellularly, but also in blood plasma which caused a very high background fluorescence.

In vivo, visualization and registration of individual, fast flowing platelets by using illumination with one single flash could only be achieved adequately with AR, and to a lesser extent with pyronin B. With AO and

mepacrine slowly flowing or adhering platelets could be observed when illumination with multiple flashes (50-200/s) was used. Leukocytes, the vessel wall and tissue cells were also labeled by these four dyes. AO especially gave strong labeling of the leukocytes and excellent orange labeling of mastcell granules. The strong labeling of the vessel wall by AO hampered the registration of individual flowing platelets, since it often caused too much blooming on the TV camera at high flash power excitation. With auramine O only weak labeling of mastcell granules was observed. Platelets were labeled only slightly with FDA and CFDA, even when the background fluorescence had disappeared.

2.3.2. Acridine Red

Events in a microvessel following injection. Through the intravital microscope, the following events were observed in a mesenteric microvessel after intravenous injection of 1-3 ml of AR working solution. Ten-15 s after starting the injection the dye became visible and during 20-40 s the whole vessel fluoresced brightly. Then plasma fluorescence diminished rapidly (see also fig. 2.3). Individual platelets and leukocytes could be observed from approximately 30 s after the end of the injection; RBC's were not labeled. The labeling of the vessel wall permitted orientation within the microvasculature. Figure 2.2.A shows platelets and fig. 2.2.B platelets and a leukocyte flowing in a small arteriole. The pictures show that platelets can

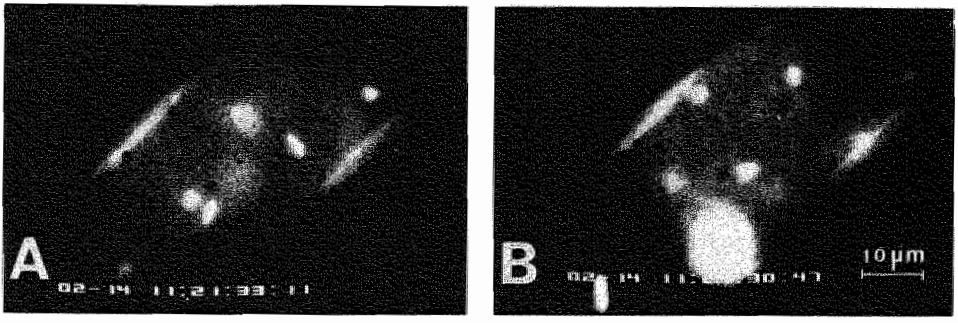


Fig. 2.2
Fluorescent platelets (A+B) and a leukocyte (B) flowing in a small arteriole. Plane of sharp focus is in the center of the vessel. Total optical magnification 200x. Bar applies to A and B.

be observed as disk shaped and homogeneously labeled particles, at least when they are within reasonably sharp focus. Sometimes a single platelet could be seen adhering to the vessel wall for a short period of time. Activation of the platelets as judged by aggregation was not observed. In most cases, leukocytes and platelets could be easily distinguished because of the difference in size and shape. The fluorescence of the platelets, varying from experiment to experiment, diminished in time. During 2-8 min, their brightness was sufficient to observe the individual platelets as 'frozen' in the blood stream by one single short flash. However, with a more frequent excitation (50-200 flashes/s), platelets adhering to a thrombus or to the vessel wall could still be observed 40-60 min later. Granules could then be seen within the platelets. When focusing in the center of the vessel, a sharp image of flowing platelets could be obtained over the full vessel width in microvessels with a diameter up to 35 μm, following an injection of 1-3 ml working solution.

Plasma concentration. In fig. 2.3, the course of the plasma concentration of AR following an injection of 1 ml working solution within 40 s and of 2 ml within 20 s is given. Following an injection of 3 ml within 40 and 30 s, a peak concentration was found of 90 and 120 μM, respectively.

Percentage of labeled platelets. The electronic and microscopic platelet counts made in the same blood sample (12 samples, 8 animals) were 396 ± 85 and 396 ± 139 x 10^9/l, respectively ($\bar{x}$ ± s.d.).

Labeling of reticulocytes,if any. In vivo, labeling of reticulocytes was not found: none of the approximately 7000 observed RBC's (3 injections, 2 animals) showed fluorescence. After in vitro labeling with AR 0.5% very weakly labeled and 1-2% weakly labeled RBC's were observed at final concentrations of AR of 180 and 360 μM, respectively. Labeling in vitro with AO yielded 2.5-5% strongly to well labeled RBC's, which corresponds with the reticulocyte values in rabbits as reported in the literature (Kozma et al, 1974).

Electron microscopy. Electron microscopic examination revealed no gross differences in ultrastructure between control platelets and platelets exposed to AR in vivo or in vitro. The platelets retained their discoid shape. Occasional pseudopod formation was seen to the same extent in both groups. The unit membrane was intact. In the interior of the cells, granules and dense bodies were still present.

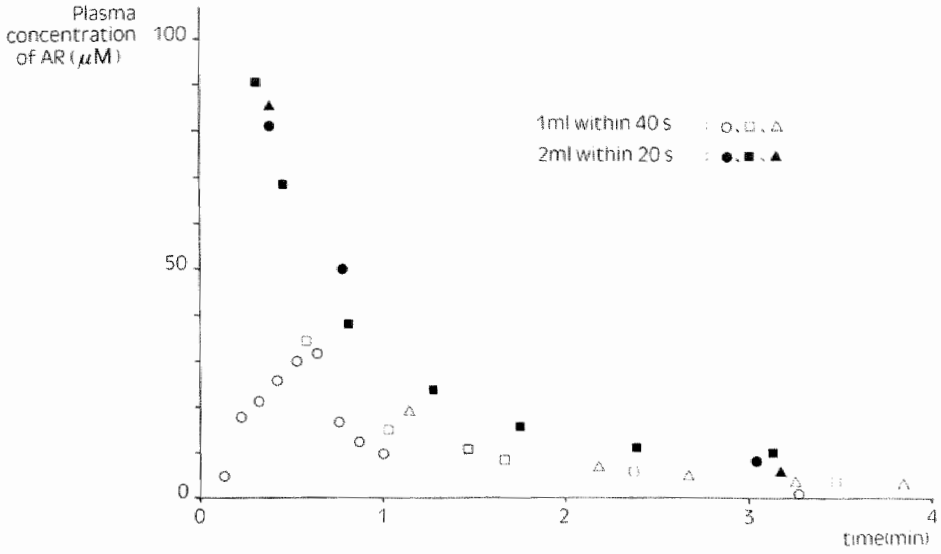


Fig. 2.3
Plasma concentrations of Acridine Red (AR) following an injection (start at time 0) of 1 ml working solution within 40 s (3 animals) and 2 ml within 20 s (3 animals). Each symbol represents the concentration halfway the sampling period. Sample times within 0-2 min and 2-5 min from time 0 were: 16 ± 15 s and 29 ± 12 s, respectively ($\bar{x}$ ± s.d.).

Influence on platelet aggregation in vitro. Tracings from the experiments on platelet aggregation in vitro are shown in fig. 2.4. PS and PS containing 5% ethanol in the same dilutions as used for the AR working solution served as controls. No differences between PS and PS containing ethanol were found. AR reduced platelet aggregation by ADP, thrombin and collagen in a dose dependent way. The greatest influence was found on collagen aggregation. At a final concentration of AR of 23 μM no aggregation response to collagen could be recorded, although at this dose the response to ADP and thrombin was practically normal. At a final concentration of AR of 186 μM, thrombin no longer induced an aggregation response, while some aggregation response to ADP was still observed. The dye itself induced no shape change or aggregation at final concentrations up to at least 186 μM, as fig. 2.4 also shows. When AR was added to a final concentration of 740 μM, an immediate and irreversible decrease in light transmission with loss of oscillations occurred following the addition of the dye.

Influence on platelet behavior in vivo. The electronic platelet counts before and at various intervals after an intravenous injection of AR working solution, as presented in table 2.2, show that disappearance of platelets from the circulation did not occur. A decrease in hematocrit was not observed.

TABLE 2.2

Electronic Platelet Counts at Various Intervals Following an Intravenous Injection of 1-3 ml AR Working Solution as a Percentage of the Control Count before Injection. At each Interval, the Mean Values and Standard Deviations are given. m = Number of Injections; n = Number of Animals. Control Count: 428 ± 87 x 10^9/l ($\bar{x}$ ± s.d.; m = 19; n = 10).

	0.2-1 min	1-5 min	30-60 min	80-120 min
$\bar{x}$ ± s.d. :	97 ± 13 %	103 ± 22 %	96 ± 14 %	98 ± 18 %
m :	5	16	9	4
n :	2	9	5	3

When a laser injury was inflicted in small arterioles either before or after injection of AR working solution, thrombus formation was seen in all cases. After the administration of AR, the reaction started immediately following the injury and looked qualitatively similar to the one in the control situation. Substantial thrombi formed from which small or large parts embolized, whereupon the thrombus grew again by adhesion and aggregation of platelets. Figure 2.5.A shows a thrombus composed of fluorescent platelets and fig. 2.5.B shows a small aggregate breaking loose to embolize.

The BT's as measured on the ear are depicted in fig. 2.6. In the AR group, the scatter increased and a shift occurred towards longer BT's. A significant difference was found among the 3 groups ($p=0.04$). The noninjection group and the PS group did not differ significantly. The AR group differed significantly from the noninjection group ($p=0.02$), as well as from the PS group ($p<0.05$). However, fig. 2.6 shows that in the AR group short BT's still occurred in a considerable number of vessels, and that bleeding stopped (within 300 s) in all but two of them.

The PHT's as obtained in the mesentery following one or more injections of 1 ml working solution of AR are presented in fig. 2.7. The most prominent feature shown in this figure is the increase in scatter after the third injection of AR. The significance levels of the differences between each

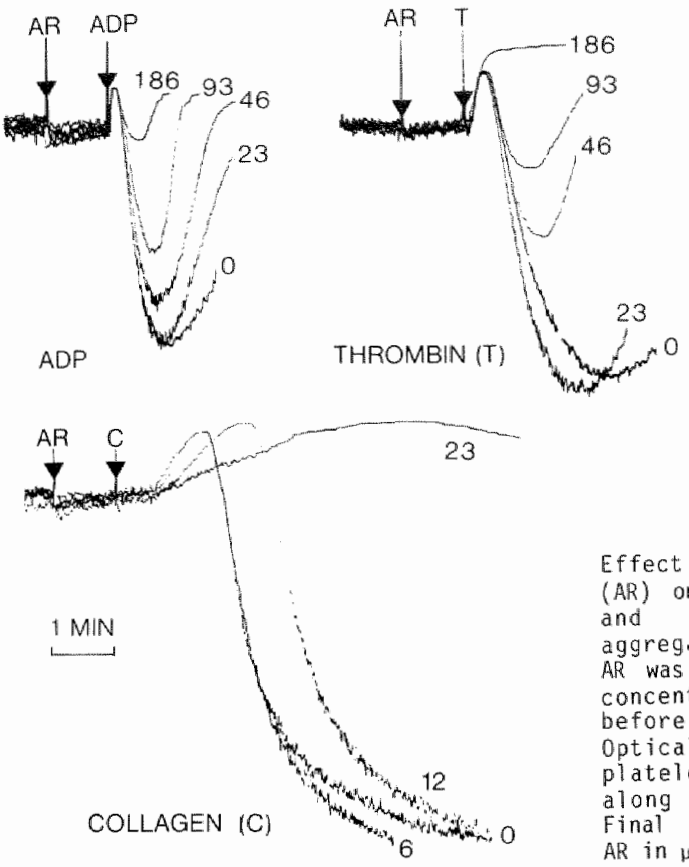


Fig. 2.4
Effect of Acridine Red (AR) on ADP-, thrombin- and collagen-induced aggregation in vitro. AR was added in various concentrations 1 min before aggregation. Optical density of the platelet rich plasma along the ordinate. Final concentrations of AR in μM.

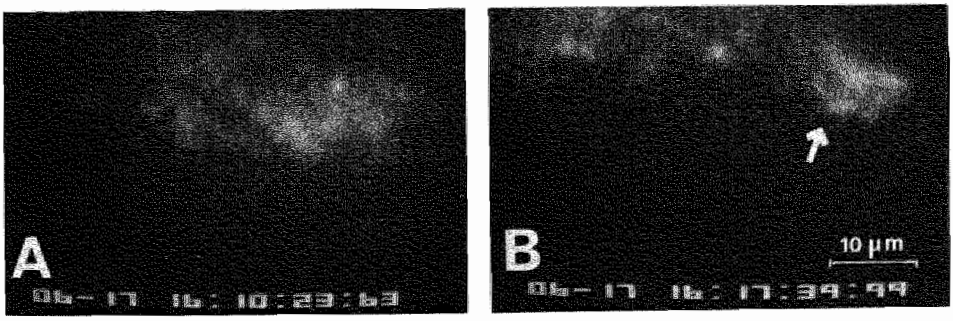


Fig. 2.5
Laser-thrombus (A) and beginning embolus (B, arrow) composed of fluorescent platelets. Total optical magnification 200x. Bar applies to A and B.

injection group and the control group are presented in fig. 2.7 as well. In one animal of the control group, platelet aggregation and plug formation were clearly depressed and bleeding did not stop in half of the vessels. Therefore, these data were excluded. In one control animal, only one PHT could be obtained for technical reasons. To test whether an increasing number of transections in a preparation would influence the PHT, for instance due to a possible deterioration of the preparation, the data in the control group were allocated to three subgroups. The first subgroup consisted of the first four PHT's obtained in each animal, and the second and the third subgroup consisted of the fifth through eighth PHT's and nine or more PHT's in each animal, respectively. No significant differences were found among these three subgroups. Although after the third injection of AR the scatter in PHT increased considerably, generally good aggregation of the platelets was observed even in the cases with a long PHT. However, parts of the plug embolized much easier and hence final cessation of blood flow was more difficult to achieve. In three vessels in which bleeding did not stop within 600 s following the fourth or fifth injection, good plug formation occurred with PHT's varying from 140-230 s when the vessel was subsequently transected more proximally.

Following multiple injections of 2-3 ml working solution of AR, qualitatively normal platelet aggregation and the formation of plugs with stop of bleeding were observed after transection of mesenteric arterioles.

2.4. DISCUSSION

The study presented in this chapter shows that during 2-8 min following an intravenous injection of the fluorochrome AR, a clear microscopic image can be obtained of individual blood platelets flowing in a microvessel. Stationary platelets can be observed during 40-60 minutes. The good agreement between the electronic and microscopic platelet counts made in the same blood sample indicates that almost all the circulating platelets are labeled. Leukocytes and the vessel wall were also labeled, but in vivo reticulocytes did not label with this dye, neither did RBC's. In most cases, leukocytes and platelets can be easily distinguished.

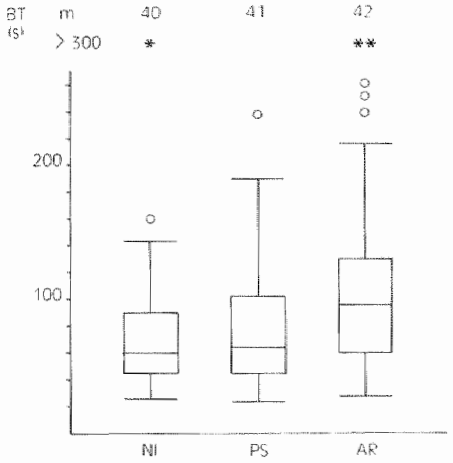


Fig. 2.6
Bleeding time (BT) as assessed in small vessels on the ear for noninjection-group (NI), PS-group (injection 3 ml) and Acridine Red (AR)-group (injection 3 ml working soluton). Schematic plot showing median, interquartile range (box) and range (horizontal bars), minus stray values (0) and BT > 300 s (*), which are indicated separately (see section 2.2.5). m = number of measurements.

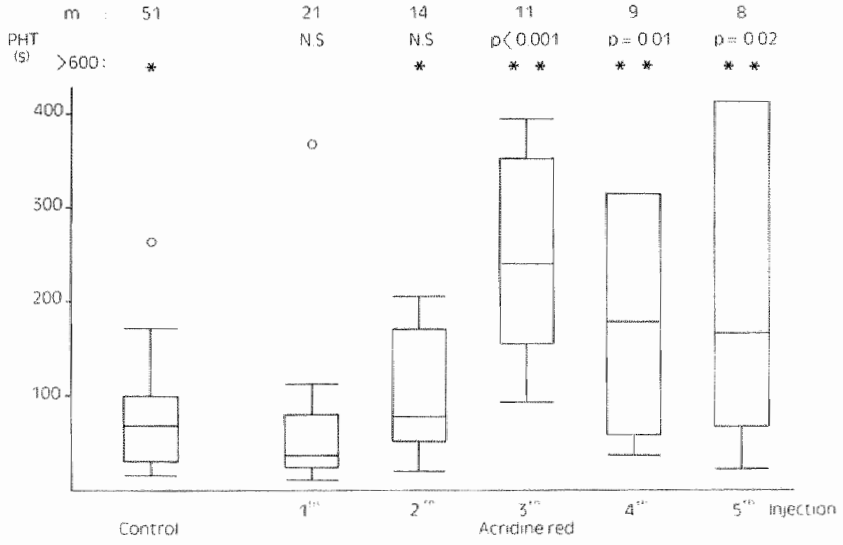


Fig. 2.7
Primary hemostatic plug formation times (PHT) of proximal arteriolar ends in the mesentery for control group (no injection) and a group of animals with 5 subsequent injections of 1 ml Acridine Red working solution. Schematic plot (see legend fig. 2.6); stray values (0) and PHT > 600s (*). m = number of measurements. The significance levels of the differences between each injection group and the control group are indicated. N.S. = not significant.

AR did not induce activation of the platelets *in vivo*. *In vitro*, the dye did not induce shape change or aggregation up to a concentration of at least 186 μM, which is much higher than the peak values measured *in vivo*. A fall in platelet count or hematocrit following the injection of AR did not occur. Electron microscopy revealed no gross ultrastructural changes.

In vitro, AR reduced platelet aggregation by ADP, thrombin and collagen in a dose dependent way. Comparing the final concentrations of the dye in the cuvette with the plasma concentrations during and following intravenous injection, the results of the aggregation experiments *in vitro* suggest an influence of AR *in vivo* on collagen aggregation starting at an injection of 1 ml working solution, and on ADP and thrombin aggregation starting at an injection dose of 2 ml. However, *in vivo* aggregation and adhesion of platelets as induced by laser injury or transection of an arteriole could be observed in all cases,not only following a single injection of 1 ml working solution of AR, but also following multiple injections of 2-3 ml. After the first and second injection of 1 ml working solution the PHT did not differ significantly from the PHT of the control group, which might indicate that quantitatively the platelets also behaved normally in these cases. On the other hand, following the third through fifth injection of 1 ml working solution the scatter increased considerably. The differences in PHT between these three groups and the control group were significant. Since no clear influence of time was found within the control group, this effect is probably due to AR. Following an injection of 3 ml working solution of AR, a relatively high dose, an increase in scatter and prolongation of BT was observed. The AR group differed significantly from both the noninjection and the PS group. However, even at this dose bleeding did stop in all but two vessels, indicating that aggregation and adhesion of platelets occurred. In the mesentery, platelet aggregation looked qualitatively normal and plug formation with cessation of bleeding did occur even following multiple injections of 2-3 ml working solution.

The observation that *in vitro* platelet aggregation by ADP, thrombin and collagen is reduced at concentrations of AR, which influence platelet function *in vivo* only to a limited extent, is in agreement with the discrepancy between the *in vitro* and *in vivo* findings on the sensitivity of platelets to drugs and dietary effects on platelet behavior (Butler and White, 1977; Hornstra, 1980). Therefore, the *in vitro* data on the influence of AR on platelet aggregation should be interpreted with care with respect to

the influence of the dye on platelet function in vivo.

The findings in this study indicate that the labeling procedure with AR is suitable to study in vivo the rheological behavior of individual platelets. For the study of this behavior the following results are important: the high percentage of labeled platelets, the little chance to mistake other blood cells for platelets, the absence of gross morphological changes of platelets, the absence of platelet activation by the dye and the absence of a fall in platelet count or hematocrit following injection of the dye. In light of these results, the rheological behavior of the platelets is presumably not influenced by an intravenous injection of 1-3 ml working solution of AR. Although less evident, the rheological behavior of the platelets may also depend on their ability to react with chemical stimuli. Since the in vivo data on laser injury and vessel transection show that even after an intravenous injection of 3 ml working solution of the dye the platelets can adhere and aggregate, it is likely that their rheological behavior is not significantly influenced by a functional depression of the platelets, if any, at these doses. Whether this technique can also be used to study functional platelet behavior in vivo, needs further investigation.

The labeling of platelets by AR can be explained by the basic nature of the dye (Lillie, 1977). Since the pH of blood plasma is much higher than the pKa of AR, less than 0.25% of the dye molecules in plasma will be dissociated. Entrance into the cell will therefore take place by simple, nonionic diffusion, depending on the lipid solubility (Goodman and Gilman, 1975) and facilitated by the hydrogen ion gradient across the cell membrane (Solomon and Zieve, 1967; Goodman and Gilman, 1975). AR readily dissolves in ethanol. Intracellularly, the basic nitrogen atom of the dissociated dye molecules can bind to negatively charged groups on macromolecules (Loeser et al, 1960; Solomon and Zieve, 1967; Lillie, 1977). Beside binding to macromolecules, small basic dyes or drugs have been reported to accumulate within platelet granules (Sokal, 1963;Solomon and Zieve, 1967; Koslov, 1975; Da Prada and Pletscher, 1975; Lorez et al, 1977). Labeling of granules was also observed with AR. Disappearance of labeled platelet granules after thrombin induced aggregation has been observed in vitro (Sokal, 1963; Koslov, 1975). To what extent monitoring of the release reaction will be possible in vivo cannot be concluded from the present study.

The binding of AR to macromolecules, for example enzymes or receptor molecules, could be responsible for a functional depression of the platelets.

The acridine derivative mepacrine has been reported to inhibit phospholipases and the binding of fibrinogen to platelets (Winocour et al, 1981). However, despite its name, one should realize that AR is not an acridine derivative, but a xanthene (Lillie, 1977). An inhibitory or activating effect on the platelets of the ethanol in the working solution seems unlikely. No effect of PS, containing ethanol, was observed in vitro. Besides, the ethanol concentration in vivo will be considerably lower than that reported to inhibit platelets (Mcgregor and Renaud, 1981) or to induce micro-aggregates (Elmer et al, 1977).

The difference between AR and the other dyes in visualizing platelets in whole blood may, apart from differences in labeling, be explained by the favorable spectral properties of the dye. In this respect, basic fluorochromes with both their absorption and emission above 600 nm wavelength are interesting dyes.

A fluorescent dye which labels the platelets for their entire lifetime may serve, in combination with rapid flow cytofluorometry (Van Dilla et al, 1969; Rotman, 1973) and electronic counting techniques, as an alternative to the labeling with isotopes in the study of platelet survival. None of the compounds used in this study seems to label the platelets long enough, not even CF which in liposomes has a halftime of weeks (Weinstein et al, 1977). However, more permanent labeling of platelets may be achieved by labeling in vitro with fluorescein-isothiocyanate or fluorescamine (Puchinger et al, 1976).

In conclusion, a clear microscopic image of individual, fluorescent blood platelets flowing in a microvessel can be obtained over the whole cross-sectional area of the vessel, following an intravenous injection of the dye AR. The findings in the present study indicate that the labeling procedure with AR allows the study of the rheological behavior of the platelets in vivo.

2.5. CONCLUSIONS

- In rabbits, a clear microscopic image of individual, fluorescent blood platelets flowing in a mesenteric microvessel could be obtained over the whole cross-sectional area of the vessel following an intravenous injection of a solution containing 5-15 mg of the fluorochrome acridine red.
- Following injection of the dye almost all the circulating platelets were labeled, while activation of the platelets or a fall in platelet count or hematocrit did not occur. Electron microscopy revealed no gross ultrastructural changes.

- In vitro, the dye reduced platelet aggregation as induced by ADP, collagen and thrombin in a dose dependent way.

- In vivo, aggregation and adhesion of platelets as induced by laser injury or transection of an arteriole was observed in all cases, even following multiple injections.

- Primary hemostatic plug formation times as measured in mesenteric arterioles were normal after the first and second injection of 5 mg acridine red, but prolonged after subsequent injections. Bleeding times as measured on the ear were prolonged following injection of 15 mg of the dye.

- The labeling with acridine red allows the study of the rheological behavior of the platelets in vivo. Whether the technique can also be used to study functional platelet behavior in vivo needs further investigation.

3. LOCALIZATION WITHIN A THIN OPTICAL SECTION OF FLUORESCENT BLOOD PLATELETS FLOWING IN A MICROVESSEL*

3.1. INTRODUCTION

Blood platelets flowing in small arterioles or venules can only be observed by normal bright-field microscopy if they are located close to the endothelial wall (Schmid-Schonbein and Zweifach, 1975). The detection of individual platelets flowing more centrally in the vessel is hampered by the excess of red blood cells (RBC's). However, when they are preferentially labeled with a fluorescent dye, individual platelets can easily be detected among the nonlabeled RBC's by fluorescence microscopy, even when they are located deep in the vessel. A smeared image due to moving of the platelet can be prevented by using illumination with a flash of short duration, yielding an image of the particle fixed in time and place.

To study the rheology of fluorescent platelets in a microvessel, it is necessary to know their position in that vessel. Perpendicular to the optical axis the position is well defined. Along the optical axis, however, the position can only be assessed accurately when the depth of field is small and the image of the particle is sharp (James, 1976). The region of sharp focus of an object is normally determined by focusing up and down several times. This is not possible when one single flash is used to fix a flowing particle. In that case, it is not precisely known whether the observed fluorescent particle is properly focused. Moreover, the sharpness of the image of particles deep in a vessel decreases due to overlying layers of RBC's. So, the position of a fluorescent platelet along the optical axis, when observed by one single flash, is uncertain. It may well be that a platelet which is observed when focusing in the center of a vessel is actually positioned more to the anterior or posterior wall of this vessel.

In this chapter of the thesis a method is described to localize fluorescent platelets, flowing in small arterioles and visualized by one single flash, within a finite optical section. The depth of this section was sufficiently shallow with respect to the vessel diameter to localize platelets

*Tangelder G.J., Slaaf D.W., Teirlinck H.C., Alewijnse R. and Reneman R.S. Microvascular Research 23, 214-230, 1982.

at various sites in the vessel along the optical axis. This allows the study of their rheological behavior *in vivo*. To obtain information about the characteristics of the optical section *in vitro* experiments were performed with latex spheres, the size of which was comparable to that of platelets. In addition, *in vitro* experiments were performed with leukocytes. A theoretical analysis was made to estimate the influence of orientation of a platelet on the depth of the optical section.

3.2. METHODS

3.2.1. Microscopic system

All observations were made with a Leitz intravital microscope adapted to telescopic imaging (Slaaf et al, 1982). The normal ocular was replaced by a projection eyepiece and a transfer lens (focal length 322 mm) to accomplish final imaging of the intermediary image. Fluorescence microscopy was performed with an incident fluorescence illuminator (Leitz Ploemopak 2.2, tube factor 1.6x) and a xenon flash arc fed by a Strobex power supply (Chadwick Helmuth 136).

The images were displayed on a TV monitor (Siemens) through a TV camera (RCA TC 1005/tube RCA 4532) coupled to a three-stage image intensifier (RCA 4550). Coupling was achieved by a 1:1 imaging lens system consisting of a Nikon lens (Macronikkor 55 mm/3.5), a PK 13 ring, and a C-mount adapter. Using white light (2858 K), this combination is 70 times more sensitive than the TV camera. The resulting signal on the TV camera is linear with respect to the light level at the front of the image intensifier. For off-line analysis, the images were recorded on a videotape recorder (IVC 801 PSM). Flashes of short duration (< 0.1 ms) were given in the blanking period of the TV camera every 9th or 10th field, so that the pictures did not contain a residual image from the previous flash.

Leitz water-immersion objectives with a high numerical aperture (NA) were used: SW 25x (NA 0.60), SW 50x (NA 1.00), and SW 100x (NA 1.20). Two Leitz projection eyepieces were used: 1x and 2x. The final magnification at the front plane of the image intensifier, and thus the TV camera, was two times the magnification of the objective lens using the 1x projection eyepiece and four times this magnification when the 2x eyepiece was used. Final magnifications of 100x, 200x, and 400x were employed.

In measuring distances along the optical axis several aspects have to be considered. With regard to the influence of differences in refractive index, only the difference between the medium surrounding the particle and the medium in contact with the objective is important, while different indices of structures in between do not affect the measurements (Françon, 1961). The refractive index of the isotonic solution superfusing the preparation (in which the SW objectives are immersed) differs only slightly from that of the blood plasma surrounding the observed particles (Barer, 1956; Ross, 1967). Calculations showed the resulting error in the depth measurement to be approximately 1%. Consequently, the influence of differences in refractive index on the measurements could be neglected.

The fine adjustment of the microscope was calibrated with two different precision micrometers (Tesa and Mitutoyo); one division was 1.0 µm. There was no dead turn, as could be checked with a lens combination with a narrow depth of field (0.7 µm).

Curvature of field was tested with a flat interference grating (d=1 µm). Settings of the fine admustment with sharpness of focus in the center and at the border of the field of view were compared. At a final magnification of 200x, no difference could be detected between these sites with the SW 100x objective and a difference of 1 µm was found with the SW 50x. At a final magnification of 100x, a difference of 2-3 µm was found with both the SW 50x and the SW 25x. Therefore, the SW 50x and the SW 25x were used only in the experiments with latex spheres, which were studied after being positioned in the center of the field of view. In all other experiments, the SW 100x was used.

3.2.2. Materials and preparation

The following fluorescent particles were used: fluorescein-isothiocyanate (FITC)-labeled latex spheres with a diameter of 1.50 ± 0.01 µm ($\bar{x}$ ± s.d.; Paesel GmbH, Frankfurt, West Germany; the first batch was kindly provided by Dr. M. Steinhausen from Heidelberg), platelets labeled with Acridine Red (AR) and leukocytes labeled with Acridine Orange (AO). AR and AO were obtained from Chroma (Stuttgart,West Germany). A working solution of AO (16.5 mM) was made in physiological saline (PS; 0.154 M NaCl) and of AR (18.2 mM) in PS containing ethanol (5 or 10% v/v; see chapter 2). The platelets and leukocytes for the in vitro experiments were labeled by adding 20 µl working solution of the dye to 1 ml platelet rich plasma (PRP). PRP was obtained by

centrifugation (20 min, 140g) of blood collected from the central ear artery of rabbits of various breeds in 0.1 vol trisodium citrate (0.129 M). For the experiments with leukocytes the top layer of the buffy coat was sucked up. For the in vivo experiments, the platelets were labeled with AR in situ, i.e. within the animal. In the incident fluorescence illuminator, the following Leitz interchangeable filter sets were used: No. I_2 for FITC and AO (excitation filter: BP 450-490; dichroic mirror: RKP 510; barrier filter: LP 515) and No. $N_{2,1}$ for AR (excitation filter: BP 515-560; dichroic mirror: RKP 580; barrier filter: LP 580).

In vitro experiments were performed in an isolated mesentery preparation of the distal ileum obtained from dogs and rabbits killed for other acute experiments. The preparation was spread on the microscope stage and superfused with a buffered Tyrode solution at 37°C (Reneman et al, 1980). Through a polyethylene catheter in the feeding artery the preparation was perfused with PS until visual bloodlessness. Then, a solution with fluorescent particles was injected. Before injection, the latex spheres and PRP were diluted with PS, approximately 1:10. The particles were studied after fluid movement in the vessels had stopped and they were motionless, sticking to the vessel wall or caught in small vessels. Latex spheres were also studied fixed on an object glass under a thin layer of nail polish (Cutex, Chesebrough-Pond's).

In vivo experiments were performed on seven rabbits of various breeds and either sex, ranging in weight from 3 to 4 kg. The animals were anesthetized with intramuscular injections of 0.2 ml/kg body wt Vetalar (100 mg/ml, Parke-Davis) and 0.4 ml/kg body wt Rompun (2% solution, Bayer). Surgery was started 5-10 min after the second injection of these compounds, which was given 15-20 min after the first one. Anesthesia was maintained by injecting the same dose of the drugs every 30 min. The animal was placed on an electrically heated (30-35°) microscope table. Through a small midline incision a short segment of distal ileum was brought outside and carefully spread over a siliconized glass plate mounted on an electrically heated (37-38°) microscope stage. The preparation was continuously superfused with the buffered Tyrode solution at 37°. The bowels were covered with moistened swabs. A polyethylene catheter was placed in a marginal ear vein and 2-3 ml of the working solution of AR was injected within 20-30 s. Following the intravenous injection of the dye, platelets were studied when sticking to the wall of venules, when caught in small vessel segments where flow had stopped, or when flowing in small arterioles (20-35 μm in diameter). Blood was collected from the central ear

artery in 0.1 vol disodium EDTA (0.027 M) and the platelets in this sample were counted in duplicate with a Coulter Counter (model ZF), using the method described by De Bruyere et al (1974).

3.2.3. Experiments and analysis

In the experiments where the particle of interest was motionless, its position and the levels at which its image disappeared were determined by focusing up and down through the preparation in steps of 0.5 or 1 μm. Using the latex spheres as model particles, the influence of NA and total magnification on this disappearing phenomenon was investigated, using different combinations of objectives and eyepieces. The influence of illumination was studied either by varying the output of the power supply, which changed flash duration and/or amplitude (flash power range 1-32x), or by placing neutral density filters in the excitation beam, which only changed the amplitude. Neutral density filters were also placed between eyepiece and transfer lens as well as between the image intensifier and the TV camera. The influence of the type of illumination, if any, was investigated by changing from incident illumination to transillumination (Condenser: NA 0.70; Excitation filter: 3 mm BG 12; Leitz filter set No. I_2 in Ploemopak 2.2). In the experiments with platelets flowing in small arterioles the level of focus in the center of the vessel, yielding the widest vessel diameter, was first established (zero focal position). Subsequently, we focused upward in small steps, the first being 5 μm, the next ones 2 or 1 μm.

Off-line analysis was done field by field with an electronic system that displayed the signal of a single TV line which was selected with a modified video-dimension analyzer (IPM 303). The signal of this TV line was fed into a 100-MHz transient recorder (Biomation 8100) with a 2-kbyte digital memory and displayed on an oscilloscope (Tektronix T922) or a physiological recorder (Schwarzer). The TV line through the center of the image of a particle was selected and the displayed intensity distribution was used to assess the presence of a particle in an objective way. By changing the sample interval of the transient recorder, the whole TV line or a selected part of it could be displayed. A particle was considered to be in sharp focus when the amplitude of the intensity distribution curve was maximal.

For symmetrical particles like latex spheres, the degree of sharpness of the image of the particle can be determined from the shape of its intensity distribution curve, at least when the signal-to-noise ratio is adequate.

This technique, however, cannot be used in the case of asymmetric particles with different orientations toward the optical axis, such as platelets flowing in a microvessel, or in the case of inadequate signal-to-noise ratios. Therefore, only the amplitude of the intensity distribution curve was used to define the optical section containing these particles.

A particle was considered to be within the section as long as the signal clearly exceeded the background noise. This was assessed by feeding the signal into one channel of a dual-trace oscilloscope, and positioning the tracing of the empty channel along the top of the background noise. As long as the signal exceeded the empty tracing, resulting in a signal-free area between the two tracings, the particle was considered to be within the section. This decision can be made by eye rather accurately. The procedure is easy and quick. Using this definition, the depth of the section was determined for the different particles. An additional advantage of this definition is that local blooming and saturation of the TV camera are no longer disturbing factors, because the particles are considered to be within the section when the above condition is fulfilled.

3.3. RESULTS

Since various optical factors influence the section depth, the effect of these factors will be described at first to be able to interpret the *in vivo* and *in vitro* findings adequately. Because of the asymmetry of the platelets these effects were studied in experiments with latex spheres.

3.3.1. Latex sphere experiments

Determination of the optical section

By focusing up or down from the level of sharp focus, the image of a fluorescent particle spread out and its brightness decreased until the particle could no longer be observed. Simultaneously the amplitude of the intensity distribution of the TV line through the center of the particle decreased, while the width of this signal increased. Finally, the signal disappeared in the noise. Figure 3.1 shows an example of the images and concomitant signals of a nonmoving latex sphere in an isolated mesentery preparation.

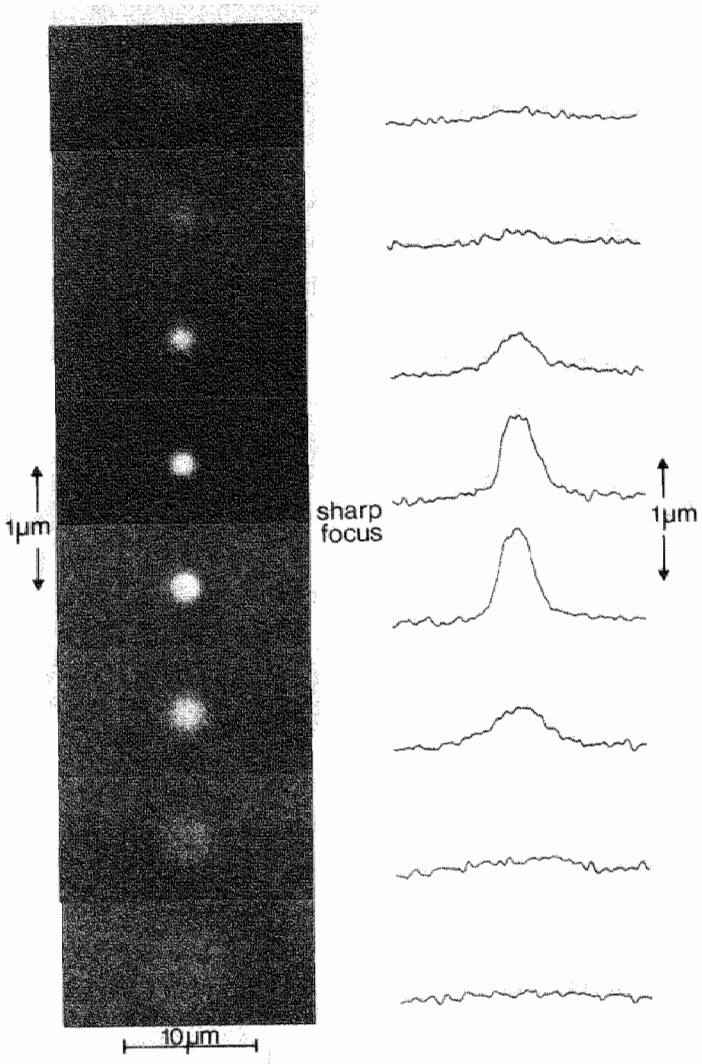


Fig. 3.1
Images and concomitant intensity distribution curves of a nonmoving latex sphere in an isolated mesentery preparation, as obtained by defocusing up and down in steps of 1µm at a total optical magnification of 200x and constant flash power.

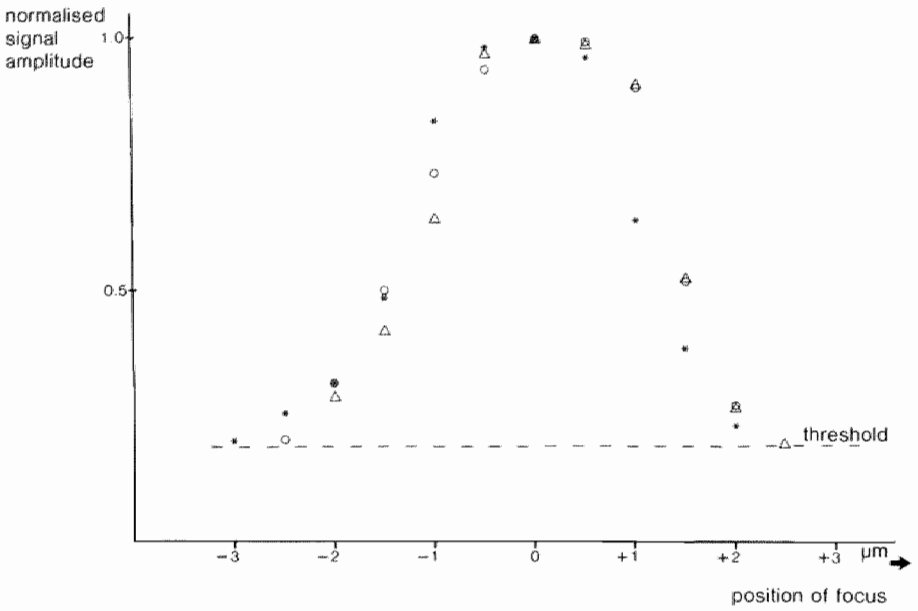


Fig. 3.2
Normalized signal amplitude as a function of position of focus, measured for three (Δ, o, *) latex spheres. Each point represents the mean of 10 measurements. Defocusing was done in steps of 0.5 μm. Zero is the point that was considered to be sharp focus. Increase in distance between particle and objective lens: +; decrease: -. The total optical magnification was 200x.

The decrease in amplitude of the intensity distribution curves of three latex spheres with defocusing is shown in fig.3.2. Recording was performed at a flash power at which no saturation of the TV camera occurred. The slight variations in position of the various graphs along the horizontal axis may result from the fact that the exact point of sharp focus was not precisely known due to the stepwise defocusing procedure. An important feature shown by this amplitude defocusing graph is that the amplitude of the signal decreases gradually as long as the plane of perfect focus stays within the object. However, as soon as this plane exceeds the object limits the amplitude drops steeply. Increasing the flash power proportionally raised the height of each point of this amplitude defocusing graph. The possible mechanism involved in the decrease in amplitude of the signal with defocusing and especially the steep drop in amplitude, is discussed in Appendixes 3.A and 3.B.

As can be seen in fig.3.1, the effect of defocusing on the image is nonsymmetric. This effect has only a minor influence on the symmetry of the section (fig. 3.2). Sections were found to be almost symmetrical around the level of sharp focus.

Factors influencing the depth of the section

Since the lighting intensity of the image at the TV camera is used to define the section, the depth of the section for a given particle is determined by the total amount of luminous flux received at the TV camera and the rate at which it is spread over an increasing surface area by defocusing. The luminous flux depends on the labeling efficiency - being the total number of dye molecules per particle -, the quantum yield of the fluorochrome, the flash power, the light gathering power of the objective lens, and the amount of absorption and scatter by structures between the object and the TV camera. The surface area receiving this flux depends on the total magnification which determines the rate of spreading of the image. Of all these factors the total magnification was found to have the greatest effect on the section depth.

Total magnification. Increasing the total magnification by a factor of 2 reduced the depth of the section by approximately the same factor. The effect of doubling the total magnification by changing the eyepiece on the section depth of latex spheres is shown in fig. 3.3.

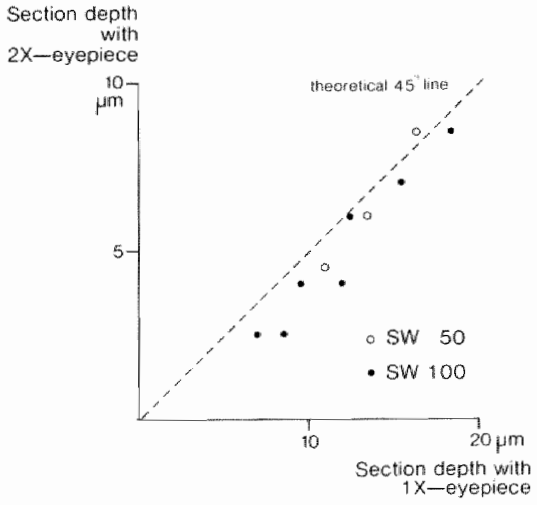


Fig. 3.3
Change in section depth of latex spheres when doubling the eyepiece magnification, using the same objective at a constant flash power. The results of 2 objectives over a wide range of flash powers are shown. Note the different scales along the axes.

At the same total magnification the section depths found with the SW 25x and SW 50x objectives were approximately the same, using flash powers corrected for the difference in light gathering power between the two objectives (see below). With the SW 50x and SW 100x these depths were similar without correction for differences in light gathering power. When the total magnification was doubled by shifting from the SW 50x to the SW 100x objective the section depth reduced by a factor of 2.

Illumination. In contrast with the effect of total magnification a twofold increase in the amount of luminous flux incident upon the TV screen did not increase the depth of the section by the same factor. Figure 3.4 shows that at an increase in illumination power by a factor of 2^{α} the section depth (in μm) of a latex sphere increases according to the straight line $f(\alpha) = f(0) + 2\alpha$ up to a section depth of approximately 9 μm and thereupon according to the straight line $f(\alpha) = f(0) + 3.2\alpha$. Also shown is the expected $x^{1/2}$ fit (where $x = 2^{\alpha}$), assuming that at a twofold increase in illumination the receiving surface area on the TV screen has to increase by the same factor in order to keep the amplitude of the intensity distribution curve the same. The figure shows that at higher values the increase in section depth is less than expected. The figure also shows that the type of illumination has no influence. Changing the flash power by varying the duration or the amplitude made no difference. Neutral density filters in the excitation beam, in the emitted light, or behind the image intensifier rendered the same effect.

A measure for the light-gathering power of an objective lens is the NA. According to theory, the brightness of a microscopic image will change with the fourth power of the NA of the objective lens when this lens is also used as a condensor for incident illumination (Ploem, 1969). At the same total magnification the amplitudes of the signal of a latex sphere in the position of sharp focus as determined with the SW 25x and the SW 50x objectives were in the proportion of 1 to 8. With the SW 50x and the SW 100x objectives these amplitudes were in the proportion of 1 to 2. At the same total magnification, the section depths of latex spheres found with the SW 50x and SW 25x objectives were approximately the same at relative flash powers of 1x and 8x, respectively. However, at the same total magnification no difference was found between the section depths with the SW 50x and SW 100x objectives, except below values of 6-7 μm where depths with the SW 50x were slightly smaller.

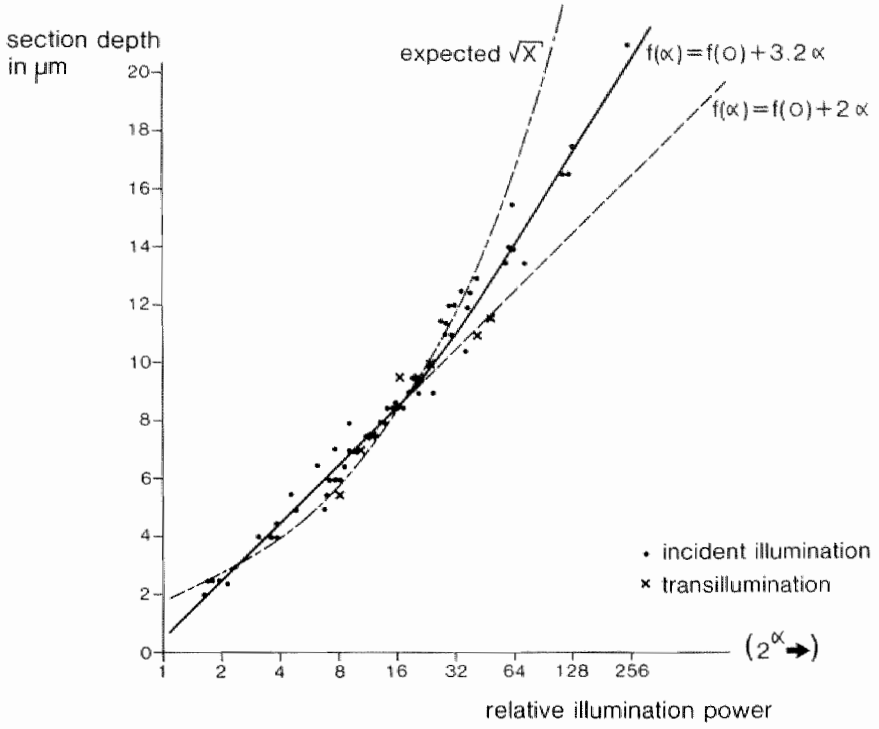


Fig. 3.4
The section depth of latex spheres as a function of illumination power. Iterative fit (two times) by eye of series of section depths of different spheres at total optical magnifications of 100x, 200x, and 400x. The series were obtained either by varying the flash power or by placing neutral density filters in the optical system. Also shown is the expected $\sqrt{x}$ fit ($x = 2^{\alpha}$).

3.3.2. Platelets and leukocytes

Defocusing produced qualitatively similar changes in the image of a platelet and a leukocyte as in that of the latex spheres. For platelets and leukocytes labeled in vitro and injected into an isolated mesentery preparation, the following sections were found at a total magnification of 200x. For platelets: 7-9 µm at the highest relative flash power (16-32x) and for leukocytes: ranging from 12 to 20 µm with a relative flash power between 1x and 4x.

After intravenous injection of AR, the dye is taken up by the platelets, the leukocytes, and the vessel wall. Leukocytes and platelets could be easily distinguished because of the difference in size and shape. The intensity of the platelets varied from experiment to experiment. Subjectively, the intensity remained approximately the same during the first 2-6 min after the end of the injection, thereafter it clearly decreased. For platelets labeled *in vivo* and sticking to the wall of venules or caught in small vessel segments, the sections found at the highest flash power ranged from 5 to 9 μm with a mean value of 6.8 μm (25 platelets in 4 animals).

Figure 3.5 shows *in vivo* labeled platelets flowing in a small arteriole. As an example the signal of one TV line and the intensity distribution curves of 10 platelets are shown as well. Platelets Nos. 2 and 10 are seen sideways, whereas platelet No. 8 is seen more from on top. Visually platelet No. 7 is clearly out of focus, but since the amplitude of its intensity distribution curve is above the noise level, it is considered to be within the optical section. On the contrary, No. 1, seen only as a diffuse spot, is not considered to be within the section. No. 9 is not part of the wall, since it had disappeared in the next image. When the system was focused in the center of the vessel, platelets could be detected over the whole vessel width. By focusing upward from the center, the images of the vessel walls came closer to each other and finally coincided. Simultaneously, the number of observed platelets decreased till they could not be detected anymore.

To estimate the section depth of flowing platelets one can localize the position of focus above the anterior wall of the vessel at which platelets can no longer be detected. Precise localization of this position, however, is rather difficult because either fluorescence of the anterior wall interferes with the fluorescence of the platelets or the anterior wall cannot be localized accurately. In two experiments in which these drawbacks were limited, platelets could no longer be detected approximately 3 μm above the anterior wall, which corresponds with a section depth of approximately 6 μm. These measurements were made within 2 min after labeling of the platelets.

In another set of experiments (6 experiments in 3 animals; 4 vessels) the section depth was estimated as follows. The platelets were observed when focusing in the center of the vessel (zero focal position) or in the +5 μm position, during 20-50 s within 2 min after labeling. Between 100 and 200 flashed TV frames were analyzed. The number of platelets observed in the

vessel over a certain length (between 20 and 40 μm in the different experiments) and considered to be within the optical section, was counted. From this number a platelet count per unit volume (PCV) can be calculated when the section depth is known and the vessel is considered to be a cylinder. A PCV was also obtained with the Coulter Counter. After correction for the 10% dilution with EDTA, these Coulter Counter PCV's ranged from 349 to 418 x 10^9/l ($\bar{x}$ = 390 x 10^9/l). At a section depth of 6 μm the calculated PCV's ranged from 280 to 501 x 10^9/l ($\bar{x}$ = 373 x 10^9/l). At a section depth of 5 μm the mean calculated PCV in these 6 experiments was $\bar{x}$ = 446 x 10^9/l.

In another animal in which no PCV with the Coulter Counter was obtained, the calculated PCVs ranged from 468 to 528 x 10^9/l ($\bar{x}$ = 505 x 10^9/l) at a section depth of 7 μm (3 vessels).

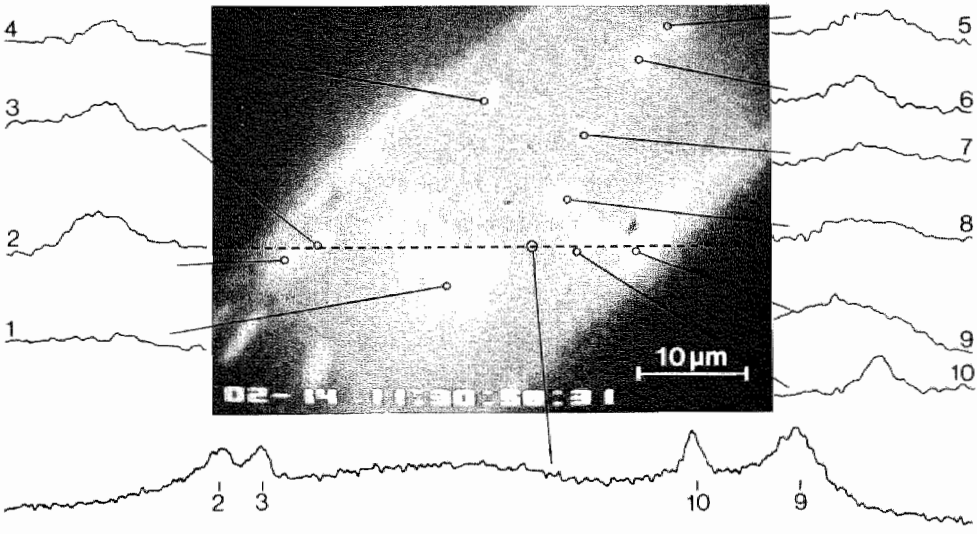


Fig. 3.5
Small arteriole with flowing fluorescent platelets and the intensity distribution curves of several of these particles. The signal of one complete TV line is also shown (dashed line). Plane of sharp focus is in the center of the vessel. Total optical magnification 200x.

In 2 experiments in which Coulter Counter data were available, focal positions above +5 μm were observed till platelets could no longer be detected. In analyzing these experiments a transparent sheet was positioned over the monitor screen. The vessel walls were drawn (in the zero focal position) and the lumen was divided into 6 segments (see table 3.1) equal in width and length. The number of platelets within the optical section was counted in each segment in the different focal positions. The results of such an experiment in an arteriole 23 μm in diameter are shown in table 3.1. The table shows the progressive disappearance of the platelets from the outer to the inner segments of the vessel with defocusing from the vessel center. This was seen in all in vivo experiments. The platelet numbers in the zero focal position at time 255 s were considerably less than the ones at time 0 s, showing the decrease in intensity and consequently in section depth with time. However, the detection of platelets in the zero focal position at time 255 s also shows that the absence of platelets in position +13 μm was not due to insufficient intensity of the platelets.

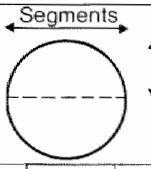
3.4. DISCUSSION

This part of the study shows that fluorescent platelets flowing in a microvessel can be localized objectively within a finite and thin optical section, using the intensity distribution of their microscopic image. Even at the highest available flash power and shortly after labeling, the depth of the section never exceeded 9 μm at the suitable magnification of 200x. The section depth is therefore sufficiently shallow to localize platelets at various sites along the optical axis in microvessels with a diameter from 20 to 35 μm.

For platelets labeled directly in vivo, the section depth found when they were motionless was in good agreement with that found when they were flowing in small arterioles. The estimation of the section depth of flowing platelets by comparing the calculated PCV with the one obtained by the Coulter Counter seems to be justified because in chapter 2 it was shown that with this labeling procedure almost all the circulating platelets are labeled, while RBC's and reticulocytes are not labeled. The calculated and determined PCV's in the present study are in good agreement with the ones given for rabbits in the literature (Zweifach, 1961; Maupin, 1969; Kozma et al, 1974).

Table 3.1

Number of platelets in each segment recalculated per 100 TV frames.



Focal position (µm)	Vessel radius in µm						Time (s)
	12	8	4	0 4	8	12	
0	42.0	34.0	21.3	18.0	23.3	34.6	0
+ 5	20.0	63.3	39.1	31.6	37.5	11.6	46
+ 7	0.7	35.0	42.1	38.5	22.1	0.7	71
+ 9	0	25.7	32.8	20.7	10.0	0	103
+10	0	15.7	27.8	16.4	1.4	0	133
+11	0	4.2	9.3	9.3	0	0	164
+12	0	0	0.7	2.1	0	0	193
+13	0	0	0	0	0	0	229
0	22.6	14.6	10.6	11.3	13.3	18.0	255

The ability to localize platelets in small microvessels at various sites along the optical axis indicates that the rheological behavior of these particles can be studied in vivo. An example of the distribution of platelets is given in table 3.1. The platelet numbers counted in the different segments in the zero focal position during a short time period represent a sample of the platelet distribution over the vessel width. The data in this table show that 255 s later the same inhomogeneous distribution was measured, which points to a rather stable flow situation in the vessel. The higher concentration of platelets near the wall is probably real, because no systematic difference in transmission over the vessel width could be detected with our system (see chapter 4).

For rheological studies it is advantageous to have a small section depth, yielding the highest resolution. At a certain total magnification the section depth can easily be varied by changing the illumination, for instance, with the flash power. This changes the height of the amplitude defocusing graph of a particle relative to the threshold.

The threshold depends on the background light level and the envelope. In our system the envelope was rather constant and mainly due to electrical noise. However, in studies which require an equal section depth in different parts of the vessel, the asymmetry of the platelets has to be considered. Differences in orientation of a platelet toward the optical axis may influence the height and shape of the amplitude defocusing graph, and hence the section depth (see, for example, the signals of platelets Nos. 8 and 10 in fig. 3.5).

To estimate the influence of a difference in orientation of a platelet on the section depth as well as to understand the decrease in amplitude with defocusing, a theoretical analysis was made (see Appendixes 3.A and 3.B). The results are presented in fig. 3.6.

At the left, calculations for a point source and 3 spheres of different size are shown. Comparison of the curves of the spheres with fig. 3.2 shows that the model describes the phenomenon as such quite well. The major difference is a shorter "plateau phase" in the model. However, the steep drop and the tail of the curve are more important with regard to the optical section.

The right part of fig. 3.6 shows the curves of an ellipsoid body in two orientations perpendicular to each other. The amplitude is maximal in the orientation with the longitudinal diameter along the optical axis. After approximately 3.5 μm of defocusing both curves coincide. Hence the influence of the orientation of a platelet on the section depth depends on the threshold level, or better on the signal-to-noise ratio in the position of sharp focus. This ratio can be varied by changing the illumination. The size of the "platelet" in the model (volume 6.7 μm^3) is in the upper part of the distribution for platelets as given in the literature (volume 5-7 μm^3; Maupin, 1969; Frojmovic and Panjwani, 1976). Consequently, the influence of differences in orientation of the platelets on the section depth is, according to the calculations of the theoretical analysis, negligible when they are observed at sufficient illumination, yielding a section depth of approximateley 6-7 μm.

At the same total magnification the section depth of leukocytes at the lowest flash power exceeded the one for platelets at the highest flash power. The difference in section depth between leukocytes and platelets can be partially explained by the influence of the size of the object on the shape

of the amplitude defocusing graph (see Appendix 3.B, fig. 3.6).Besides, the section depth is influenced by the greater amount of luminous flux coming from leukocytes. Since basic dyes form complexes with different intracellular components (Albert, 1966; Da Prada and Pletscher, 1975), the labeling of cells with these dyes is generally a volume labeling. Hence the labeling efficiency will approximately increase to the third power with an increase in diameter. Moreover, leukocytes are spherical while platelets are disk-shaped. In contrast to platelets, leukocytes have a nucleus and basic dyes have a high affinity for nucleic acids. Also the quantum yield of intracellular AO is probably higher than that of AR (Forster, 1951). The section depths for leucocytes found in this study are too large to allow the investigation of their rheological behavior in small vessels.

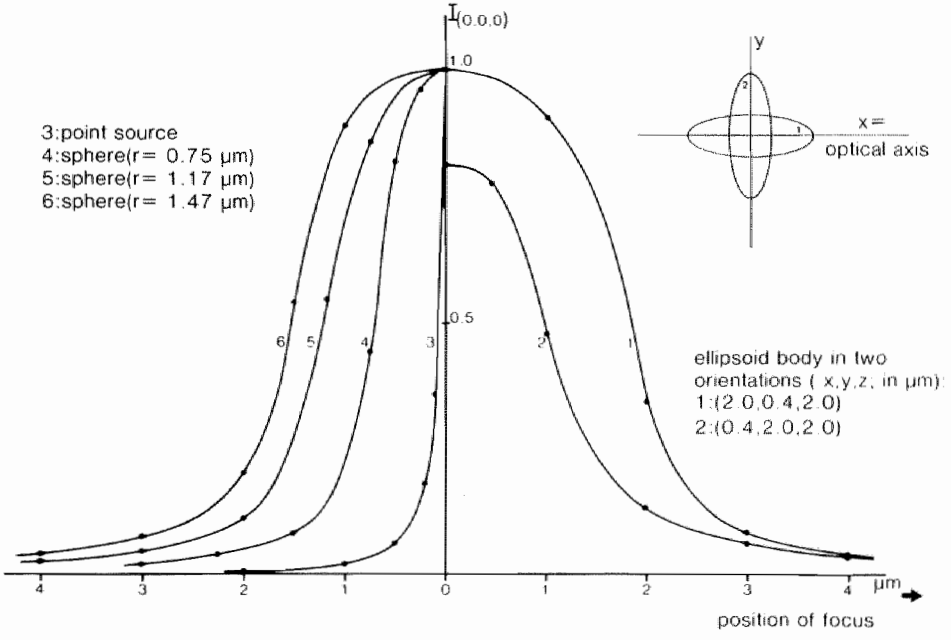


Fig. 3.7
Calculated amplitude defocusing graphs (see Appendix 3.B) for an ellipsoid in two orientations perpendicular to each other (1 and 2), a point source (3), and spheres of different size (4, 5, 6). The z-axis is perpendicular to the plane of the diagram.

3.5. CONCLUSIONS

- Blood platelets labeled with the fluorescent dye acridine red could be localized objectively within a thin optical section using the height of the intensity distribution of their microscopic image relative to the background noise.

- Latex spheres (1.5 μm o.d.) were used as model particles to assess the influence of various optical factors on the depth of the optical section. These experiments showed that 1) the section depth was almost symmetrical around the level of sharp focus, 2) it halved by doubling the total magnification and 3) it increased according to a straight line with an exponential increase in illumination power.

- Theoretical analysis showed that in case of the disk-shaped platelets the amplitude of the signal of their microscopic image depends on the orientation of the platelet with respect to the optical axis. It was calculated that the influence of orientation was negligible at a section depth of 6-7 μm.

- At the suitable magnification of 200x and using the highest available flash power, a section depth ranging from 5 to 9 μm was found for platelets sticking to the wall of venules or caught in small vessel segments. For platelets flowing in small arterioles (diameter 20-35 μm) a section depth was estimated ranging from 5 to 7 μm, allowing the investigation of their rheological behavior in these vessels.

3.6. APPENDIXES

Appendix 3.A:
descriptive analysis of the smearing of the image with defocusing

A fluorescent object can be considered to be composed of a large number of self-luminous pinpoints. For simplicity, let us consider one point source (for instance, the arrow point in fig. 3.7.A). When the point is in sharp focus the image is formed in the intermediary image plane. All light rays contributing to the image formation form a cone of light, described by the objective image aperture. From wave optics it is known that the final spatial image is an ellipsoid body. However, in the intermediary image plane the image is the so-called Airy disk (Françon, 1961). Shifting the point source toward and away from the objective lens along the optical axis increases and decreases the image distance, respectively. The axial shift of the image away from the intermediary image plane (Δi) is greater with an equal shift of the point source (Δo) toward the lens than away from it (Wayland, 1979). Also the lateral position of the image changes in such a way that with defocusing the lateral position falls, in first approximation, along the line "optical center lens - center Airy disk in the intermediary image plane" (thick line in fig. 3.7.B).

Consider a fixed screen in the intermediary image plane. This plane serves as an "object" for the final image on the TV screen. With the point source in sharp focus the image on the fixed screen is the Airy disk. Neglecting changes in interference pattern as they occur with defocusing and considering only geometrical optics, the following can be said about the change of the image. With defocusing and increasing Δi, the light incident on the fixed screen is smeared over a circle of increasing size (fig. 3.7.B). The center of this "circle of smear" coincides with the center of the Airy disk. Hence the image is not changing its position. Only the light is smeared over a larger region, thus decreasing the maximum intensity seen on the fixed screen. As can also be been in fig. 3.7.B, this effect is in first approximation symmetric.

Appendix 3.B: theoretical analysis of the optical sectioning

In Appendix 3.A, the smearing of the image is described when a self-luminous point is defocused in the object space. For the analysis, all light entering the aperture of the perfect objective lens is supposed to be equally distributed over the Airy disk and consequently over the circle of smear. For a point source, Δo out of focus, the resulting circle of smear has a radius

$$r_{sm}(\Delta o) = |\Delta i|.\tan \alpha + r_A \qquad \text{(see fig. 3.7.C)},$$

where

r_{sm} = radius circle of smear,
Δo = defocusing distance,
Δi = shift of the image from the intermediary plane,
α = image aperture of the objective lens,
r_A = radius of the Airy disk.

The change in image distance is in first approximation $\Delta i = -M^2.\Delta o$, where M is the lateral magnification of the objective lens (Wayland, 1979). Substituting this into the equation for the radius of the circle of smear gives

$$r_{sm}(\Delta o) = M^2.\Delta o.\tan \alpha + r_A.$$

For the analysis it is assumed that the center of the fluorescent particle is on the optical axis, the volume exists of self-luminous points, and none of these points exerts mutual interference. The intensity of the light on the optical axis in the intermediary image plane $I_{(0,0,0)}$ is calculated, because at this place the intensity is maximal. The lighting intensity I is defined as the luminous flux received per unit area. The lighting intensity was assumed to be constant over the circle of smear. Therefore, the lighting intensity to be calculated is the sum of the intensities of the circles of smear of all points within the particle in the object space that extend their circle of smear over the optical axis. However, the contribution of each of these points has changed proportional to the rate of smearing of its image in the intermediary plane. The magnification of the particle, however, is nonisotropic. Laterally the magnification is a factor of M, axially of M^2. By dividing all axial measures by a factor of M, the ellipsoid spatial image body of each point source becomes a sphere and the light-density distribution in the image space becomes isotropic, which simplifies calculations. However,

the radius of the circle of smear does not change. After this operation, the image aperture has changed according to tan β = M.tan α, where β is the new image aperture. This new image aperture is equal to the object aperture of the objective lens. In these modified (isotropic) coordinates, the equation for the radius of the circle of smear becomes

$$r_{sm}(\Delta o) = M.\Delta o.\tan \beta + r_A.$$

Within the Airy disk, each point has the same intensity I_0. The intensity of

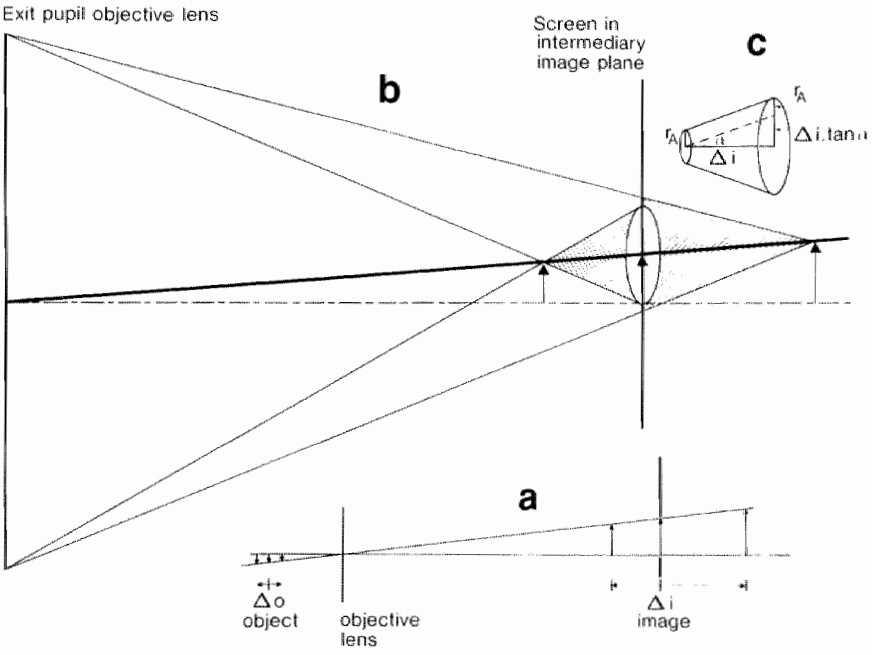


Fig. 3.7
A. Change in image distance (Δi) with an equal change in object distance (Δo) B. Formation of the circles of smear in the intermediary image plane from a point source at a distance, + or - Δo, out of focus. C. Relation among the radius of the circle of smear, the change in image distance (Δi), the image aperture of the objective lens (α) and the radius of the Airy disk (r_A). (See Appendixes 3.A and 3.B).

the circle of smear becomes

$$I_{sm} = I_o \cdot \frac{\pi (r_A)^2}{\pi (r_{sm})^2} = I_o \cdot \frac{(r_A)^2}{(M \cdot \Delta o \cdot \tan \beta + r_A)^2}$$

All points that contribute with their circle of smear to $I_{(0,0,0)}$ lie in the object space within a cone with top angle U. Since points outside this cone can also contribute to $I_{(0,0,0)}$ due to the extra extension by the Airy disk, the corresponding cone in the isotropic image space increases by r_A over its entire volume. This results in a frustum in the image space with a top angle $\beta = U$ and a top circle with radius r_A. Thus $I_{(0,0,0)}$ becomes

$$I_{(0,0,0)} = \iiint_{vol} I_o \cdot \frac{(r_A)^2}{(M \cdot \Delta 0 \cdot \tan \beta + r_A)^2} \cdot dV,$$

where vol = the volume of the isotropic spatial image of the particle within the frustum (β, r_A).

With this equation, the decrease in intensity with defocusing was calculated for a point source and for 3 different spheres (diameters 1.50, 2.34, and 2.94 μm, respectively). To estimate a possible difference in section depth for platelets with different orientations toward the optical axis, an ellipsoid body was used with a longitudinal diameter of 4 μm and a thickness of 0.8 μm. Calculations were made with its longitudinal diameter along the optical axis as well as perpendicular to it. All calculations were done for a perfect objective lens with M = 100 and NA = 1.20. The results are presented in fig. 3.6.

4. CONCENTRATION DISTRIBUTION OF BLOOD PLATELETS FLOWING IN SMALL ARTERIOLES

4.1. INTRODUCTION

Blood platelets play a key role in the response of blood to vessel wall injury (see chapter 1). Beside their well established role in hemostasis and thrombosis, an increasing number of observations points toward platelet-endothelial interactions in the normal circulation (Johnson, 1971; D'Amore, 1978; Nalbandian and Henry, 1978; Gingrich and Hoak, 1979). These data suggest that substances released from the platelets support and regulate the maintaining of the endothelial lining. For these functions platelets have to come close by or in contact with the vessel wall.

The distribution of the platelets in blood flowing through a vessel not only depends on their own rheological behavior in a shear field but also on their interaction with neighboring red blood cells (RBC's). On the basis of rheological theories and *in vitro* studies with model particles an inward radial migration was predicted for RBC's flowing in the smaller blood vessels, while a tendency for inward migration of the blood platelets, if any, would be considerably smaller (Jeffery, 1922; Segre and Silberberg, 1962; Chaffey et al, 1965; Goldsmith and Mason, 1967; Karnis and Mason, 1967; Goldsmith, 1972). Inward migration of individual RBC's (Goldsmith, 1971, 1973) resulting in an increase in hematocrit in the tube-center (Palmer, 1965; Goldsmith, 1967; Palmer and Betts, 1975) was found *in vitro*. Because of the observed inward migration of the RBC's with resulting central crowding, it was assumed that the lighter platelets would disperse from the center of the vessel, resulting in a higher platelet concentration near the wall. Experiments *in vitro* have provided direct (Palmer, 1967) or indirect (Turitto et al, 1972; Blackshear et al, 1977; Eckstein and Beck, 1978; Beck and Eckstein, 1980) evidence for a higher platelet concentration near the wall.

In vivo, studies on the distribution of blood platelets flowing in a blood vessel have not been performed. Moreover, only a limited number of studies, and none in microvessels, has been performed on the distribution in a blood vessel of RBC's (Phibbs and Burton, 1968; Wiederhielm and Billig, 1968), leucocytes (Phibbs, 1966) or microspheres (Phibbs et al, 1967; Phibbs and Dong, 1970). In addition, these studies were done *ex vivo* in quickly frozen vessels.

Recently, it has become possible to visualize individual blood platelets flowing in a microvessel (see chapter 2) and to localize them objectively within a thin optical section (see chapter 3). By these techniques the concentration distribution of blood platelets flowing in a microvessel, i.e. the arrangement in the vessel of the number of platelets per unit volume, can be studied. In this part of the thesis this distribution was determined in small arterioles using the rabbit mesentery preparation and intravital fluorescence microscopy with position of sharp focus in the center of the vessel. Platelets localized within an optical section were counted in equal segments over the vessel width, which yields a concentration profile.

4.2. METHODS

4.2.1. Experimental set-up

For a detailed description of anesthesia, tissue preparation, the microscope and recording system used in this part of the study the reader is referred to chapter 3, and for details of blood collection, hematocrit measurement, electronic platelet counting and fluorescent labeling of the platelets to chapter 2.

In short, 10 rabbits of various breeds and either sex, ranging in weight from 2.5-4.5 kg were anesthetized with intramuscular injections of 0.2 ml/kg body wt Vetalar (100 mg/ml ketaminehydrochloride, Parke Davis) and 0.4 ml/kg body wt Rompun (2% solution, Bayer). Following induction of anesthesia blood was collected from the central ear artery and the hematocrit and platelet count per unit volume (PCV) were measured in duplicate. Through a small midline incision a short segment of distal ileum was brought outside and the mesentery was carefully spread without stretching over a siliconized glass plate mounted in a heat-controlled (37-38^{o}) stage on a Leitz intravital microscope. Viability of the preparation was maintained by superfusion with a prewarmed (37^{o}) Tyrodes solution. The gut was kept moist with overlying wet gauze.

Arterioles with a diameter ranging from 21 to 35 μm were selected, except in one experiment where a vessel-tree, including daughter branches with a diameter of 10-20 μm was studied. To investigate the platelet distribution a vessel was observed through a Leitz SW 100X water-immersion objective (numerical aperture 1.20) with position of sharp focus in the median plane of

the vessel, i.e. the focal position yielding the widest vessel diameter. Then the platelets were labeled with the fluorochrome Acridine Red (Chroma) by injecting within 20-30 s, 2-2.5 ml working solution of the dye (18.2 mM) through a polyethylene catheter in a marginal ear vein. In 6 animals one or two injections were given, in 3 animals three injections and in one animal four. Injections were given at an interval of 15-30 min.

Following each injection the circulating platelets were examined during two to six minutes by fluorescence microscopy, using incident illumination (Leitz Ploemopak 2.2, interchangeable filter set No. N 2.1) with flashes of short duration (< 0.1 ms). Usually several sites along the vessel were studied following each injection, for example the different sites around a branch point. Each site was recorded for at least 30-40s and always with the vessel diameter in sharp focus. Flashes were given every 9th field in the blanking period of the TV camera by a xenon flash arc, corresponding to a flash interval of 180 ms. This interval is sufficiently long to insure that each instantaneous picture contains no information from the previous flash and platelets cannot be counted twice. On the other hand, the flash frequency is high enough to obtain a sufficient number of pictures within a relatively short period of time and too far away from the heart rate (110-160/min; three animals) and the respiratory rate (30-60/min; 3 animals) of the anesthetized animal to obtain pictures which are randomly dispersed throughout both cycles. In all experiments the highest flash power range was used. This range was adjusted prior to the experiment with the vernier intensity control (continuous from 1-2x) to avoid blooming of the TV-camera.

The images were projected on either a 1-inch TV camera coupled optically (1:1) to a 3-stage image intensifier, or a 1-inch SIT camera (Bosch TYC 9A/SIT-tube RCA 4804 HP). The final magnification at the front plane of the camera was 200x. The images were displayed on a TV-monitor and stored on videotape for final analysis. In most vessels RBC-velocity was measured shortly following fluorescence microscopy by means of the dual slit method (Wayland and Johnson, 1967; Intaglietta et al, 1970) using photo-diodes (IPM) and a velocity tracking correlator (IPM/MOD 102).

4.2.2. Analysis of the data

Off-line analysis was done field by field with a transparent sheet positioned over the monitor screen. Each field contains only the even or odd TV lines of a complete frame. The first field following each flash was used.

In the case of one field the calculated interline-spacing in the object plane was 0.17 µm. The vessel walls were drawn and the lumen divided into six segments equal in width and length (27-45 µm). The number of platelets that could be localized objectively within a thin optical section (see chapter 3 for definition and determination) was counted in each segment. In short, to assess whether a platelet was within the optical section the amplitude of the signal of one TV line through the center of its microscopic image was used. The TV line was selected with a modified video dimension analyzer (IPM 303) and displayed on a dual-trace oscilloscope. The empty tracing of the oscilloscope was placed along the top of the background noise. A platelet was considered to be within the optical section as long as the signal clearly exceeded the empty tracing, resulting in a signal-free area between the two tracings. As described in chapter 3 a section depth ranging from 5 to 9 µm was found in vivo for stationary platelets at the highest flash power. When a platelet was positioned in two segments, it was counted in the segment that contained the largest part of it. To assess the platelet distribution over the vessel width 130-160 TV-fields were analyzed which covers a relatively short period of time (20-30s). The walls of each vessel were defined as left or right in accordance with the direction of flow and each distribution was classified from left to right.

Because illumination consisted of flashes of short duration, each flashed TV field represents an instantaneous picture. The use of instantaneous pictures eliminates the time factor. Consequently, the velocity factor is also eliminated. Hence, differences in velocity over the vessel width have no influence on the number of platelets counted in each segment, in contrast to the situation when the number of platelets passing a line is counted during a certain time interval. Therefore, the platelet concentration profile within the optical section can be determined if the counting volume is equal in all segments.

4.2.3. Equality of the six counting volumes

The volume of each segment depends on its length, width and depth. The error made in drawing segments equal in width and length was neglegted.

The depth of the segment depends on the depth of the optical section, see fig. 4.1. In each experiment the PCV of the animal as determined with the Coulter Counter was used to estimate, from the total platelet number counted within the six segments, the total volume in which this platelet number was counted. From this volume and the length and width of the segments the mean depth of the optical section was calculated.

Differences in depth of the optical section, and hence in counting volume, if any, between the six segments may occur as a result of 1) differences in orientation of the platelets from the center toward the wall and 2) a systematic difference in light-transmission over the vessel width, or both. In addition, a segment near the wall may have a smaller volume as compared to the other segments due to irregularities of the wall and the cylindrical shape of the vessel, see fig. 4.1.

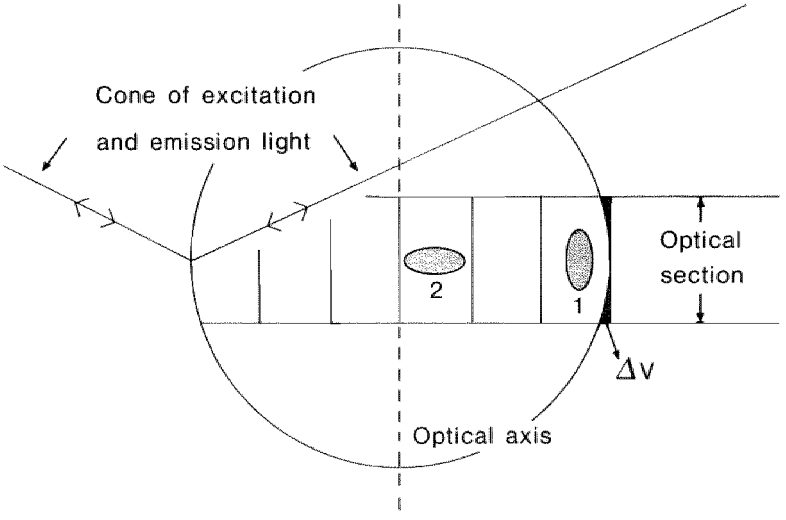


Fig. 4.1
Schematic drawing of a cylindrical vessel with an optical section around the median plane of the vessel. The width of the vessel is divided into six equal segments. The volume of a wall segment is diminished as compared to the other segments by a volume fraction, ΔV (black area), due to the cylindrical shape of the vessel. Two platelets (1, 2) are shown with their diameter along and perpendicular to the optical axis, respectively, i.e. the two extreme orientations. The course of the outermost light rays between the objective lens and a spot on the vessel wall have been constructed. The rays are not parallel but form a cone of light. Because of the incident illumination the cone of the excitation and emission light coincide. The top angle of this cone is large due to the high numerical aperture of the objective lens.

Cylindrical shape of the vessel. As shown in fig. 4.1 the cross-section of each segment is a rectangle, except in case of the wall segments. Due to the cylindrical shape of the vessel the volume of the wall segments is diminished as compared to the other segments with a volume fraction depicted as ΔV in fig. 4.1. To estimate this volume fraction for each experiment and hence the resulting difference in platelet numbers counted in the wall segments and the other segments, the vessel was assumed to be a perfect cylinder. Using the diameter of the vessel and the estimated mean depth of the optical section (see above) this volume fraction ΔV was obtained by simple calculation.

Orientation. As shown in the theoretical analysis in chapter 3, fig. 3.6 the orientation of the longitudinal diameter of the platelet with respect to the optical axis influences the amplitude of the signal of its microscopic image and hence the depth of the optical section. The analysis showed that for a big platelet (volume 6.7 μm^3) the difference between the amplitude-defocusing graphs of the two extreme orientations, i.e. longitudinal diameter along and perpendicular to the optical axis, decreased progressively between 2-3 μm. They almost coincided following 3.5 μm of defocusing. Therefore, to minimize orientation-effects only experiments with an estimated mean section depth equal to or exceeding 4.5 μm were used.

In chapter 5 it will be shown that more platelets are oriented with their longitudinal diameter along the optical axis near the wall than in the center of the vessel. For each experiment the extent of the remaining orientation-effect on the platelet numbers counted near the wall and in the center of the vessel was assessed using the estimated depth of the optical section, the two (extreme) amplitudedefocusing graphs of the platelet as presented in fig. 3.6 and the difference between the wall and the center in percentage platelets present in these two extreme orientations. The latter was estimated from the data in chapter 5.

All platelets with their diameter at an angle of 0°to 45° with regard to the optical axis were regarded as parallel to this axis (platelet No. 1 in fig. 4.1). The remaining platelets were regarded as perpendicular to the optical axis (platelet No. 2 in fig. 4.1).

Light transmission. Absorption and scatter of both the excitation and emission light by structures lying between the platelets and the objective lens, largely RBC's but also tissue components, will influence the lighting intensity of the image and hence the section depth (see fig. 4.1).

Since this effect may be more pronounced in the central segments, where more RBC's are present, the intensities in a small spot positioned in the center of the images of 30 platelets were measured in the central segments and wall segments, respectively. This was performed in four arterioles (diameters 25-32 μm) in three animals. In each experiment the measurements were made shortly following labeling within a time period of approximately one min, while the time to collect the 30 platelets in both groups differed less than 10s.

Because the orientation of a platelet influences the intensity of its microscopic image (see above), only platelets within an optical section and with a certain orientation were used. The long and short axes of the image were measured and the analysis restricted to platelets with a short to long axis ratio equal to or exceeding 0.8, i.e. the image being round or almost round (see chapter 5). At this orientation the influence of platelet thickness, which is unknown, on the short to long image axis ratio can be neglected.

To obtain more accurate measurements of these axes another definition for the optical section than the one described above was used . Only platelets with a reasonably sharp image were included. Otherwise the procedure to determine the optical section was the same. To narrow the section depth down a signal-to-noise ratio of 2:1 was used.

A window was placed over the center of the platelet image and the average video signal amplitude within this window was determined with a video-photometric analyzer (IPM, 202) and displayed on a voltmeter (Fluke 8020A). The window consisted of a small part of one TV line, with a calculated length in the object plane of 1.3 μm. To measure the background intensity the window was left at the same spot and the intensity in the following or preceding flashed image was recorded. The latter value was substracted from the first to obtain the image intensity. In this way systematic spatial differences in background due to fluorescence of wall and tissue components as well as systematic spatial differences in camera sensitivity were eliminatd.

The effect of the measured difference in light transmission on the section depth and hence on the counted platelet numbers in the different segments was estimated using the relationship between the section depth and the illumination power as described in chapter 3 (fig. 3.4).

4.2.4. Statistical methods

The image intensities measured to assess a difference in light transmission between segments near the wall and segments near the center showed positively skewed distributions which became symmetric following logarithmic transformation. To obtain a 95%-confidence interval of the intensity difference between center and wall, analysis of variance (Scheffe, 1956) was performed on the logarithmically transformed data.

Platelet concentration profiles of individual vessels are presented from the left to the right wall as frequency distributions. To estimate a general concentration profile the assumption was made that within each segment the number of platelets per TV field could be considered as counts from independent Poisson distributions. This assumption was confirmed by inspection of the data and application of the chi-square test for goodness of fit (Rao, 1973; Dixon and Brown, 1979) on 8 separate segments in 2 experiments as well as simultaneously on 6 segments in 3 experiments. Therefore, a log-linear model (Fienberg, 1980) was used to calculate a general concentration profile from wall to wall, taking into account the direction of flow. The 95%-confidence limits of this profile were calculated using the asymptotic standard-error (Fienberg, 1980).

4.3. RESULTS

The hematocrits of the 10 animals ranged from 28 to 39% ($\bar{x}$ = 33%) and the electronic platelet counts from 302 to 519 x 10^9/liter ($\bar{x}$ = 405 x 10^9/liter). Thirty-six measurements were made at 30 sites in 17 vessels. After excluding the samples with an estimated section depth < 4.5 µm, 32 measurements at 28 vessel sites in 17 vessels were used for the analysis. In these samples the estimated section depth ranged from 4.5 to 9.0 µm ($\bar{x}$ = 5.8 µm). Disregarding the few daughter branches with a diameter < 20 µm, the mean vessel diameter was 27 µm. The RBC velocity in various vessels ranged from 1.5 mm/s to 9 mm/s ($\bar{x}$ = 4 mm/s) and remained constant within about 1 mm/s during minutes. Vasomotion was observed in none of the vessels.

4.3.1. Equality of the six counting volumes

The calculated influence of the cylindrical shape of the vessel on the platelet counts in the wall segments ranged from 1-7% ($\bar{x}$ = 2.6%).

The estimated influence of differences in orientation varied between 0 and 4% ($\bar{x}$ = 2.2%). Since the cylindrical shape of the vessel and the difference in orientation between wall segments and central segments have opposing effects, the combination of these effects was assessed for each experiment by subtracting the effect on the platelet count due to the cylindrical shape of the vessel from that due to orientation. The combined effect ranged from -7 to +3% ($\bar{x}$ = -0.3%). Therefore, when combined the influence of these two factors is minimal.

The 95%-confidence interval of the ratio of the intensities in the central segments to the intensities near the wall was 1.00-1.06. Even at a difference of 6% in light transmission the difference in depth of the optical section will only be approximately 0.17 μm, resulting in a difference in the platelet counts of 2-4%. Therefore, the influence of differences in light transmission over the vessel width is also small.

4.3.2. Concentration profiles

In all measurements the concentration distribution was non-uniform, the wall segments containing the highest platelet numbers, except for some wall segments at the side of branching immediately following a branch point.

Sites in a vessel without preceding branch points. These sites were considered to be parts in a vessel without upstream branch points within at least 10 vessel diameters. A distance of 10 vessel diameters was the lower limit and in most cases measurements were made further downstream from a branch point. Approximately symmetric as well as rather asymmetric profiles were found (see fig. 4.2). In most cases the platelet numbers decreased progressively from the wall segments toward one of the more centrally located segments.

A reason for asymmetry might be the presence of curves in a vessel. A profile as determined 35-65 μm distal to the center of a sharp curve to the left is shown in fig. 4.3. The vessel part preceding this curve was rather straight over a relatively long distance. Likewise concentration profiles recorded distally to or in the center of other curves did not show a striking difference from those recorded in straight parts of the vessel. Therefore, no distinction was made between straight and winding vessels. Besides, most arterioles in the unstretched mesentery meander with curves in opposite directions shortly following each other.

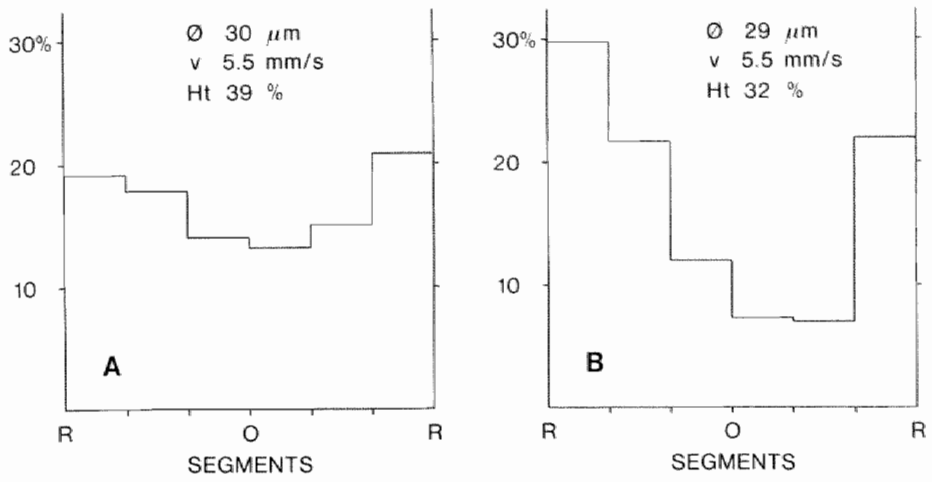


Fig. 4.2
Frequency distributions of the number of platelets counted in each segment at two sites in a vessel without upstream branch points. Left wall segment is depicted on the left side. Ø = vesseldiameter (µm), v = measured RBC velocity (mm/s) and Ht = systemic hematocrit (%). R = vesselradius, O = center of the vessel. Rather symmetric (A) and asymmetric (B) concentration profiles are shown. Note that both vessels have approximately the same diameter and RBC velocity, but that the animals differed in systemic hematocrit.

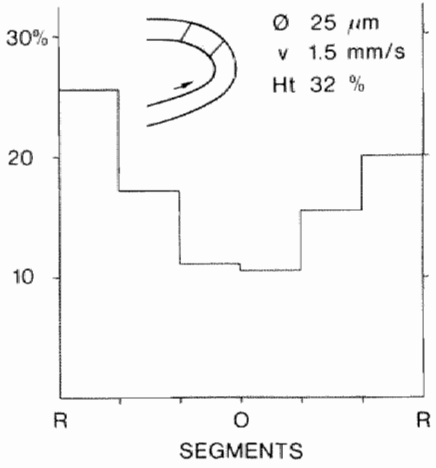


Fig. 4.3
Effect of a curve (40-50°) to the left. Position of observation 35-65 µm downstream of the center of the curve. Arrow: direction of flow. For symbols see legend to fig. 4.2.

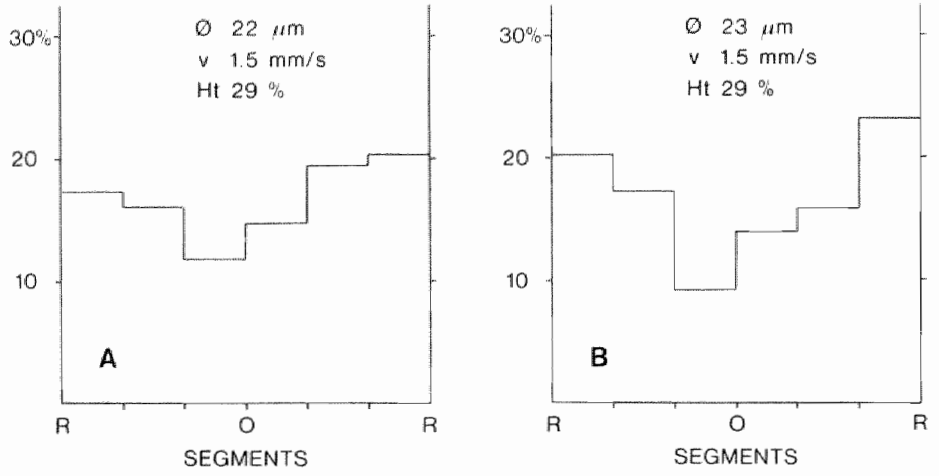


Fig. 4.4
Two near-by spots from the same vessel. The profile as depicted in B was approximately 100 μm upstream of the one shown in A. The former was analyzed 20 minutes later after a new labeling-injection. For symbols see legend to fig. 4.2.

When two samples were analyzed from the same spot or from two neighboring spots in the same vessel, either at an interval of some minutes during the same post-injection period or following the next injection, the concentration profiles, although mostly not quite the same, generally showed the same trend. In fig. 4.4 the profiles are given from two neighboring spots. The profile in fig. 4.4.A was determined following the first injection and the one in fig. 4.4.B approximately 100 μm upstream after the second one, twenty minutes later.

In addition to vessel geometry, the platelet concentration profile might be influenced by the local hematrocrit, which, among other things depends on

the flow rate in the vessel since the inward migration of RBC's increases with the flow rate (Goldsmith, 1972). In contrast to the systemic hematocrit, the local hematocrit could not be measured. In the vessels of animals having a higher systemic hematocrit a clear tendency for a more pronounced profile, i.e. increasing platelet concentration near the wall with decreasing concentration in the center, was not found. Often the opposite occurred (compare, for example, the concentration profiles depicted in fig. 4.2). Even so, no clear relation was found between concentration profile and RBC velocity.

In fig. 4.5 two vessels in the same mesentery and of similar diameter are shown with a RBC velocity of 3 mm/s and 9mm/s, respectively. The vessel with the lower velocity had a more pronounced concentration profile. It should be noticed that on the basis of these experiments no definite conclusions can be drawn about the effect of velocity and hematocrit on the concentration profile because the actual local hematocrit is not known and the number of experimental data is limited.

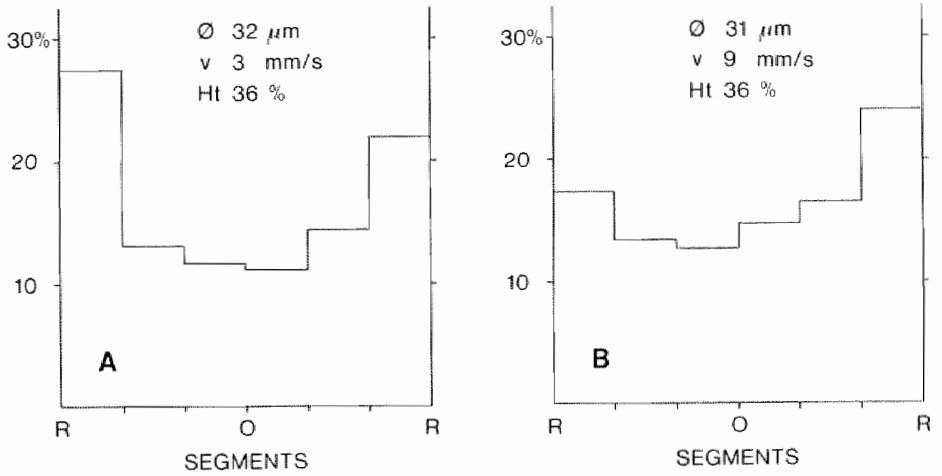


Fig. 4.5
Two vessels from the same animal and of similar diameter, but with a different RBC velocity. For symbols see legend to fig. 4.2.

Branch points. In contrast to curves, branch points had a more pronounced influence on the concentration profile. In fig. 4.6 the profiles are given just before and after a sidebranch at an angle of approximately 90^{0}. Distal to the sidebranch the platelet concentration in the part of the vessel adjacent to this sidebranch has been reduced, as may be expected from the course of the streamlines, showing that the sidebranch draws its blood mainly from the adjacent segments (Charm and Kurland, 1974). A similar result was obtained distal to three other 90°-sidebranches.

In fig. 4.7 a vessel tree is shown as well as the concentration profiles around four different branchpoints. In all daughter branches the outer wall segment contained a higher platelet number than the inner wall segment, except in branch 3C. Note the effect of the little upstream sidebranch (diameter 7μm) on the profile of spot 4A.

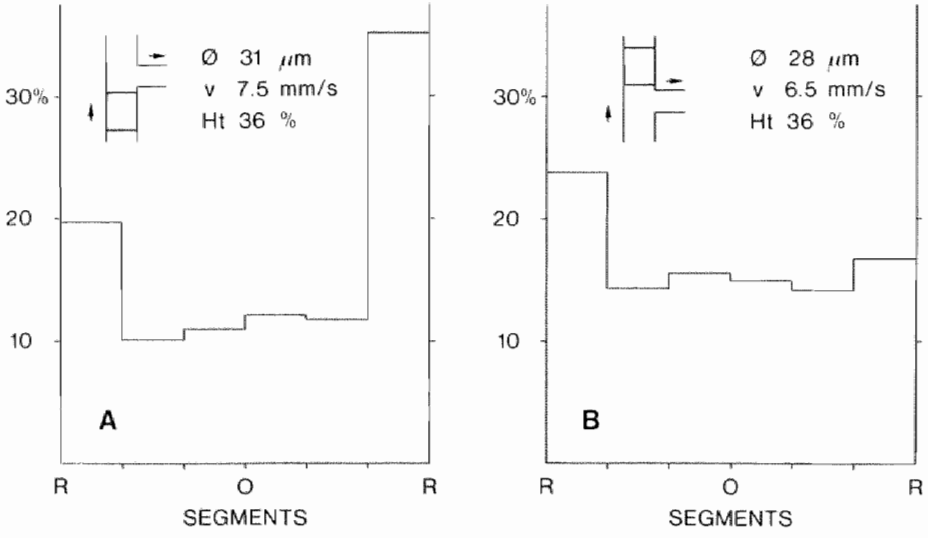


Fig. 4.6
The effect of a 90°-sidebranch on the concentration profile. The profiles as shown are determined just before (A) and after (B) a sidebranch with a diameter of 18 μm. For symbols see legends to fig. 4.2 and fig 4.3.

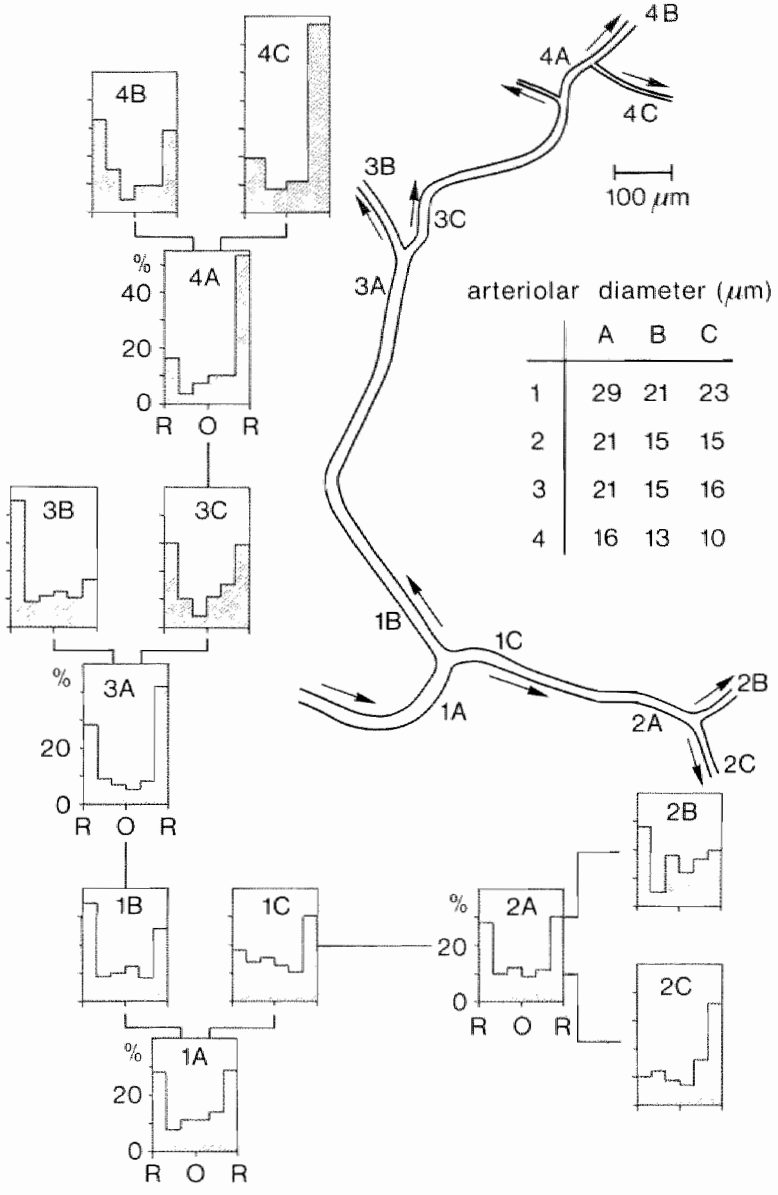


Fig. 4.7
A vessel tree with bifurcations. The corresponding concentration profiles are depicted in a schematic tree. On spots 1-3 the RBC velocity ranged from 2-3 mm/s. On 4 the RBC velocity was less than 1 mm/s. The diameter of the side branch upstream of spot 4A was 7 µm. Vessel 4C was an arteriolar-venular anastomosis. In contrast to the other spots, the optical section was divided in only four segments on spot 4C, because of the small diameter of the vessel. For symbols see legends to fig. 4.2 and fig. 4.3.

General profile. The general concentration profile as calculated from 19 measurements at 15 sites without upstream branch points in 13 vessels (diameter 21-35 μm) in 10 animals (total platelet number: 6571) was found to be: 1.44-0.91-0.78-0.76-0.85-1.51. This profile and its 95%-confidence limits are depicted in fig. 4.8. Measurements at the same site in a vessel were added prior to the calculation. Because a log-linear model was used multiplication of the values of the six segments yields unity, a value which could be found for each segment in case of a uniform concentration distribution. The calculated profile is rather symmetric with a platelet concentration in the wall segments of about twice the concentration in the central segments.

4.4. DISCUSSION

In the study presented in this chapter the concentration distribution of blood platelets flowing in small arterioles was determined. In all cases a non-uniform distribution was found. At vessel sites without a preceding branch point the highest concentration was always found near the wall. In general, the platelet concentration decreased from the wall toward the vessel center with a concentration near the wall of approximately twice the central concentration.

The concentration distribution was assessed by taking instantaneous pictures with the position of sharp focus in the median plane of the vessel and by subsequently counting in the six adjacent segments the number of platelets that could be localized objectively within a thin optical section. As a result of the use of instantaneous pictures the number of platelets counted in each segment is independent of differences in velocity over the vesselwidth.

The chance that at the flash interval used a platelet is counted twice, i.e. the samples are not independent, is likely to be negligible, even in the experiments with the lowest RBC velocity. In the experiments with the lowest velocity one can often observe a platelet just entering a wall segment and moving close to the wall, while no detectable platelets in this segment are seen in the next picture or even in the whole field of view.

As shown in the section on results the error made in determining the concentration distribution due to differences between the volumes of the six segments is small. In the worst case the number of platelets in the wall

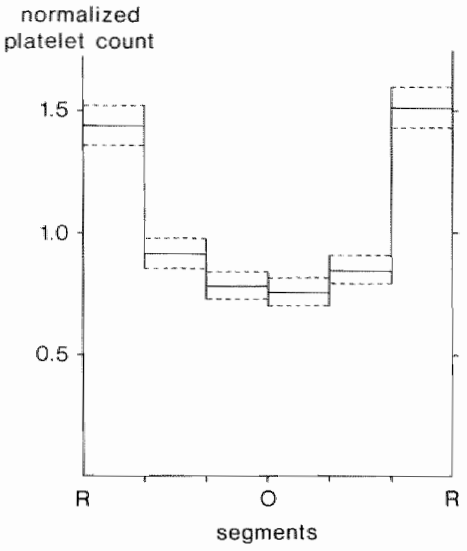


Fig. 4.8
General concentration profile (solid line) with 95%-confidence limits (dashed line) as calculated with a log-linear model from 19 measurements at 15 sites without upstream branch points in 13 vessels in 10 animals (total platelet number: 6571).

segments was over- or underestimated with respect to the central segments by 3% or 11%, respectively. The similarity in image intensity of platelets in the wall and central segment may seem surprising. However, one should realize that the rays of excitation and emission light are not parallel, but form a cone of light with its top in the platelet and its base on the objective lens (see fig. 4.1). Even for a platelet in contact with the wall (in the median plane of the vessel) half of this cone has to cross the blood vessel. Due to the high aperture of the objective lens the majority of the light rays in this half cone crosses the blood vessel over a length exceeding the radius of the vessel.

The total optical density (OD) of blood may be regarded as the sum of the OD due to scatter, which appears to be the major component (Lipowsky et al, 1980), and absorption (Twersky, 1970; Lipowsky et al, 1980). Simple calculation shows a difference in transmission due to absorption of 3% between the wall and the center of a vessel with a diameter of 20 μm and an oxyhemoglobin concentration of 7 mM (Kozma et al, 1974), using extinction coefficients (Van Kampen and Zijlstra, 1967) at the peaks of the absorption and emission spectrum of Acridine Red (see fig.2.1). The effect of scatter is difficult to calculate, since the orientation of the RBC's (Phibbs and Burton, 1968;

Wiederhielm and Billig, 1968) will have a marked influence and the contribution of scatter by tissue components cannot be neglected (Lipowsky et al, 1980). However, the similarity in image intensity of platelets in the wall and central segment as found in this study suggests little difference between center and wall segment as far as scatter is concerned. This is in agreement with the findings of Wiederhielm and Billig (1968). In arterioles they found that the OD in the center was even less than the OD in the rest of the vessel, confirming the well-known observation of a central light streak in vessels with sufficiently rapid flow.

Branch points had a marked influence on the concentration profile. In order to reduce the influence of branch points only measurements, made at vessel sites located at least 10 vessel diameters downstream from a branch point, were used to calculate the general profile. The length needed to restore a disturbed platelet concentration profile is not known. It might be longer than 10 vessel diameters, offering an explanation for the asymmetry found in different individual profiles. Nevertheless, in all wall segments at these sites except in one, the relative platelet concentration clearly exceeded the relative concentration found in the adjacent segments distal to a side branch. This indicates that at most vessel sites without preceding branch points as observed in this study a disturbance in the profile caused by an upstream branch point already had lessened considerably. The general concentration profile calculated from all individual profiles was rather symmetric.

It will be interesting to study in arterioles the development of the platelet concentration profile downstream from a branch point, because such data will yield information about lateral movements of the platelets. In the microcirculation the Reynolds numbers are less than unity and inertia forces are negligibly small. Therefore, it is assumed that the length needed to restore a disturbed velocity profile is about one vessel diameter (Caro et al, 1978). Consequently, away from there restoration of a disturbed platelet concentration profile depends on lateral displacements of the platelets across the streamlines. In vitro large sideway movements of small latex spheres flowing through a tube in a ghost cell suspension have been observed (Goldsmith, 1972). The lateral displacements of the spheres were due to an interaction with the surrounding RBC's, which showed continuous deformation and small sideway displacements. Distorsions and alterations in position of the RBC's were also observed in vivo (Bloch, 1962). However, at present no

direct information is available about the movement of individual platelets in microvessels outside the capillary region.

In contrast to branch points, the concentration profiles recorded in or distal to curves did not show an obvious difference from those recorded in straight parts of a vessel. This finding is supported by in vitro experiments. In glass tubes of 45 μm diameter with a 90°-curve and perfused with whole blood at a RBC velocity of 2-3 mm/s the shifts in the peak of the RBC velocity profile due to the curvature were small (Hung et al, 1980).

The finding in the present study that in arterioles with a diameter of 20-35 μm the platelet concentration in wall segments is approximately twice the concentration in the center is in agreement with the in vitro results of Palmer (1967). With whole blood flowing through a rectangular slit with a short diametric axis of 30 μm and ending into three branches he found a platelet concentration approximately twice as high in the peripheral branches as in the central branch. Indirect support for platelet peripheralization in whole blood is given by three other in vitro studies, although these studies were conducted in flow systems with much larger diameters (Turitto et al, 1972; Blackshear et al, 1977; Eckstein and Beck, 1978; Beck and Eckstein, 1980).

The higher concentration of platelets near the vessel wall as compared to a random distribution seems favorable with regard to their functioning. It implicates an increased collision frequency with the wall and with each other (Goldsmith, 1972), an increased local concentration of substances secreted by the platelets (Niewiarowski, 1981) and a more effective scavenging (Gordon, 1976) of substances released from the wall. Moreover, in the case of wall injury and liberation of activating substances a higher percentage of the platelets will come into contact with these activators or will be subjected to increased shear stresses following vessel transection (Schmid-Schonbein et al, 1976), since these stresses are highest near the wall. Because the flow velocity decreases toward the wall these platelets have more time available to become activated and to adhere to an area of wall injury.

In calculations of the kinetics of hemostatic plug- or thrombus-formation, for instance following laser injury (Poliwoda et al, 1973; Arfors et al, 1976), the non-uniform distribution of the blood platelets must be taken into account. However, the rate of delivery of the platelets depends not only on the concentration profile, but also on the velocity profile of the platelets in a vessel. At present only the velocity of the RBC's has been

measured in these vessels and the precise nature of the RBC velocity profile is unknown (Berman and Fuhro, 1969; Goldsmith, 1972; Baker and Wayland, 1974; Schmid-Schonbein and Zweifach, 1975; Hung et al, 1980).

4.5. CONCLUSIONS

- The concentration distributions of blood platelets flowing in small arterioles (21-35 μm diameter) were non-uniform.

- At vessel sites without upstream branch points for at least ten vessel diameters the highest concentration was always found near the wall.

- In general, the platelet concentration progressively decreased from the wall toward the vessel center.

- The mean concentration near the wall was about twice the mean concentration in the center.

- In contrast to curves, branch points had a marked influence on the concentration profile. Distal to sidebranches the platelet concentration was reduced in the part of the vessel adjacent to the side branch.

5. ORIENTATION AND DIAMETER-DISTRIBUTION OF RABBIT BLOOD PLATELETS FLOWING IN SMALL ARTERIOLES

5.1. INTRODUCTION

The blood cells which, during evolution, specialized in sealing a wound in a vessel by means of cellular aggregation, have developed in time to lenticular or disk-shaped cells without a nucleus: the mammalian platelet. In the equatorial plane of the platelet a circumferential band of microtubules is present (see section 1.2.2.). It is assumed that this tubular system acts as a cytoskeleton to support the lenticular shape. It is interesting to know that these microtubules are already present in the thrombocytes of the nonmammalian vertebrates, which still contain a nucleus. It is believed that the tubules likewise play a role in maintaining the thrombocytes ovoid disk shape (Belamarich, 1976).

Therefore, it is tempting to assume that the lenticular shape of these cells does have a specific advantage. However, a few and only speculative remarks can be found in the literature about the meaning of this prominent feature of the platelet, such as: an increase in surface area per unit volume with a concomitant increase in surface receptors (Belamarich, 1976), or the disk-shape facilitates the passage of the platelets through capillaries (Crawford, 1976). The latter seems unlikely because platelet diameters are generally less than the diameter of even the smallest capillaries.

In case of a cell which spends its whole life in a flowing liquid one may think in the direction of streamlining, either to reduce the amount of wear and tear on the cell, or to favor its rheological behavior. The shape of the cell is one of the factors that influences the movement of individual platelets flowing in whole blood, and consequently their function. Adhesion and aggregation can only occur if platelets come into contact with the wall and with each other, and therefore depend on the rheological behavior of the individual platelets.

The present knowledge of the movement of individual platelets in flowing blood is solely based on the findings in theoretical models and *in vitro* experiments, employing rigid spheres and disks (Jeffery, 1922; Goldsmith and Mason, 1962, 1967; Goldsmith, 1972; Goldsmith and Marlow, 1972). Most of these experiments were performed in dilute suspensions. These studies show

that rigid disk-shaped particles or cells which are flowing through a tube in a dilute suspension rotate with a periodic angular velocity due to the torque on the particle resulting from the velocity gradient in the surrounding fluid. The angular velocity of the particle is maximal when the major diameter of the particle is perpendicular to the axis of flow and minimal when aligned with flow. Because of the differences in angular velocity during the period of rotation a particle spends more time in orientations near alignment with the direction of flow than in orientations with its major diameter perpendicular to the axis of flow. The period of rotation decreases when the velocity gradient in the fluid surrounding the particle increases. In contrast to deformable disk-shaped particles, rigid disks did not show an increase in orientation toward the flow axis with an increase in velocity gradient or at increasing distance from the center of the tube.

In this chapter the orientation of platelets flowing in small arterioles in the rabbit mesentery was investigated to evaluate the existing knowledge and to obtain more information about the movement of individual platelets in whole blood. Simultaneously a frequency distribution of the diameter of rabbit platelets was obtained in vivo.

5.2. METHODS

5.2.1. Experimental part

For this study some of the experiments as described in chapter 4.2 were used. Five straight arterioles (diameter 21-32 μm; $\bar{x}$ = 26 μm) without upstream branch-points within at least 10 vessel diameters were selected from 4 animals. The red blood cell (RBC) velocity in these vessels as measured with the dual-slit technique (section 4.2.1.) ranged from 1.5 to 3.5 mm/s ($\bar{x}$ = 2.5 mm/s).

In short, platelets flowing in these arterioles were observed by fluorescence microscopy following labeling of the platelets in vivo with the fluorochrome acridine red. Incident illumination was used with flashes of short duration (< 0.1 ms) at an interval of 180 ms. Each vessel was examined through a Leitz 100x water-immersion objective (numerical aperture 1.20) with position of sharp focus in the median plane of the vessel. The images were recorded on videotape through a low light level TV camera. Final magnification on the front plane of the TV camera was 200X.

Off-line analysis was done field by field. The calculated interline spacing in the object plane was 0.17 μm. In a way similar to that described in chapter 4.2 the vessel walls were drawn on an overlying transparent sheet and decribed as left or right wall according to the direction of flow. The lumen was divided into 6 equal segments and the platelets in these segments were localized within an optical section. To be able to define the boundaries of the platelet-images clearly, only platelets with a reasonably sharp image were used. Platelets were considered to be within the section at a signal-to-noise ratio of at least 2:1.

To determine the orientation of a platelet observed in a segment and present within the optical section, three measurements were made (see fig. 5.1): 1) the length of the major axis of the image (=a), 2) the length of the short axis of the image (=b), 3) the angle between the direction of the major axis of the image and the axis of flow (clockwise, 0-180^{o}). To measure this angle a second transparent sheet was taped over the first one. On this second sheet the axis of flow was drawn as a straight line exactly in

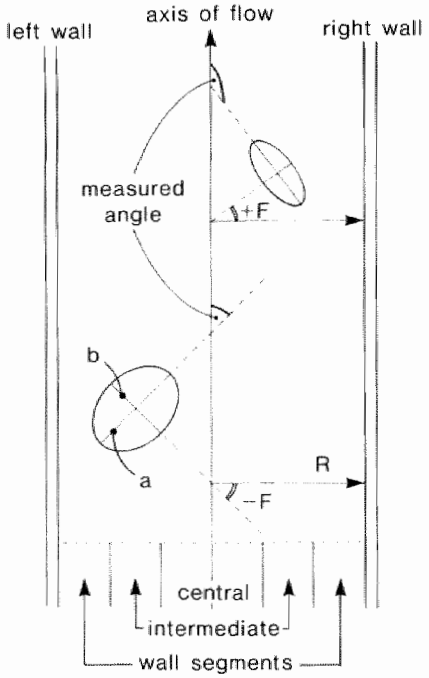


Fig. 5.1
Schematic drawing of the microscopic images of an arteriole (radius = R) and two platelets. The vessel walls were designated as left or right according to the direction of flow. a = major axis of the image of a platelet, b = short axis. The angle between the direction of a and the axis of flow was measured as shown. From this measured angle the angle F was calculated. F = angle between R and the projection on the image plane of a vector coinciding with the platelet axis of rotational symmetry (see fig. 5.2).

between both vessel walls. The angle was measured with a protractor (scale division 1^{o}) and the lengths with a vernier calipers (scale division 0.1 mm, which was less than one fifth of the interline spacing on the monitor). In each vessel a time period ranging from 20 to 40 s was analyzed. Since the platelet concentration is lowest in the center of the vessel (see chapter 4), in some experiments more fields had to be analyzed to obtain a sufficient platelet number in the central segments. Of platelets with $b/a > 0.9$, i.e. platelets observed as (almost) round, no angle could be measured with any accuracy. The number of platelets analyzed in wall, intermediate and central segments (corresponding segments were pooled in the analysis, see below) were 681, 389 and 354, respectively.

To assess the error made in determining a and b, the length of each of these axes was measured in 100 platelets by three independent observers. To determine the error made in assessing the angle between a and the direction of flow, this angle was measured by two independent observers in three groups of ten platelets, with $b/a < 0.4$, $0.6 < b/a < 0.7$ and $0.8 < b/a < 0.9$, respectively. In this case the axis of flow was drawn by one observer. To assess the error made in drawing the axis of flow, this axis was drawn in each of the five arterioles by two independent observers and the angle between both lines was measured.

5.2.2. Theory and analysis

The majority of the platelets in the circulation have a lenticular shape (Frojmovic and Milton, 1982). In the analysis of the data a platelet is assumed to be perfectly symmetrical around its axis of rotational symmetry. To each platelet a vector is designated coinciding with its (platelet) axis of rotational symmetry. This vector is called PARS (see fig. 5.2).

A Cartesian coordinate system (x, y, z) is constructed (see fig. 5.2) with the x-axis radially directed, the y-axis along the axis of flow and the z-axis along the optical axis. To describe the orientation of a platelet the origin of this coordinate system is positioned in the center of gravity of that platelet.

The orientation of a platelet is defined by two angles: 1) the angle of PARS with one of the axes, which will be called the principal axis, and 2) the angle of the projection of PARS on the plane perpendicular to the principal axis with one of the other axes.

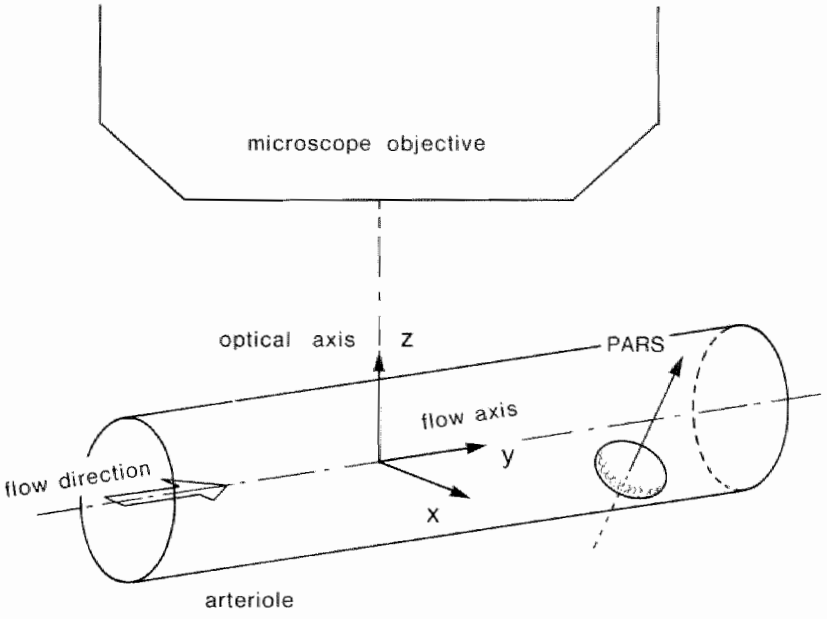


Fig. 5.2
Arteriole with Cartesian coordinate system (x,y,z). x = radially directed, y = along the flow axis and z = along the optical axis. A platelet within the vessel is shown. PARS = a vector coinciding with the platelet axis of rotational symmetry. To describe the orientation of a platelet the origin of the coordinate system was positioned in the center of gravity of the platelet.

Optical axis as principal axis: angles F and T

The image of a platelet as observed through the microscope is the projection of the platelet on the image plane. Since the image plane coincides with the median plane of the vessel (xy-plane), the image can be regarded as the projection of the platelet on the xy-plane. As shown in chapter 3 all platelets within the optical section can in first approximation be considered as localized in the xy-plane. The projection of the PARS on the xy-plane coincides with the short axis of the image. The angle of the xy-projection of the PARS with the x-axis is called F (see fig. 5.1), defined from $+90^{o}$ to -90^{o}. As is clear from fig. 5.1 the angle F has the same value as the measured angle with a negative sign, or the supplement of the measured angle with a positive sign. Since the images of platelets located in the vessel on the left hand side of the yz-plane are mirror symmetric with those

on the right hand side, data can be pooled by converting the images on the left hand side to images on the right hand side simply by changing the sign of the angle F. In the case of platelets with b/a > 0.9 where no angle was measured (the numbers in the central, intermediate and wall segments being 34, 17 and 29, respectively), a random value of the angle F was attributed to these platelets.

The orientation of a platelet with respect to the Cartesian coordinate system is defined when in addition to the angle F, the angle of PARS with the z-axis, called T, is known (see fig. 5.3). The angle T describes the tilting of the platelet with respect to the xy-plane. Since no distinction is possible between images of platelets with a positive or negative value of the angle T, the angle T was defined from 0-90°. The true diameter of the platelet is called α and the true thickness ρ (see fig. 5.3). The axis ratio of the platelet (ρ/α) is defined as K. The image of a disk-shaped platelet with angle T = 0°, i.e. PARS coinciding with the optical axis, is a circle. With tilting of the platelet the circular image becomes an ellipse. From the lengths of the major axis (a) and short axis (b) of the ellipsoidal image (see fig. 5.1) the angle T can be calculated (see appendix 5.A) provided the axis ratio of the platelet, K, is known. Since the axis ratio of each individual platelet cannot be deduced from the present measurements, a frequency distribution of the axis ratio of glutaraldehyde hardened rabbit platelets as published by Frojmovic and Panjwani (1976) was used to calculate the angle T from a and b (see appendix 5.A).

Flow axis as principal axis: angles P and Q

Theoretical studies and in vitro experiments have shown an orientation of disk-shaped particles with respect to the axis of flow. For the interpretation of the present data a coordinate system having its symmetry around the axis of flow will yield physically directly interpretable results. Therefore, the axis of flow is an optimal principal axis for the description of platelet orientation with respect to flow. The angle of PARS with the axis of flow (y-axis) is called P, defined from 0-90° (see fig. 5.4). The angle of the xz-projection of PARS with the z-axis (optical axis) is called Q, defined from $+90^{\circ}$ to -90°. For the calculation of the angles P and Q from the angles F and T the reader is referred to appendix 5.B. The transformation is schematically shown in fig. 5.4. As can be seen from fig. 5.4, the angle P is analogous to the angle F and the angle Q to the angle T.

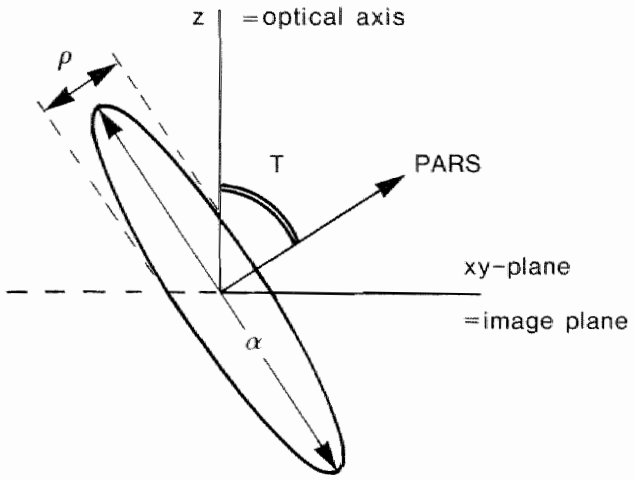


Fig. 5.3
Cross-section of a lenticular shaped platelet tilted with respect to the image plane. T = angle between PARS and the optical axis. α = diameter and ρ = thickness of the platelet.

OPTICAL AXIS COORDINATE SYSTEM FLOW AXIS COORDINATE SYSTEM

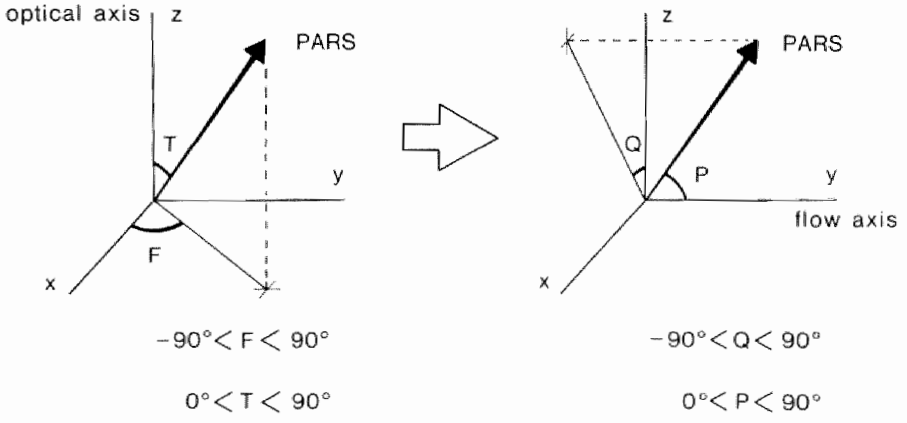


Fig. 5.4
Coordinate transformation: from a description of platelet orientation with the optical axis as principal axis (angles F and T) to a description with the flow axis as principal axis (angles P and Q). P = angle between PARS and the flow axis. Q = angle between xz-projection of PARS and the optical axis. The angles P and Q were used in this study.

The meaning of the angles P and Q with respect to the orientation of the platelets toward the planes of shear in the vessel is as follows. When P = 0°, PARS coincides with the axis of flow and the diameter of the platelet is perpendicular to the flow axis, i.e. position across the flow and spanning as much shear as possible. A platelet is maximally aligned with flow, i.e. spans minimum shear, irrespective of its position within the vessel if P = 90° and PARS points toward the axis of flow simultaneously. Whether PARS points toward the axis of flow is described by the angle Q and the xz-position of the platelet within the vessel.

The rotation of a platelet in a shear field as described by theory and observed in vitro for rigid disk-shaped particles (Jeffery, 1922; Goldsmith and Mason, 1962; Goldsmith and Marlow, 1972) can be illustrated as follows. Consider a platelet at the right hand side and in the median plane of the vessel. The platelet is maximally aligned with flow, i.e. the angle P = 90°. When the platelet rotates from this maximally aligned orientation through the orientation opposite to flow toward a new maximally aligned orientation, the angle P changes from 90°to 0° and back to 90°. During the first half of its path, i.e. from maximally aligned toward the opposite orientation, the angle Q is negative. During the second half of its path, i.e. from the opposite orientation back toward alignment, the angle Q is positive.

5.2.3. Statistical methods

Errors and worst case analysis

To assess the error made in determining the angles P and Q, and hence in the percentages of the platelets present within different orientations a worst case analysis was performed (see appendix 5.C).

The error made in determining the angle F has two components. The error made in drawing the reference axis of flow on the transparent sheet was neglected since it appeared to be less than 1°. The standard deviations of the differences in the angle F between both observers in the three groups of ten platelets with $b/a < 0.4$, $0.6 < b/a < 0.7$ and $0.8 < b/a < 0.9$ were 1.8, 2.7 and 5.2^{o}, respectively.

Therefore, in the worst case analysis these values were attributed to platelets having $b/a < 0.45$, $0.45 < b/a < 0.75$ and $0.75 < b/a < 1.0$, respectively.

The major errors in assessing a and b, and hence in the angle T, result from the uncertainty in defining the edges of the image of a platelet. Localization of the edge of the image is influenced by such factors as diffraction, smearing of the image with defocusing, irregularities in shape and fluorescent labeling of the platelet, differences in background fluorescence and the interline spacing on the TV monitor. To assess the error in determining the angle T due to the combined influence of all these factors, the standard deviations of the errors made in a and b were determined from the measurements on 100 platelets by 3 observers.The standard deviations in a and b were used in combination with two extreme values of K (platelet axis ratio) to obtain worst case limits for the angle T (see appendix 5.C).

For each platelet worst case limits of the angles P and Q were obtained from the limits of the angles F and T (see appendix 5.C). In case of the angle P the differences between the limits of each individual platelet were small in most cases: with a median of 6° and an interquartile range (see chapter 2.2.5.) from 4 to 10° and range from 0 to 53°. In contrast to the angle P, the differences between the limits in the angle Q were much greater: with a median of 26° and an interquartile range from 16 to 33° and range from 6 to 66°. In case of an estimation of the percentage of platelets presented within certain values of the angles P and Q the worst case limits of the individual platelets were combined in a way that yielded the maximal variation.

Presentation and test of significance

For each segment a picture is presented showing simultaneously the angles P and Q of each individual platelet. For the presentation of these combined data points the center of gravity of each platelet was considered to be at the origin of the Cartesian coordinate system. The orientation of each platelet is represented by the intersection of its PARS with the surface of a sphere which has its center at the origin of the Cartesian coordinate system. Since the angle P was defined from 0-90° and the angle Q from +90°to -90°all data points can be positioned on the concomitant quarter sphere. For the presentation, this quarter sphere and its data points were projected on the xz-plane in such a way that the surface density in the projection was maintained, yielding a semi-circle with projected data-points.

In addition, the cumulative frequency distributions of the angle P are presented for each segment, as well as the concomitant curve which one expects to find in case of a random orientation of the platelets.

In case of a random orientation the intersections of the PARS of the platelets with the surface of the above mentioned sphere are equally distributed over this surface. Therefore, the percentage of the platelets expected within certain values of the angles P and Q at a random platelet orientation is equal to the percentage of the surface area of the sphere within those limits.

The percentages of the platelets within certain values of the angles P and Q describing different orientations, are given. Variations in the number of platelets within these values based on the worst case analysis are presented within parentheses.

To test a difference between the observed and expected frequencies the chi-square test for goodness of fit was used (Rao, 1973).

5.2.4. Influence of the depth of the optical section

In section 5.2.2. it has been mentioned that in case of complete alignment the angle Q depends on the localization of the platelet within the vessel, in contrast to the angle P. Within the optical section the precise position of the platelet along the optical axis is not known. Therefore, in case of complete alignment in a certain segment a range for the angle Q will be found, depending on the depth of the optical section and the diameter of the vessel. This range for the angle Q increases toward the center of the vessel.

To select values of the angle Q for the description of complete alignment, the depth of the optical section was assumed to be 5 μm and the vessel diameter to be 25 μm. In this case a platelet observed within the wall, intermediate and central segments could still be aligned with flow at an angle Q equal to or exceeding about 70°, 60° and 0°, respectively. However, because of the error made in assessing the angle Q a margin of 10° was added and values of the angle Q equal to or exceeding 60°, 50° and 0° were taken for the description of complete alignment in the wall, intermediate and central segments, respectively.

5.3. RESULTS

In fig. 5.5 the combinations of the angles P and Q of all individual platelets in the 3 segments are depicted. Each semi-circle is the projection of the surface area of a quarter-sphere (see section 5.2.3.) The angle Q runs around the circumference of the semi-circle from -90° to +90°and the angle P along the radius from the center to the outside (0 - 90°).

Here it should be stressed that this semi-circle should not be regarded as the half of a cross-section through the vessel. It represents the orientation-distribution of the platelets within a segment of the optical section as is schematically shown in fig. 5.5.D.

In the center of the semi-circle, i.e. the angle P = 0°, the diameter of a platelet is perpendicular to the axis of flow. While viewing along the flow axis such a platelet is observed face-on, as is schematically shown in fig. 5.5.E. Platelets on the circumference of the semi-circle have their diameter parallel to the axis of flow. While viewing along the flow axis these platelets are observed side-on, as is shown in fig. 5.5.E. Whether a platelet on the circumference is completely aligned or not, depends on its position in the vessel (see section 5.2.4.).

In case of the wall segments the distribution of the points within the semi-circle is non-uniform with a preference toward an angle P = 90° and an angle Q = ± 90°, i.e. toward alignment of the platelets with flow. In the central segments the distribution of the points seems more random.

To assess a tendency of the platelets toward alignment with flow in the different segments, an area on the semi-circle which was considered to represent alignment was selected and the percentage of the platelets present within this area was determined. Values for the angle P were chosen to be equal to or exceeding 70°. For the angle Q values equal to or exceeding 60°, 50° and 0° were chosen in the wall, intermediate and central segments, respectively (see section 5.2.4.).

The percentages of the platelets within these values were 62% (46 - 72%), 49% (38 - 55%) and 53% (38 - 62%) for the wall, intermediate and central segments, respectively. Since unequal values of the angle Q were used for the different segments these percentages as such cannot be compared, but they have to be compared with the percentages as expected in each area of the semi-circle in case of a random orientation of the platelets.

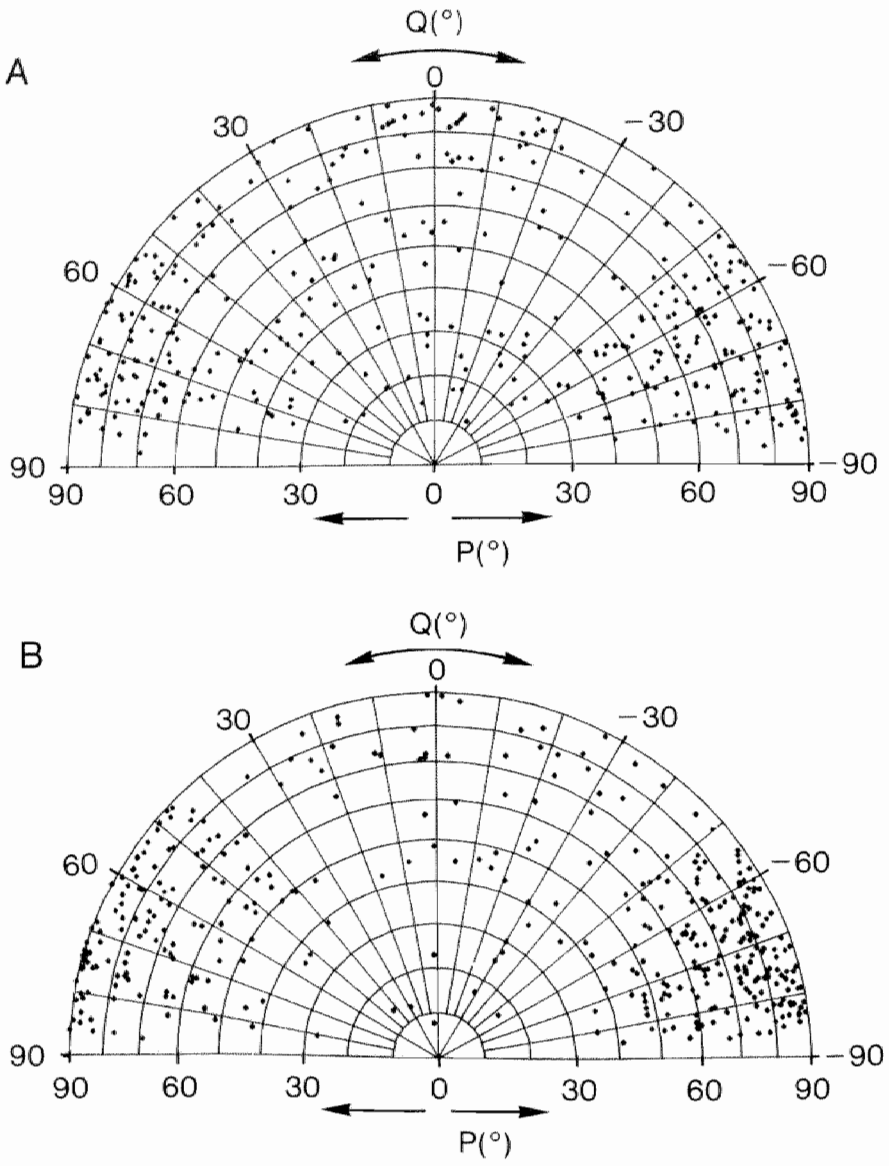
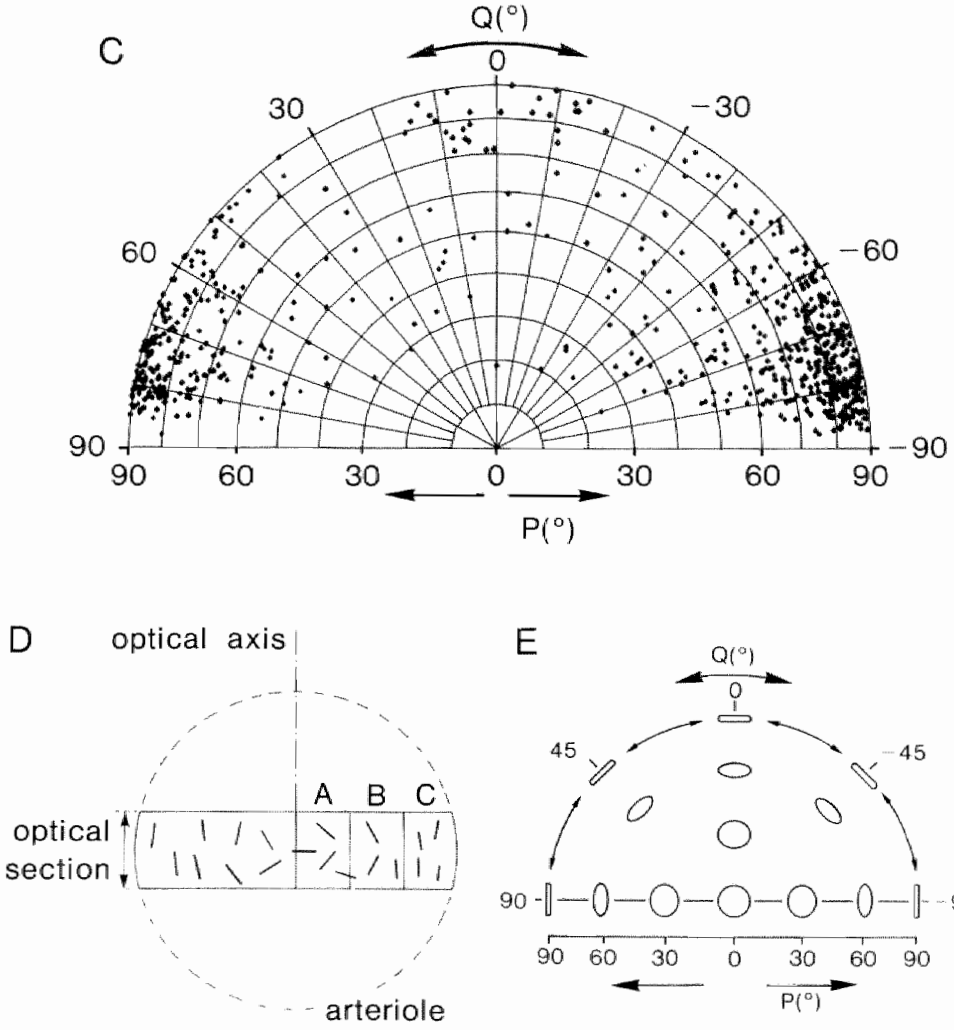


Fig. 5.5
Combinations of the angles P and Q of all individual platelets in the central (A), intermediate (B) and wall segments (C). Each semi-circle represents the orientation distribution of the platelets within a segment of the optical section as is schematically shown in D. The semi-circle is the projection of the surface area of a quarter sphere. Each point is the projection of the intersection of the PARS of an individual platelet positioned in the center of the sphere with the surface of the quarter sphere (see section 5.2.3.).



The angle Q runs around the circumference from -90° to +90°, and the angle P along the radius from the center toward the outside (0-90°). In the center of the semi-circle, i.e. the angle P = 0°, the diameter of the platelet is perpendicular to the axis of flow. While viewing along the flow axis, such a platelet is observed face-on as is schematically shown in E. Platelets on the circumference of the semi-circle have their diameter parallel to the axis of flow. While viewing along the flow axis, such a platelet is observed side-on as is shown in E.

The expected frequencies are 11, 15 and 34% for the angle Q equal to or exceeding 60°, 50° and 0°, respectively. Thus, the percentage of platelets in the wall, intermediate and central segments aligned with flow in excess of the percentage expected in case of random orientation was 51, 37 and 19%, respectively. These figures suggest that the tendency for alignment of the platelets in the planes of shear increases from the center of the vessel toward the wall.

In fig. 5.6 the cumulative frequency distributions of the angle P in the three segments (1 = center, 2 = intermediate, 3 = wall) as well as the curve for random orientation (0) are shown. It is clear from this figure that for each segment the curve of the angle P differs from a random orientation and that the shift of the curve toward complete alignment, i.e. P = 90°, increases from segment 1 to 3.

Another feature shown by this figure is that orientations of the platelet with its diameter perpendicular to the axis of flow, i.e. with the angle P near 0°, are almost absent. For platelet orientations with the angle P < 30° the expected value in case of a random orientation is 13,4%. The values found for the segments 1,2 and 3 were 5.6% (2.3-9.3%), 2.3% (1.5-5.1%) and 1.0% (0.4-2.5%), respectively. Even for the maximal value in segment 1 the difference was significant ($p < 0.025$). Therefore, a random orientation of the platelets can be rejected for each segment.

Figure 5.7 shows the histogram of the diameter of the platelets. The mean and standard-deviation of this distribution were 3.15 ± 0.72 μm. The differences between the diameter of the platelets in the various segments were much smaller than the experimental error made in measuring this diameter.

5.4. DISCUSSION

The study in this part of the thesis shows that the orientation of blood platelets flowing in small arterioles is not random, but that they tend to align themselves in the planes of shear. The data suggest that this tendency for alignment increases from the center of the vessel toward the wall. Of the platelets present within a distance from the wall of one third of the vessel radius only 1% of the platelets was found lying with their equatorial plane ± 30° perpendicular to the flow axis, while about 60% was aligned with flow within ± 20-30°.

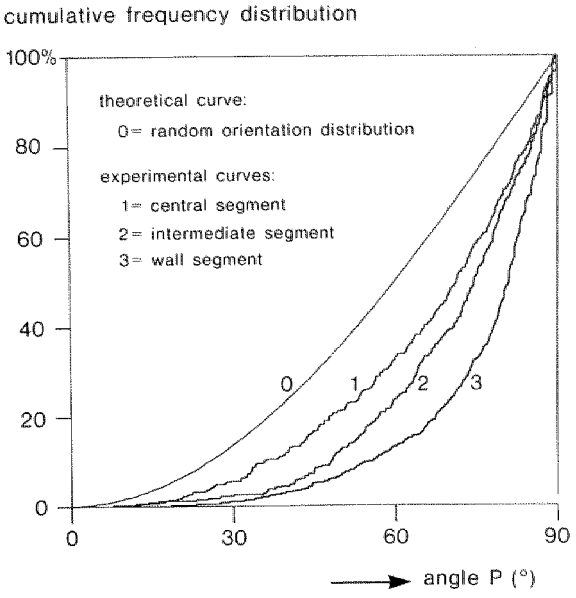


Fig. 5.6
Cumulative frequency distributions of the angle P.

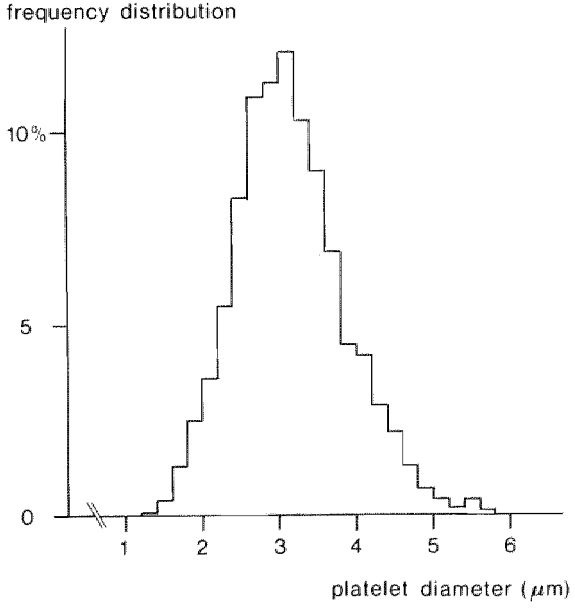


Fig. 5.7
Histogram of the diameter of rabbit platelets flowing in small arterioles as determined from the major axis of their microscopic image.

Only platelets flowing within a shallow optical section in the median plane of the vessel were studied. To describe the orientation of the platelets two angles were used, P and Q. The most important one is the angle P, since it is the angle of the platelet axis of rotational symmetry with the axis of flow. It allows testing for a random orientation of the platelets and an estimation of the percentage lying across the flow irrespective of the localization of the platelets within the vessel.

The error made in assessing the angle P was small and hence allowed a rather accurate estimation of the percentage of platelets lying across the flow. In addition, a random orientation of the platelets could be rejected even on the basis of the frequencies obtained in the worst case analysis. Moreover, the values of the angle P suggest an increase in alignment of the platelets with flow from the center toward the wall. However, for complete description of alignment with flow the angle Q has to be known in addition to the angle P.

The angle Q is the angle between the projection of the platelet axis of rotational symmetry on the plane perpendicular to the flow axis and the optical axis. In contrast to the angle P the error made in assessing the angle Q was great. This error, however, was neutralized to a considerable degree by the fact that for the angle Q a range of 30° or more had to be selected due to the depth of the optical section with regard to the small vessel diameter. In the wall segment a range of 30° could be used, which is small enough for reasonable description of alignment.

The estimation of the percentage of platelets near the wall aligned with flow seems to be accurate within about ± 10%, as is shown by the worst case analysis. At increasing distance from the wall the range for the angle Q that could be expected in case of alignment increased. Consequently the alignment with respect to the angle Q is less well defined toward the center. Moreover, figures for alignment obtained with different values for the angle Q cannot be compared as such. However, when each figure was corrected for the number of platelets expected in case of a random orientation, they also showed an increase in alignment from the center toward the wall. Therefore, the conclusion of an increasing tendency for alignment from the center toward the wall seems justified.

Comparison of our data with the literature is hampered by the fact that in all previous studies orientation has been described by one angle only. As discussed the orientation of platelets cannot be described completely by

measuring only one angle. In the in vitro studies of Goldsmith and Mason (1962) and Goldsmith and Marlow (1972) an angle which is analogous to the angle P in our study has been used. In contrast to theory and the findings of these in vitro studies the cumulative frequency distribution of the angle P changed with radial position in the vessel, suggesting an increase in alignment with an increase in velocity gradient. This increase in alignment was also suggested by the data combining the angle P and the angle Q. However, theory and the in vitro studies were restricted to freely rotating rigid disks in parabolic flow.

Therefore, the aforementioned discrepancy might indicate either that platelets cannot be regarded as purely rigid cells or that platelet orientation is influenced by the excess of flexible RBC's.

At present few and only speculative remarks can be made about the implications of these results with regard to platelet function. Since platelets occupy less than half a percent of the total blood volume, their rheological behavior, when they are not activated, is likely not important for the flow characteristics. Therefore, the disk shape of the platelets and/or the rheological consequences have to be considered in the light of platelet functioning itself or their survival.

An effect of shear stresses as low as 2 - 3 N/m2 on platelets has been noticed following exposure times of 20 min (Stein and Sabbah, 1980). Although little is known about the effect of lower shear stresses during relatively long exposure times, it might well be that alignment toward the planes of shear protects the "explosive" platelets (see chapter 1) and reduces the wear and tear on the cell membrane.

Alignment in the planes of shear allows platelets flowing near the wall to come close by or in contact with the endothelium with their flat side, and hence with a greater surface area. This might favor the exchange of substances between platelet and endothelium (see chapter 4).

The diameter of rabbit blood platelets flowing in vivo as determined in this study by fluorescence microscopy is 3.15 ± 0.72 μm ($\bar{x}$ ± sd), and the diameter distribution is rather symmetric. Using phase contrast microscopy Frojmovic and Panjwani (1976) found in vitro a diameter of 3.0 ± 0.40 μm for normal and of 3.1 ± 0.43 μm for glutaraldehyde hardened rabbit platelets. These values were obtained by measuring midway between inside and outside diffraction rings. For the outside diffraction rings the diameter found, was 3.9 ± 0.70 μm. Therefore, our data obtained in vivo are in agreement with the

data obtained *in vitro* within the error due to diffraction. Moreover, it indicates that in our study the influence of smearing due to defocusing must have been small, and that, on the other hand, venepuncture, handling of the platelets *in vitro* and glutaraldehyde fixation did not alter platelet diameter significantly.

5.5. CONCLUSIONS

- The orientation of blood platelets flowing in small arterioles is not random. They tend to align in the planes of shear, i.e. toward a position with minimal span of shear.

- The data suggest that the tendency for alignment increases from the center toward the wall of the vessel.

- Within a distance from the wall of one third of the vessel radius only 1% of the platelets are lying with their flat side ± 30° across the flow, while about 60% are aligned with the planes of shear within ± 20-30°.

- The distribution of the diameter of rabbit platelets flowing *in vivo* is rather symmetric. The mean diameter and standard deviation being 3.15 ± 0.72 μm.

5.6. APPENDIXES

Appendix 5.A: calculation of the angle T

The angle T is defined as the angle of PARS with the z-axis (optical axis), see fig. 5.3. This angle describes the tilting of the platelet with respect to the xy-plane (image plane). The image of a tilted disk is an ellipse. The angle T of an infinitely thin platelet with true diameter α can be obtained by measuring the major axis (α) and the short axis (β) of the image of the platelet

$$\beta = \alpha \cos T. \qquad (1)$$

However, for a platelet with a thickness ρ or axis ratio $K = \rho/\alpha$ equation (1) becomes

$$\beta = \alpha \sqrt{\cos^2 T + K^2 \sin^2 T} \qquad (2)$$

Equation (2) gives implicitly the angle T as a function of β/α and K. Rearranging equation (2) gives

$$T(\beta/\alpha, K) = \arccos \sqrt{\frac{(\beta/\alpha)^2 - K^2}{1 - K^2}}, \quad 0 \leq K \leq \beta/\alpha. \qquad (3)$$

The axis ratio, K, of each individual platelet cannot be deduced from the present measurements. A frequency distribution of the axis ratio of glutaraldehyde hardened rabbit platelets has been published by Frojmovic and Panjwani (1976). Since they found no difference in geometry between hardened and normal platelets this empirical frequency distribution of K was used to determine the expected value of the angle T, T_{exp}, according to equation (4)

$$T_{exp}(\beta/\alpha) = \int_0^1 T(\beta/\alpha, K)\, F_K(K|\beta/\alpha)\, dK \qquad (4)$$

where $F_K(K|\beta/\alpha)$ is the conditional density function of K given β/α.

$F_K(K|\beta/\alpha)$ is given by equation (5)

$$F_K(K|\beta/\alpha) = \frac{P_K(K)}{\int_0^{\beta/\alpha} P_K(K)dK}, \quad 0 \leqq K \leqq \beta/\alpha$$

$$= 0 \quad \text{otherwise} \qquad (5)$$

where $P_K(K)$ is the marginal density function of K. It can be seen that F_K is the normalized part of P_K within the interval $0 \leqq K \leqq \beta/\alpha$. The empirical frequency distribution of K (Frojmovic and Panjwani, 1976) was substituted for P_K.

The true values α and β are not known. In order to estimate the angle T using equation (4) the corresponding measured values, a and b, have to be substituted, yielding

$$\text{estimated angle } T = T_{exp}(b/a) \qquad (6)$$

The transfer function $T_{exp}(b/a)$ is shown in fig. 5.8. This figure also shows the functions T(b/a,0) and T(b/a,0.35). These are the transfer functions for an infinitely thin platelet (K = 0) and a platelet at the 97.5%-limit of the frequency distribution P_K (K = 0.35). The latter transfer functions were used to obtain worst case limits for the angle T (see appendix 5.C).

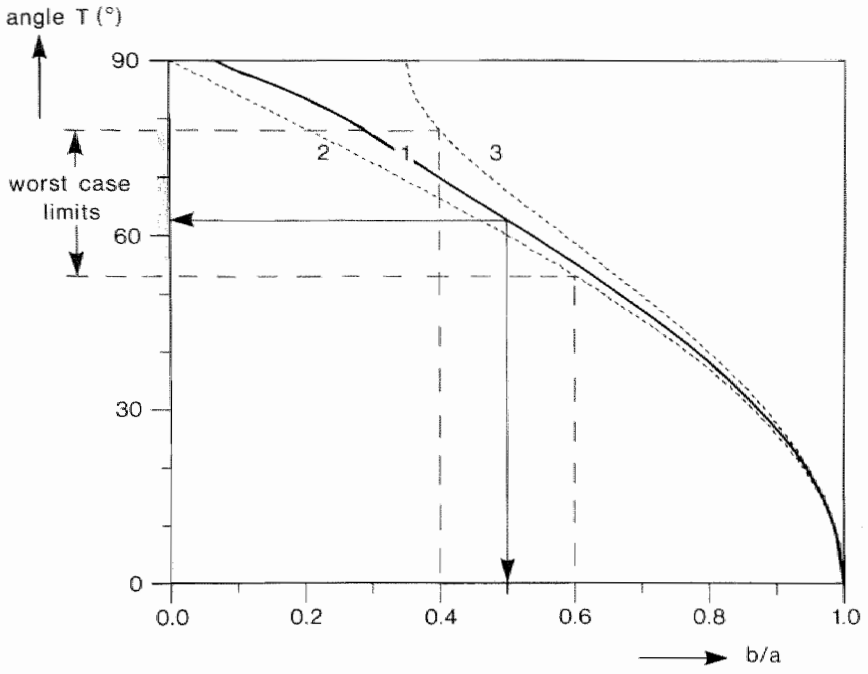


Fig. 5.8
Transferfunction, $T_{exp}(b/a)$, used to estimate the angle T (1). Also shown are the functions for an infinitely thin platelet (2) and a platelet at the 97.5%-limit of the frequency distribution of P_K (3). Functions 2 and 3 were used to obtain worst case limits for the angle T. An example is given for the determination of the worst case limits from b/a = 0.5 ± 1 sd.

Appendix 5.B: calculation of the angles P and Q

The angles P and Q are calculated from the angles F and T using the following equations:

$$\cos P = \sin F \cdot \sin T \qquad (7)$$

$$\tan Q = \cos F \cdot \tan T \qquad (8)$$

The transformation is shown schematically in fig. 5.4

Appendix 5.C: worst case analysis

The measured values of the major axis (a) and the short axis (b) of the image of a platelet differ from the true values α and β by an error λ and ℓ, respectively: $a = \alpha + \lambda$ and $b = \beta + \ell$. The variance of the error made in determining a (var λ) and in b (var ℓ) were determined from the measurements on 100 platelets by 3 observers. Consequently the variance of b/a (var b/a) for each individual platelet was estimated using equation (9) in which the second order terms have been neglected

$$\text{var } b/a = 1/\alpha^2 \, [\text{var } \ell + (\beta/\alpha)^2 \, . \, \text{var } \lambda] \qquad (9)$$

and approximating α by a and β by b. As can be seen from equation (9) var b/a decreases with an increase in diameter of the platelet or a decrease in β/α, i.e. increased tilting of the platelet.

To obtain worst case limits of the angle T for each platelet, b/a plus and minus one standard deviation were combined with the transfer functions T(b/a,0) and T(b/a,0.35), (see appendix 5.A). The combination yielding the greatest interval in the angle T was selected. An example is given in fig. 5.8 for a platelet with a diameter of 3 μm and b/a = 0.5, in which case the standard deviation was 0.1.

Worst case limits of the angle F were defined as F plus or minus one standard deviation (see section 5.2.3.).

Worst case limits of the angles P and Q were obtained from the worst case limits of T and F. From the different combinations the two values of each angle yielding the greatest interval were selected. The validity of this operation is based on the fact that the angles P and Q are monotonically rising functions of the angles F and T.

6. SUMMARY AND CONCLUSIONS

The aim of this thesis was to study *in vivo* the rheological behavior of blood platelets. Blood platelets are small disk-shaped cells that play an important role in the response of blood to vessel wall injury (hemostasis, thrombosis) and in maintaining the endothelial supportive function. For these functions platelets have to come close by or in contact with the vessel wall. A survey of the literature (chapter 1) showed that information about the rheology of platelets is limited, in contrast to the rapidly growing knowledge of platelet structure and biochemistry. Moreover, the information presently available is mainly derived from models and *in vitro* studies.

Studies on platelet rheology *in vivo* are necessary to test the correctness of *in vitro* findings which may be different from the results obtained in intact blood vessels. It is well known that platelets quickly respond even to slight changes in their environment, while in the body they function in close connection with other cells and functional systems. This, for example, may explain the discrepancy between the effects of drugs or diets on platelet function *in vitro* and *in vivo*.

In the present investigation the microcirculation (blood vessels with a diameter $< 100\ \mu m$) was selected to study platelet rheology *in vivo*, because these small vessels yield the opportunity to observe the platelets directly using intravital fluorescence microscopy. The microcirculation in the surgically exposed mesentery of anesthetized rabbits was used.

The first part of the study concerns the visualization of individual platelets flowing in a microvessel amidst an excess of red blood cells (RBC's). This was achieved by labeling the platelets *in vivo* with a fluorescent dye. Illumination with flashes of short duration was used to prevent a smeared image due to movement of the platelets. To study the rheology of these labeled platelets the position of all platelets in the cross-sectional area of the vessel has to be known during the same flash. This is impossible because with a depth of field equal to or exceeding the diameter of the vessel, the position of the platelets along the optical axis is not known, while with a shallow depth of field many platelets outside this region of sharp focus cannot be detected. However, the median plane of the vessel can be regarded as a representation of the cross-sectional area of the vessel because of rotational symmetry. Therefore, the rheological behavior of

platelets located within this plane will represent the rheological behavior of the platelets over the cross-sectional area of the vessel. The position of a platelet within this plane, i.e. perpendicular to the optical axis, is well defined. In this study the median plane was approximated by localizing the platelets objectively within a thin optical section. Consequently the optical section was divided into six equal segments and the concentration distribution of the platelets was determined by counting the number of platelets within each segment. Finally the orientation of the platelets in the different segments was estimated.

The search for a fluorescent dye that labels the platelets directly in vivo but not the RBC's is described in chapter 2. Twenty fluorochromes and eleven fluorogenic enzyme substrates were at first tested in vitro. Several basic dyes labeled the platelets in vitro. Of these dyes only acridine red (AR) allowed the visualization and recording of fast flowing platelets in vivo. Following an intravenous injection of a solution containing 5-15 mg AR a clear microscopic image of individual platelets flowing in a small arteriole could be obtained over the whole cross-sectional area of the vessel during 2-8 minutes, using fluorescence microscopy. Incident illumination was used with flashes of short duration. Flashes of short duration were required to prevent a smeared image due to movement of the platelets. The images were recorded with a low light level TV-camera and stored on videotape. Leukocytes and the vessel wall were also labeled, but in vivo reticulocytes did not label with AR, neither did the RBC's. Generally leukocytes and platelets can be easily distinguished because of the difference in size and shape. Both the absorption and emission spectrum of AR with peaks at wavelengths of 525 and 625 nm, respectively, are favorably positioned with respect to the absorption spectrum of blood.

Following the injection of AR, almost all the circulating platelets were labeled, while activation of the platelets or a fall in platelet count or hematocrit did not occur. Electron microscopy revealed no gross ultrastructural changes. In vitro, the dye reduced platelet aggregation in a dose dependent way. However, in vivo aggregation and adhesion of platelets as induced by laser injury or transection of an arteriole was observed in all cases, even following multiple injections of AR. Primary hemostatic plug formation times as measured in mesenteric arterioles were normal after the first and second injection of 5 mg AR, but were prolonged after subsequent

injections. Bleeding times as measured on the ear were prolonged following injection of 15 mg of the dye. In this light and because 10 mg AR were usually injected to visualize the platelets, it was concluded that labeling with AR could be used to study the rheological behavior of the platelets in vivo.

In chapter 3 the method is described to localize fluorescent platelets flowing in small arterioles within a thin optical section. The images recorded on videotape were analyzed field by field with an electronic system that displayed the signal of a single TV-line on a dual-trace oscilloscope. To define the optical section the amplitude of the signal of a TV-line through the center of the microscopic image of a platelet was used. The amplitude of the signal decreased with defocusing. The empty tracing of the oscilloscope was placed along the top of the background noise. A platelet was considered to be within the optical section as long as the signal clearly exceeded the empty tracing, and hence the background noise. This decision can be made by eye rather accurately. Beside experiments with AR-labeled platelets, experiments were performed with leukocytes labeled with acridine orange and with latex spheres (outer diameter 1.5 μm) labeled with fluorescein-isothiocyanate. Information about the characteristics of the optical section was obtained in vitro using latex spheres as model particles, because of the asymmetry of the disk-shaped platelets.

Sections were found to be almost symmetrical around the level of sharp focus. The depth of the section halved by doubling the total optical magnification and increased according to a straight line with an exponential increase in illumination power. To estimate the influence of a difference in orientation of a disk-shaped platelet on the section depth as well as to understand the decrease in amplitude with defocusing, a theoretical analysis was made. The influence of the orientation of a platelet with respect to the optical axis was calculated for a rather big platelet and was found to be negligible at a section depth of approximately 6-7 μm. At the suitable total optical magnification of 200x and using the highest available flash power in vivo a section depth ranging from 5-9 μm was found for platelets sticking to the wall of venules or caught in small vessel segments. For platelets flowing in small arterioles the section depth was estimated to range from 5-7 μm. The section depths found for leukocytes were much larger. It was concluded that the depth of the optical section was sufficiently shallow with respect to the

diameter (20-35 μm) of small arterioles to localize the platelets at various sites in the vessel along the optical axis, allowing the investigation of their rheological behavior in these microvessels.

Chapter 4 of the thesis deals with the concentration distribution of blood platelets flowing in small arterioles. Experiments were performed with position of sharp focus in the center of the vessel. For the analysis of the data a transparent sheet was positioned over the monitor screen and the optical section within the lumen of the vessel was divided into six segments equal in width and length. In each segment the number of AR-labeled platelets that could be localized within the optical section was counted. In each measurement 130-160 TV-fields were analyzed which means a counting time of 20-30s. Because of the illumination with flashes of short duration, instantaneous pictures were used so that it is unnecessary to correct for velocity differences along the cross-section. Therefore, in this way the platelet concentration profile within the optical section is determined if the depth of the optical section, or better the counting volume, is equal in all segments. To assess the differences in counting volume between the six segments and to estimate the resulting error made in assessing the number of platelets in each segment, experiments were performed to determine the difference in light transmission between the wall and central segments. In addition, the error made due to the cylindrical shape of the vessel and the differences in orientation of the platelets was calculated. It was found that the influence of all these factors on the number of platelets counted in each segments was minimal.

In all measurements (32 in 10 animals) a non-uniform concentration distribution was found. At vessel sites without proximal branch points at a distance of at least ten vessel diameters the highest concentration was always found near the wall. In general, the platelet concentration progressively decreased from the wall toward the vessel center. Symmetric as well as rather asymmetric concentration profiles were found, both in straight and winding vessels. The profiles as recorded in or distal to curves did not differ significantly from those recorded in straight parts of the vessel. In contrast to curves, branch points had a marked influence on the concentration profile. Distal to a sidebranch, the concentration was reduced in the part of the vessel adjacent to the side branch. From 19 measurements in 13 vessels without proximal branch points at a distance of at least 10 vessel diameters

in 10 animals (total platelet number:6571) a general concentration profile was calculated. From wall to wall, taking into account the direction of flow the following profile was found: 1.44-0.91-0.78-0.76-0.85-1.51. It was concluded that in small arterioles the platelet concentration near the wall is about twice the concentration in the center of the vessel. This is in agreement with the concept of an increase in hematocrit in the center of the vessel, resulting in a dispersal of platelets from the center and a higher platelet concentration near the wall.

Chapter 5 reports about the orientation of the blood platelets flowing in small arterioles. Simultaneously a frequency distribution of the diameter of rabbit platelets *in vivo* was obtained. Five straight arterioles were selected from the experiments described in chapter 4. The platelets were localized within an optical section which was divided into six equal segments. The length of the major and short axes of the microscopic image of a platelet and the angle of the direction of the major axis of the image with the direction of flow were measured. From these measurements the orientation of the platelets was estimated. Of each type of measurement the error was estimated and these errors were used in a worst case analysis (worst case limits given within parentheses; see below).

The orientation of the platelets differed significantly from a random orientation in all segments. The platelets tend to align themselves with flow, i.e. toward a position with minimal span of shear. Moreover, the data strongly suggested that the tendency for alignment increases from the center of the vessel toward the wall. The increase in alignment from the center toward the wall as found in this *in vivo* study is in contrast to theory and the findings *in vitro* for freely rotating, rigid disks in parabolic flow. Of platelets present in the wall segment only 1% (0.4-2.5%) was found lying with with their flat side ±30° across the flow (expected for random orientation: 13.4%), while 62% (46-72%) of the platelets was found aligned in the planes of shear within ±20-30° (expected for random orientation: 11%).

The distribution of the diameter of the rabbit platelets was rather symmetric. The mean value and standard deviation were 3.15 ± 0.72μm. These data on the diameter of the platelets as obtained *in vivo* are in agreement with the data obtained *in vitro*.

In conclusion:

- in rabbits a clear microscopic image of individual, fluorescent blood platelets flowing in a microvessel can be obtained following intravenous injection of the fluorochrome acridine red.

- blood platelets labeled with acridine red can be localized objectively within a thin optical section using the height of the intensity distribution of their microscopic image relative to the background noise.

- the depth of the optical section is sufficiently shallow with respect to the diameter of small arterioles (20-35 μm) to allow the investigation of their rheological behavior in these microvessels.

- the concentration distribution of blood platelets flowing in small arterioles is non-uniform: the concentration decreases from the wall toward the center.

- the mean platelet concentration near the wall is about twice the mean concentration in the center of the vessel.

- the orientation of blood platelets flowing in small arteioles is not random: they tend to align in the planes of shear, i.e. toward a position with minimal span of shear.

- the tendency for alignment of the platelets increases from the center toward the wall of the vessel.

7. SAMENVATTING

De kleinste levende bouwsteen van de verschillende weefsels en organen in ons lichaam is de cel. Om gezond te blijven heeft iedere cel voedingsstoffen en zuurstof nodig en moeten zijn afvalproducten worden afgevoerd. Aanvoer en afvoer gebeuren door het bloed. Via zich vertakkende, steeds kleiner wordende bloedvaten bereikt het bloed de weefsels. Aangekomen in de allerdunste bloedvaatjes, de haarvaten of capillairen, geeft het bloed voedingsstoffen en zuurstof af, en neemt het afvalstoffen op. Via samenvloeiende en steeds groter wordende vaten verlaat het bloed het weefsel. De kleine aanvoerende bloedvaatjes voor de haarvaten worden arteriolen genoemd, de kleine afvoerende vaatjes venulen. Dit deel van de bloedsomloop of circulatie waarin de bloedvaatjes kleiner zijn dan een tiende millimeter wordt microcirculatie genoemd. Het bloed zelf is een vloeistof waarin drie soorten cellen aanwezig zijn: rode bloedcellen, witte bloedcellen en bloedplaatjes. De rode bloedcellen dienen voor het transport van zuurstof. De witte bloedcellen hebben een taak bij de afweer tegen infecties. Over de derde bloedcel, het bloedplaatje, gaat dit proefschrift.

Het bloedplaatje is een kleine, schotelvormige cel. De belangrijkste taak van de bloedplaatjes is het dichten van gaten in de wand van beschadigde bloedvaten, zodat het bloedverlies beperkt blijft (bloedstelping of hemostase). Wanneer een klein bloedvat wordt aangeprikt of doorgesneden, hechten langsstromende bloedplaatjes zich aan de beschadigde vaatwand (adhesie) en aan elkaar (aggregatie). Door aanhechting van meer en meer bloedplaatjes ontstaat een snel groter wordende plaatjesprop welke uiteindelijk het gat in de wand geheel afdicht. De plaatjesprop wordt verstevigd door een netwerk van fibrinedraden die eerst rond en vervolgens ook in de prop ontstaan. De fibrinedraden ontstaan als eindproduct van een keten van chemische reacties tussen verschillende soorten eiwit-moleculen in het bloed (bloedstolling). Ook bij de bloedstolling spelen de bloedplaatjes een belangrijke rol doordat een aantal reactiestappen versneld verloopt aan het oppervlak van geactiveerde bloedplaatjes. Bij ziekte van de vaatwand (b.v. slagaderverkalking) kan het voorkomen dat bloedplaatjes zich hechten aan een beschadiging in de binnenbekleding van het vat zonder dat er sprake is van een gat in de wand. Wanneer dan meer bloedplaatjes aanhechten ontstaat er een thrombus (dit proces heet thrombose), welke het vat vernauwt of zelfs geheel kan afsluiten. In dit laatste geval wordt het achterliggende weefsel niet meer van bloed voorzien

en treedt weefselversterf (infarct) op.

Bloedplaatjes kunnen alleen hechten aan een beschadigde vaatwand wanneer ze hiermee in contact komen. Het in contact komen met de wand en met elkaar hangt af van de manier waarop de bloedplaatjes zich bewegen in het stromende bloed: hun stromingsgedrag of rheologisch gedrag. Uit het literatuuroverzicht gegeven in hoofdstuk 1 blijkt dat er op dit moment weinig over het rheologisch gedrag van de bloedplaatjes bekend is. Bovendien is het merendeel van de thans beschikbare informatie afkomstig van theoretische studies en experimenten, welke verricht zijn buiten het levende organisme (in vitro). De resultaten van onderzoek verricht buiten het levende organisme kunnen verschillen van de bevindingen verkregen bij onderzoek binnen het levende organisme (in vivo). Bloedplaatjes reageren snel op veranderingen in hun omgeving. Zulke veranderingen kunnen al optreden bij de afname van bloed. In het lichaam werken de bloedplaatjes bovendien nauw samen met andere celtypen en functionele systemen, zoals bijvoorbeeld het stollingssysteem. Deze verschillen kunnen er mede de oorzaak van zijn dat (kandidaat) geneesmiddelen welke de aggregatie van bloedplaatjes in vitro remmen dit in vivo vaak niet doen. Hetzelfde geldt voor het effect van veranderingen in het dieet.

Het doel van dit proefschrift was het rheologisch gedrag van de bloedplaatjes te bestuderen in vivo, in intacte bloedvaten.

In de bloedvaatjes van de microciculatie is het mogelijk de stroming van het bloed waar te nemen door de microscoop. Een weefsel dat zich goed leent hiervoor is het darmvlies van een proefdier. Het darmvlies is een dun vlies dat de darm verbindt met de achterwand van de buikholte. Wanneer via een kleine snede in de buikwand een stukje darm naar buiten wordt gebracht, kunnen de bloedvaatjes in dit vlies bestudeerd worden onder de microscoop. In deze studie is dit gebeurd bij konijnen die onder narcose waren gebracht.

In de allerkleinste vaatjes, de haarvaten, stromen de bloedcellen in ganzepasformatie, waardoor het mogelijk is de afzonderlijke cellen te zien. In de wat grotere vaten (arteriolen, venulen) is het echter door de dichte concentratie van verschillende lagen rode bloedcellen onmogelijk om de kleine, kleurloze en transparante bloedplaatjes waar te nemen met de gewone vorm van microscopie. Daarom is allereerst gezocht naar een methode om de individuele bloedplaatjes stromend in arteriolen en venulen zichtbaar te maken onder de microscoop (hoofdstuk 2). Door de bloedplaatjes selectief te

merken met de fluorescerende kleurstof Acridine Rood konden deze met behulp van fluorescentiemicroscopie gemakkelijk onderscheiden worden temidden van de grote massa ongelabelde rode bloedcellen. Het labelen van de bloedplaatjes gebeurde in vivo door intraveneuze injectie van een kleine hoeveelheid van deze fluorescerende kleurstof. Onderzoek naar de eventuele effecten van deze kleurstof op de bouw, het aantal en het functioneren van de bloedplaatjes toonde aan dat het geoorloofd was deze labelingsmethode te gebruiken om het rheologisch gedrag van de bloedplaatjes te bestuderen.

Om "uitsmering" en daarmee onscherpte van het beeld van de snelstromende bloedplaatjes te voorkomen, is gebruik gemaakt van belichting met telkens een korte flits, waardoor het beeld van de bloedplaatjes wordt "vastgevroren" in plaats en tijd. Ieder beeld is derhalve een momentopname. Per seconde werden vijf van zulke momentopnamen verkregen. Vanwege de geringe intensiteit van het bij fluorescentie uitgestraalde licht en de zeer korte belichtingstijd is voor de weergave van deze momentopnamen gebruik gemaakt van een beeldversterker geplaatst voor een TV-camera.

Voor de bestudering van het rheologisch gedrag van de bloedplaatjes moet de positie van een bloedplaatje in het vat bekend zijn. Wanneer we van boven af door de microscoop naar een bloedvat kijken, dan is de positie van een bloedplaatje in de lengte- en breedterichting van het vat duidelijk. Het probleem van het bepalen van de positie van een bloedplaatje in de diepte van het vat is in deze studie ondervangen door alleen te meten in een dunne "optische plak" op een vastgestelde diepte. Als diepte is gekozen het midden (centrum) van het vat, omdat daar alle bestanddelen van de dwarsdoorsnede van het vat aanwezig zijn (centrumgedeelte, wandgedeelte etc.).

In hoofdstuk 3 is de mogelijkheid onderzocht om fluorescerende bloedplaatjes waargenomen met een enkele korte flits, te localiseren binnen een dunne optische plak. Gevonden werd dat bij defocusseren vanuit het centrum van een fluorescerend deeltje de intensiteit van het microscopische beeld zeer snel afneemt zodra het vlak van scherpstelling buiten het deeltje komt. Hierdoor was het mogelijk om gebruik makend van de hoogte van hun intensiteitssignaal de bloedplaatjes op objectieve en nauwkeurige wijze te localiseren binnen een optische plak. De optische plak bleek nagenoeg symmetrisch rond het vlak van scherpstelling. Bij een juiste keuze van de verschillende optische parameters, met name de totale vergroting, was de dikte van de optische plak dun genoeg om met scherpstelling in het midden

van het vat het rheologisch gedrag van de bloedplaatjes te bestuderen in arteriolen en venulen.

Gebruik makend van de hierboven beschreven methodieken is vervolgens in arteriolen de concentratieverdeling over het vat en de orientatie van de bloedplaatjes onderzocht. Hiertoe werd de optische plak verdeeld in zes segmenten van gelijke lengte en breedte.

Het onderzoek naar de concentratieverdeling van de bloedplaatjes staat beschreven in hoofdstuk 4. Voor elk experiment werden in 130-160 momentopnamen het aantal bloedplaatjes geteld in ieder van de zes segmenten waarin de optische plak was verdeeld. Door het gebruik van momentopnamen hebben verschillen in stroomsnelheid over de breedte van het vat geen invloed op het aantal bloedplaatjes, geteld in ieder segment. Daarom wordt op deze wijze binnen de optische plak het concentratieprofiel van de bloedplaatjes over de breedte van het vat bepaald indien de dikte van de optische plak, of beter gezegd het telvolume, gelijk is in alle zes segmenten. Verschillen in telvolume tussen de zes segmenten bleken verwaarloosbaar klein.

In alle experimenten (32 in 10 dieren) werd een ongelijkmatige concentratieverdeling gevonden. In vaatstukken welke stroomopwaarts vrij van zijtakken waren binnen tenminste tien vatdiameters, was in alle gevallen de concentratie het hoogst aan de wand. In het algemeen daalde de concentratie zonder onderbreking van de wand tot in het midden van het vat. De concentratieprofielen in of vlak na een bocht verschilden niet duidelijk van de concentratieprofielen in een recht vaatstuk. Zowel in rechte alsook in slingerende vaten werden symmetrische en tamelijk asymmetrische concentratieprofielen gevonden. In tegenstelling tot een bocht had een aftakking een duidelijke invloed op het concentratieprofiel. Stroomafwaarts van een zijtak was aan dezelfde kant in het vat de concentratie verlaagd. Uit de resultaten van 19 experimenten in 13 vaatstukken, welke stroomopwaarts vrij waren van zijtakken binnen tenminste tien vatdiameters, is een algemeen concentratieprofiel berekend (totaal aantal getelde bloedplaatjes: 6571). Van links naar rechts, gelet op de stroomrichting in ieder bloedvat, werd het volgende algemene concentratieprofiel gevonden: 1.44-0.91-0.78-0.76-0.85-1.51. Dit resultaat laat zien dat in arteriolen de concentratie van de bloedplaatjes in de buurt van de vaatwand ongeveer tweemaal zo hoog is als in het midden van het vat.

In hoofdstuk 5 wordt het onderzoek naar de orientatie van de bloedplaatjes beschreven. Gemeten werden de lengte van de lange en korte as van het microscopisch beeld van een bloedplaatje alsmede de hoek tussen het verlengde van de lange as en de stroomrichting in het vat. Uit deze drie metingen werd de orientatie van het bloedplaatje berekend. De lengtemeting van de lange as leverde tevens een frequentieverdeling van de diameter van konijnen bloedplaatjes bepaald in vivo.

In elk van de zes segmenten waarin de optische plak was verdeeld, vertoonden de (schotelvormige) bloedplaatjes de neiging zich met hun platte kant te richten naar de stroomlijnen in het vat. Met andere woorden, bloedplaatjes proberen een orientatie in te nemen waarin zij zo weinig mogelijk effect ondervinden van de afschuifspanningen in de stromende vloeistof. De neiging zich te richten naar de stroomlijnen nam duidelijk toe vanuit het midden van het vat naar de wand.

Samenvattend zijn de belangrijkste conclusies uit dit onderzoek:

- na intraveneuze injectie van de fluorescerende kleurstof acridine rood kan met behulp van fluorescentie microscopie een duidelijk beeld verkregen worden van de individuele, fluorescerende bloedplaatjes stromend in arteriolen.

- bloedplaatjes, gelabeled met acridine rood kunnen op objectieve en nauwkeurige wijze gelocaliseerd worden binnen een dunne optische plak door gebruik te maken van de hoogte van het intensiteitssignaal van hun microscopisch beeld.

- de optische plak is voldoende dun om het rheologisch gedrag van de bloedplaatjes te kunnen bestuderen in arteriolen.

- in arteriolen is de concentratie van de bloedplaatjes het hoogst aan de wand en daalt van de wand tot in het midden van het vat.

- gemiddeld is de concentratie van de bloedplaatjes in de buurt van de vaatwand ongeveer tweemaal zo hoog als in het midden van het vat.

- bloedplaatjes stromend in arteriolen vertonen de neiging zich met hun platte kant te richten naar de stroomlijnen in het vat, dit wil zeggen de orientatie waarin ze het geringste effect ondervinden van de afschuifspanningen in de stromende vloeistof.

- de neiging van de bloedplaatjes zich te richten naar de stroomlijnen in het vat neemt toe vanuit het midden van het vat naar de wand.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AO	Acridine orange
AR	Acridine red
ATP	Adenosine triphosphate
BT	Bleeding time
CFDA	4(5)-carboxyfluoresceindiacetate
DTS	Dense tubular system
FDA	Fluoresceindiacetate
FDB	Fluoresceindibutyrate
FITC	Fluorescein-isothiocyanate
NA	Numerical aperture
OCS	Surface connected open canalicular system
OD	Optical density
PARS	Vector coinciding with the platelet axis of rotational symmetry
PCV	Platelet count per unit volume
PHT	Primary hemostatic plug formation time
PRP	Platelet rich plasma
PS	Physiological saline
RBC	Red blood cell

NAWOORD

Aan de tot stand koming van dit proefschrift heb ik met erg veel plezier gewerkt. Het is een voorrecht interessant en stimulerend werk te mogen doen. Even belangrijk als de aard van het werk is de sfeer, het sociale klimaat waarin gewerkt moet worden. Ik ben dankbaar dat ik in een prettige, ontspannen sfeer dit werk heb kunnen doen. Hiervoor dank ik allen met wie ik in deze tijd heb samengewerkt en contact heb gehad.

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CURRICULUM VITAE

9 oktober 1948	Geboren te Hilversum.
1961 - 1967	Gymnasium-b gevolgd aan het St.-Janscollege te Den Haag.
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1975 - 1976	Werkzaam geweest als waarnemend huisarts.
1 september 1976	In dienst getreden als wetenschappelijk medewerker van de capaciteitsgroep Fysiologie van de Rijksuniversiteit Limburg, alwaar, na het opstarten van het laboratorium voor Microcirculatie, in de loop van 1978 begonnen werd met het onderzoek dat geleid heeft tot dit proefschrift.

POSTSCRIPTUM

...the other night I overheard two platelets talking as they drifted in some venous backwater.

"You know," said one, "I've got the strangest feeling that I've been here before."

"What nonsens!" said the other, "Everyone knows that endothelium stretches to infinity. One doesn't go backwards - one just goes on."

"Yes - until we stop."

"One doesn't think about that - it's morbid."

"I can't help it sometimes. One day endothelium will stop passing - everyone else will go on, but I shall be stuck there - dead. I say, I wonder what's on the other side?"

"What *do* you mean? On the other side of what?"

"On the other side of endothelium, of course."

"It's a meaningless question. Endothelium encloses everything there is - it's the time-continuum. How can there *be* another side? It's a logical impossibility!"

"Well, I met a lymphocyte just now. He's certain he's been through the endothelium and has come back to another life on this side."

"Lymphocytes! One doesn't pay any attention to *them*! All that mystical nonsense about re-incarnation. Next thing they'll be saying we're all part of some Higher Organisation....!"

From: The art of the relevant
R.G. Macfarlane
Thromb. Haemost. 32: 1-8, 1974.