PLATELET ADHERENCE TO COLLAGEN CONTAINING SURFACES

By

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PLATELET ADHERENCE TO COLLAGEN

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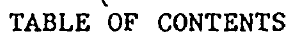
ABSTRACT

The first step in hemostasis and thrombosis is the adherence of platelets to the damaged vessel wall. The factors involved in platelet adherence are not well understood, mainly because of the lack of a suitable method to measure quantitatively platelet adherence to surfaces. The aim of the present studies was to develop a quantitative method to measure platelet adherence to collagen containing surfaces. The following results were obtained with this method: (1) In this quantitative method for measuring platelet adherence to collagen-coated glass surfaces and to subendothelium the platelelets are labeled with ^{51}Cr and adherence can be measured over a large area without sampling bias. Platelets are suspended at pH 7.35, at 37°C in an artificial medium which can be modified as required. The medium contains physiological concentrations of calcium and magnesium and platelet aggregation is prevented by the inclusion of apyrase to degrade any ADP released. There is no plasma present and thus no thrombin generation or fibrin formation occurs. Adherence is measured using a rotating probe device under controlled flow conditions and various surfaces can be tested (collagen-coated glass, everted aorta damaged to expose the subendothelium). (2) Platelet adherence to collagen-coated glass or to subendothelium is greatly reduced in the absence of divalent cations. Thus, methods which measure platelet adherence in the presence of chelating agents are difficult to interpret. (3) Increasing the albumin concentration of the medium decreases platelet adherence, but there is

less variation among replicates. (4) Increasing the hematocrit increases the number of platelets adhering to the surface. (5) Platelets adhere tightly to collagen or to subendothelium and are not dislodged by agents which readily deaggregate platelets such as EDTA, EGTA, or PGE_1. (6) Exposure of undamaged endothelium to thrombin increases the number of platelets adherent to the surface and this is blocked by heparin. (7) This method of measuring platelet adherence to collagen can be used to screen drugs which may be useful as antithrombotic agents. The best system is to test the effect of a drug in the presence of 4% albumin and 40% hematocrit, however, for initial screening a simpler system can be used (0.35% albumin, zero hematocrit). (9) Several treatments which modify the platelet surface have been tested for their effect on adherence to collagen. Removal of sialic acid with neuraminidase do not affect platelet adherence whereas treatment with sodium periodate and the proteolytic enzymes thrombin, plasmin and chymotrypsin decrease adherence. (10) UDP and UDPG do not have an effect on platelet adherence to collagen and this observation does not support the collagen: glucosyltransferase theory of platelet adhesion to collagen. (11) C1q, a subcomponent of the first component of complement specifically inhibits platelet adhesion to collagen and may compete with the collagen receptor on the platelet membrane. (12) A wide variety of agents inhibits platelet adherence to collagen-coated surfaces and to subendothelium. Among the inhibitors which decrease platelet adherence in a system containing 4% albumin and 40% hematocrit are agents which

chelate divalent cations (EGTA, EDTA, citrate), indomethacin, agents which increase cAMP levels (prostaglandin E_1, dipyridamole, RA 433), methylprednisolone, penicillin G and cephalothin. (13) Aspirin inhibits platelet adherence to collagen-coated surface or to subendothelium, but its inhibitory effect is not evident in the presence of 40% hematocrit or citrated plasma indicating that the conditions of the experiments are important in determining the effect of drugs. (14) Modifications of platelelets by treatment with thrombin, penicillin G or cephalothin which inhibit platelet adherence to collagen or subendothelium do not affect platelet survival. Modification of the platelet surface sialic acid by neuraminidase or periodate is followed by rapid clearance of platelets from the circulation. Thus there is no correlation between platelet adherence and platelet survival. (15) Treatments which decrease platelet adherence to collagen and to the subendothelium also reduce the effectiveness of platelets in hemostasis.



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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
cAMP	cyclic adenosine monophosphate
ASA	acetylsalicylic acid
CP	creatine phosphate
CPK	creatine phosphokinase
DFP	di-isopropyl phosphofluoridate
EDTA	disodium ethylene diaminetetraacetic acid
EGTA	ethylenebis(oxyethylenenitrilo)-tetraacetic acid
Hepes	N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid
5-HT	serotonin
MDA	malondialdehyde
PGE_1	prostaglandin E_1
PGG_2, PGH_2	prostaglandin endoperoxides
PRP	platelet-rich plasma
SBTI	soya bean trypsin inhibitor
TAMe	[p-tosyl-L-arginine methyl ester (HCl)]
UDP	uridine diphosphate
UDPG	uridine diphosphoglucose

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A Anne-Lise

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Qui m'ont entouré de leur amour,

de leur compréhension, de leur

patience et de leur aide.

CHAPTER ONE

INTRODUCTION

The mammalian blood platelets are derived from the megakaryocytes in the bone marrow (Behnke and Pedersen, 1974; Penington and Streatfield, 1975; Breton-Gorius and Reyes, 1976). Platelets circulate in the blood as disc shaped elements. They have a plasma membrane which encloses cytoplasm containing various constituents (storage granules, lysosomes, mitochondria, vesicular structures, microfilaments and microtubules) but lack a nucleus (David-Ferreira, 1964; Hovig, 1968; White, 1971b). Platelets are metabolically active and can synthesize fatty acid, glycogen and some proteins. Platelets derive their energy from glycolysis and the effect of Krebs cycle and oxidative phosphorylation (Marcus, 1969; Mustard and Packham, 1970). Platelets have a mean life in circulation of about 10 days in man (Mustard et al., 1966b; Paulus, 1971, 1974).

Although the platelet was originally thought to be primarily concerned with the processes of hemostasis and thrombosis, there is increasing evidence that it plays a critical part in almost all responses of blood to injury (Packham et al., 1968; Mustard and Packham, 1970; 1971). Platelets are involved in immunological and inflammatory reactions (Des Prez and Marney, 1971; Osler and Siraganian, 1972; Pfueller and Luscher, 1972; Becker and Henson, 1973; Zawilska and Izrael, 1973; Zucker, 1974), in the response to injury of the endothelium of blood vessels (French, 1966; Hugues, 1969; Spaet and Gaynor, 1970; Jørgensen, 1971; Moore, 1974; Mustard et al., 1974; Mustard and Packham; 1975b; Ross and Glomset, 1976), in the phagocytosis of foreign materials introduced into the blood stream (Mustard and Packham, 1968), in the

interaction with natural and artificial surfaces (Mustard et al., 1967a; Salzman, 1971a,b,c; Berger and Salzman, 1974; Nossel, 1975), in the transport of biogenic amines (Sneddon, 1973) and in cancer metastasis (Gasic et al., 1973).

There have been a number of general reviews, books and proceedings of conferences published on platelet function (Marcus and Zucker, 1965; Johnson and Seegers, 1967; Marcus, 1969; Michal and Firkin, 1969; Mustard and Packham, 1970; 1971; Brinkhous et al., 1971; Hirsh and Doery, 1971; Zucker, 1971; Holmsen, 1972; Weiss, 1972, 1975; Smith and Macfarlane 1974; Ciba Foundation Symposium 35, 1975; Cooper et al., 1976; Gordon, 1976), on drugs affecting platelet function (Mustard and Packham, 1970, 1975; Weiss, 1972; Genton et al., 1975; Harker et al., 1975; Weiss, 1976) and on inherited or acquired abnormalities of platelet function (Stuart 1975, Weiss, 1972, 1975; Caen et al., 1977). No attempt to give an exhaustive review of the knowledge relating to platelets will be made, but rather a few selected areas pertaining to the interaction of platelets with collagen containing surfaces will be presented as an introduction to the experimental work in this thesis. The present review will, therefore, be concerned with (1) the role of platelets in hemostasis and thrombosis, (2) platelet structure and metabolism, (3) collagen structure and adhesion of platelets to collagen, (4) the interaction of platelets with endothelium and subendothelium of blood vessels, and (5) platelet interaction with other surfaces.

I. ROLE OF PLATELETS IN HEMOSTASIS AND THROMBOSIS

Platelets play a major role in hemostasis and thrombosis. This subject has been reviewed thoroughly by Mustard (Mustard et al., 1962; 1974 et al.; Mustard, 1968, 1976; Mustard and Packham, 1970, 1975a, 1975b; Jørgensen, 1971; Weiss, 1976) and will only be summarized here.

Following transection of blood vessels, the arrest of bleeding is brought about by the formation of a hemostatic plug composed of platelets with fibrin and some red cells around the periphery. A detailed morphological description of the hemostatic plug can be found in French et al. (1964); Hovig et al., (1967) and Hovig and Stormorken, (1974).

Thrombus formation occurs in flowing blood and results in the accumulation of blood constituents, including platelets, on the surface of the vessel walls and of the heart. The factors involved in thrombus formation are similar to those which occur in hemostatic plug formation. Blood flow is important in determining the site and the size of thrombi.

1. Mechanisms involved in hemostasis and thrombosis.

The mechanisms involved in the response of platelets to vessel injury are: (a) platelet adherence to the constituents of the vessel wall; (b) release of platelet constituents and platelet aggregation and (c) the interactions of platelets and blood coagulation.

(a) Platelet adherence to subendothelium.

When the endothelium of an artery is disrupted, the platelets can interact with the components of the subendothelial connective tissue which are collagen, basement membrane and microfibrils [see

later in this section, paragraph IV and Stemerman (1974)].

When platelets adhere to collagen fibrils and are examined by transmission or electron microscopy they show evidence of morphological alterations with pseudopod formation and loss of organelles (Hovig 1968; Hovig et al., 1968). The distance between the platelet surface membrane and the surface of collagen may be less than the distance between two platelets in a platelet aggregate. In in vivo experiments, a pattern of breaks in the platelet membrane have been observed matching the cross striations of the collagen fibers (Hovig et al., 1968). Electron microscopic examination of the early stages of platelet interaction with the surface of a damaged artery has shown that platelets adherent to the subendothelium are not disc shaped and have long pseudopods (Sheppard and French, 1971). Warren and Vales (1972) have presented morphological evidence that the pseudopods frequently contained microfilaments and were terminated by multivesicular membranous sacs. The nature and significance of the multivesicular sacs is unknown, although Warren and Vales have suggested that they may represent the "morphological basis of the release reaction." However, their importance seems doubtful since other investigators have not found these multivesicular sacs. The formation of pseudopods and the loss of disc shape when platelets adhere to collagen (Hovig, 1970; White, 1974) or to damaged vessel wall during hemostasis (Shoop et al., 1970) has been confirmed and visualized in three dimensions with the scanning electron microscope.

Kinlough-Rathbone (personal communication) has demonstrated

that collagen can induce, by itself, platelet shape change independently of the release of ADP and of the formation of prostaglandin endoperoxides PGG_2, PGH_2 and thromboxane A_2.

(b) Release of platelet constituents and platelet aggregation.

When the platelets adhere to collagen they are stimulated to release the contents of their granules which include serotonin, mucopolysaccharides, ATP and ADP, lysosomal enzymes, a cationic protein which can increase vascular permeability and an anti-heparin factor, platelet factor 4 (Hovig, 1963a,b; Holmsen et al., 1969; Holmsen, 1975). There is no evidence that microfibrils or basement membrane can cause platelets to discharge their granule contents (Huang et al., 1974; Baumgartner and Muggli, 1976).

In addition to the release reaction, when platelets come in contact with collagen-containing surfaces they release arachidonate from membrane phospholipids. The arachidonate is converted to the prostaglandin endoperoxides PGG_2, PGH_2 and to thromboxane A_2 (Smith and Silver, 1976).

The released ADP and the endoperoxides and thromboxane A_2 can cause platelet shape change (Kinlough-Rathbone et al., 1976b) and platelet aggregation. There is also evidence that in the presence of low concentrations of collagen there is synergism between collagen and ADP. Trace amounts of ADP added to concentrations of collagen that cause only slight aggregation and release will cause extensive release and aggregation. There is also synergism between sodium arachidonate

and ADP (Packham et al., 1977). The formation of a platelet mass can initiate the coagulation process (Walsh, 1974).

(c) Platelets and blood coagulation.

The relationships between the coagulation mechanism and the formation of a platelet plug have not been fully elucidated, but recently Walsh (1974) has demonstrated that platelets participate in reactions with blood coagulation factors at every stage from contact activation to fibrin formation.

The exposed collagen of the vessel wall can directly activate factor XII, this reaction is dependent on the free carboxyl groups of collagen (Wilner et al., 1968a). Interaction of platelets and collagen can activate directly factor XI, bypassing factor XII (Walsh, 1972a). Platelets also have the capacity when stimulated by ADP to trigger the contact phase of the intrinsic coagulation by activating factor XII (Walsh, 1972b). Subsequent coagulation reactions (involving platelet factor 3 and factor IX and platelet factor 3 and factor X) occur on the platelet surface which provides a catalytic surface and protects activated clotting factors (XIa, Xa) from inactivation by naturally occurring inhibitors.

In addition to the activation of the intrinsic pathway of coagulation, activated factor XII (XIIa) can activate the kinin-forming system and plasminogen to plasmin (Kaplan, 1974). Plasmin can activate the complement system through its action on C1 (Kaplan, 1974). Plasmin has also been reported to induce the platelets to release their

constituents (Niewiarowski et al., 1973).

The local formation of minute amounts of thrombin, prior to the formation of fibrin might be very important in hemostasis. Endothelial cells may contribute to hemostasis by their synthesis and/or release of tissue factor (Nemerson and Pitlick, 1972) and factor VIII (Jaffe et al., 1974). Minimal trauma to the endothelium might initiate thrombin formation via the extrinsic pathway of coagulation (Nemerson and Pitlick, 1972). Thrombin can "activate" factor VIII and factor V. Minute amounts of thrombin will also induce platelet aggregation and the release reaction which can lead to acceleration of clotting. Thrombin catalyses the splitting of fibrinopeptides A and B from fibrinogen, leading to fibrin polymerization. It has been shown that platelets readily adhere to polymerizing fibrin (Niewiarowski et al., 1972), but not to fully polymerized fibrin in which the thrombin has been neutralized (Hovig et al., 1968). The effects of thrombin may be of considerable importance in the growth of thrombi. If extensive fibrin formation occurs it will trap red blood cells forming what looks like a red clot. Aggregated platelets generate chemotactic substances (derived from complement component C5 (Weksler and Coupal, 1973) and from arachidonate metabolism) which attract polymorphonuclear leukocytes. If the vessel wall is damaged, activators of plasminogen are released (Nilsson and Pandolfi, 1970). If fibrin formation is not extensive this may lead to a local activation of the fibrinolytic mechanism that will weaken the fibrin around the thrombus and lead to thrombus disruption.

2. Role of blood flow in thrombosis.

The fate of a thrombus depends upon its composition, its site and the factors leading to its stabilization and dissolution. As pointed out by Mustard and Packham (1971), thrombi are in a continuous state of formation and dissolution. Blood flow plays an important role in thrombosis. In regions of disturbed flow the amount of thrombus material which accumulates is more extensive than in regions where flow is laminar (Mustard et al., 1972a). The regions of disturbed flow include bifurcations, stenoses and sharp bends. These regions of disturbed flow are areas where platelet aggregates readily accumulate and where there is vessel injury (Geissinger et al., 1962; Mustard et al., 1962; Jørgensen et al., 1972); endothelial cell turnover is increased (Wright, 1972; Caplan and Schwartz, 1973), plasma components accumulate in the vessel wall (Packham et al., 1967a; Somer and Schwartz, 1972; Bell et al., 1974) and early atherosclerotic changes in the vessel wall tend to occur (Fry, 1976).

Blood flow is equally important in the fragmentation of a thrombus, especially in areas of high flow rate (Mustard et al., 1972a). The fragments from arterial thrombi can break up and platelets return to the circulation. However, if these fragments pass into the microcirculation and do not break up immediately, they may cause organ dysfunction and tissue injury (Moore, 1974).

The effect of blood flow on the behaviour of blood cells and its relation to thrombogenesis have been studied in several laboratories

(Goldsmith, 1972, 1974a,b; Goldsmith et al., 1975; Leonard et al., 1972; Meiselman and Goldsmith, 1973). Their studies have shown: (1) platelet interaction with the vessel wall is increased by the presence of red cells in the blood (Goldsmith, 1972); (2) in a region of flow separation and vortex formation platelet aggregates can spend a long time before rejoining the mainstream fluid (Yu and Goldsmith, 1973); (3) high shear stress increases the platelet release reaction induced by thrombin (Goldsmith et al., 1976).

3. Role of intravascular stimuli inducing platelet aggregation.

A number of intravascular stimuli can interact with platelets inducing platelet aggregation and disseminated intravascular coagulation (Mustard et al., 1974). Among these stimuli are immunological reactions, bacteria and viruses which interact with platelets causing the platelets to release and to aggregate.

Since platelet adhesion to collagen is one of the first steps in hemostasis and thrombosis, the acquisition of knowledge on the nature of this fundamental reaction is clearly important.

II. PLATELET STRUCTURE AND METABOLISM.

The ultrastructure of the resting platelet has been described and reviewed in detail by David-Ferreira (1964), Hovig (1968) and White (1971b) and will not be duplicated in this thesis. Only, the morphologic aspects necessary to understand some of the biochemical aspects of platelet function will be given.

1. Platelet plasma membrane.

(a) Structure.

When examined with the electron microscope, the outer membrane of platelets has a thickness of 70 to 90Å and a trilaminar appearance similar to the plasma membrane of other cells. On the outer surface of the membrane there is a fluffy coat 150 to 250 Å in thickness. This surface coat is considered to be an integral part of the membrane and is composed of proteins, glycoproteins and mucopolysaccharides. The function of the amorphous coat is unknown, but has been inferred in platelet surface-mediated events such as aggregation and adhesion. The outer surface coat can be stained with substances staining carbohydrates. Some plasma proteins, including clotting factors (fibrinogen, IgM, factors V, VII, XI) are adsorbed on the surface of the platelets (Weiss, 1976). More recently Okumura and Jamieson (1976 a, b, c) have described the presence in the glycocalyx of a soluble glycoprotein, with mobility and apparent molecular weight of 150,000 similar to glycoprotein I: this glycocalicin is cleaved by trypsin. Glycocalicin is probably a receptor site for the lectins wheat germ agglutinin and Agaricus bisporus (Greenberg and Jamieson, 1974) and for thrombin and ristocetin

(Okumura and Jamieson, 1976c).

A number of enzymes are also associated with platelet plasma membranes (Jamieson and Smith, 1976). One of the membrane associated enzymes, a nucleoside diphosphate kinase has been proposed as a candidate for the ADP receptor (Mustard et al., 1975a).

(b) Biochemistry.

The role of the platelet in hemostasis and thrombosis involves a variety of interactions of the platelet membrane surface with other platelet membranes, other solid surfaces and a large number of soluble agents introduced into the platelet's environment. Thus it has become imperative to develop knowledge about the structure, biochemical composition and orientation of the components of the platelet membrane. These studies have been possible with the use of new techniques such as polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; lactoperoxidase iodination and other labeling techniques; enzymatic hydrolysis; binding studies with lectins, specific antiglycoprotein antibodies and thrombin (for review see Cooper et al., 1976). These studies have involved the use of both intact platelets and isolated plasma membranes (for review see Jamieson and Smith, 1976).

Platelets interact with a large number of agents through specific receptor mechanisms. A great deal of information is now available on the possible nature of the platelet receptors for low molecular weight agonists (for review see Mills and Macfarlane, 1976) such as ADP, serotonin, catecholamines and for macromolecular components (for review

see Michaeli and Orloff, 1976) such as thrombin (Mills and Macfarlane, 1976), collagen, immunoglobulins. At the present time no platelet receptor has been identified, isolated and purified.

(i) Characterization of surface glycoproteins.

(Nurden and Caen, 1976; Jamieson and Smith, 1976; Mills and Macfarlane, 1976). The protein composition of the platelet membranes consists of some 20 polypeptides ranging in molecular weight from 10,000 to 200,000 (Barber and Jamieson, 1971; Nachman and Ferris, 1972; Phillips, 1972) when studied in 5% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. PAS staining of the gels for carbohydrate revealed the presence of three major glycoproteins (Nachman and Ferris, 1972; Phillips, 1972).

Lactoperoxidase-catalyzed iodination of intact platelets has been used in an attempt to label proteins externally oriented in the membrane. Only seven polypeptides are labeled (Phillips, 1972). The labeled polypeptides include the three major glycoproteins which can be hydrolysed by trypsin (Phillips, 1972). The external orientation in the membrane of the three major glycoproteins has been confirmed by George et al., (1976b) with diazotized (^{125}I)-diiodosulfanilic acid.

Recent studies by Jenkins et al. (1976) suggests that glycoprotein II can be resolved into 2 components IIa and IIb. George (personal communication) has demonstrated that at least 7 glycoproteins are exposed externally and that they can be identified by gel electrophoresis by modifying the concentration of polyacrylamide used in the gels.

(ii) Role of surface glycoproteins.

The possible role of the major surface glycoproteins has been reviewed by Nurden and Caen (1976) and is discussed in more detail in the present thesis (see Discussion, II).

Although no major glycoprotein has been identified yet with certainty as a platelet receptor, some light has been recently shed as to their function.

Glycoprotein I is decreased in Bernard-Soulier syndrome (Nurden and Caen, 1975; Jenkins et al., 1976) and has been implicated in platelet adhesion to subendothelium (Weiss et al., 1974; Caen et al., 1976) and in ristocetin-induced aggregation (Caen et al., 1976; Jenkins et al., 1976; Okumura and Jamieson, 1976a; Nachman et al., 1977) and may serve as a receptor for the plasma von Willebrand factor (Jenkins et al., 1976). Glycoprotein II can be hydrolysed by high concentrations of thrombin (Phillips and Agin, 1972), although it has been demonstrated that the thrombin binding site on the platelet membrane (Ganguly, 1974; Ganguly and Sonnichsen, 1976; Tollefsen et al., 1974) is different from the proteolytic site.

The concentration of glycoprotein II (Nurden and Caen, 1974,1975) or IIb (Phillips et al., 1975) is reduced in Glanzmann's thrombasthenia.

2. Platelet contractile system (Cohen and Lüscher, 1975; Adelstein et al., 1975; Lüscher, 1976) and microtubules.

The presence in human platelets of an actomyosin-like contractile protein (originally named thrombosthenin) was first described by Bèttex-Galland and Lüscher. Platelet actomyosin represents 15% of

the total platelet protein content.

(a) The components of platelet actomyosin.

Platelet actin (molecular weight (M.W.) of 43,000) filaments are rarely seen in resting platelets. Filaments that have the dimension of actin and form "arrowheads" with external heavy meromyosin can be seen in aggregated platelets (Bettex-Galland et al., 1972). Platelet actin stimulates the myosin ATPases of platelet origin. Finally, platelet actin is cleaved by thrombin (Muszbek et al., 1976).

Platelet myosin consists of 2 heavy chains (M.W. 200,000) and 2 pairs of light chains (M.W. 20,000 and 17,000 respectively) (Adelstein et al., 1975). It is an ATPase. Recently, Daniel and Adelstein (1976) have reported that a platelet myosin light chain kinase, activated by Mg^{2+}, specifically phosphorylates the 20,000 dalton light chain of myosin, and this phorphorylation is linked to an increase in activity of the actin-stimulated myosin ATPase.

The contractile system is controlled by regulatory components; troponin and tropomyosin which are bound to actin.

(b) Role of the contractile system.

In the resting platelets, there is no evidence of cytoplasmic filaments of actomyosin. However, upon activation thin filaments of F-actin appear throughout the cytoplasm or in the form of submembranous filaments adhering to the inner surface of the plasma membrane, suggesting that they have a role in the morphological alterations of the cell surface. Activation of the contractile system is thought to be

brought about by the availability of Ca^{2+}-ions in the cytoplasm (Massini and Luscher, 1976). This is achieved by the mobilization of internal, organelle-bound calcium (residues of the dense tubular system) or by the influx of Ca^{2+}-ions through the plasma membrane. It is possible that the availability of Ca^{2+} in the cytoplasm might be regulated by cAMP. During the shape change, the circumferential ring of microtubules disappears. Microtubules are not contractile elements. They are depolymerized by Ca^{2+} and their disappearance may mediate the release of Ca^{2+} into the cytoplasm.

The platelet contractile system is involved in: (1) platelet shape change and pseudopod formation. Cytochalasin B has been shown to block contractile functions in many cells and to keep platelets in disc shape (White, 1971a); (2) Contraction and relaxation of platelet aggregates, which are dependent on the availability of external calcium ions (Cohen et al., 1974); (3) Clot retraction (Cohen and Luscher, 1975). De Clerck et al. (1975) have demonstrated that if fibrinogen is clotted by reptilase, the clot will not retract, because the platelets do not change shape; (4) The return of platelets to a disc shape. White (1974) has presented evidence that platelets adherent to collagen can detach from it and recover a disc shape after addition of cytochalasin B. Similarly, cytochalasin B can detach adherent mouse peritoneal macrophages from glass (Helentjaris et al., 1976).

3. Platelet energy metabolism.

The energy metabolism of platelets has been extensively reviewed in the past few years (Mustard and Packham, 1970; Doery et al., 1970; Gross and Schneider, 1971; Holmsen and Day, 1971).

Platelets require energy to carry out their functions of shape change, aggregation, release reaction, phagocytosis and clot retraction. The continuous production of ATP in resting platelets is provided by glycolysis and oxidative phosphorylation.

Two pools of adenine nucleotides have been described in platelets (Holmsen and Day, 1971): (1) a storage pool, containing ATP and ADP, accounting for 60% of the total nucleotides. This pool, stored in the dense granules is extruded during the release reaction. (2) a metabolic pool of ATP and ADP which supplies energy and can be labeled in vitro by incubation of platelets with radiolabeled adenosine or adenine. The labeled adenine nucleotides of the metabolic pool do not exchange readily with those in the storage granules. However, recent experiments by Reimers et al., (1975, 1977) have demonstrated that in vivo or after prolonged incubation in vitro, there is equilibration between the two pools by transfer of ATP across the storage organelle membranes. The ATP and ADP of the metabolic pool are located in the cytoplasm, the mitochondria and membranes and are not extruded during the release reaction.

When platelets are stimulated ATP is consumed and broken down irreversibly to inosine monophosphate and hypoxanthine. Holmsen et al.,

(1974) have shown that a critical level of metabolic energy is required for shape change or aggregation or the release reaction and that each step in the sequence can be inhibited if the basal level of ATP falls below this critical level. The release reaction is the most sensitive to depletion of ATP. Holmsen et al., (1974) have found that most of the ATP is consumed during the shape change, rather than during aggregation, but that a higher basal level of ATP is necessary for aggregation than for shape change. Mills (1973) found that shape change was accompanied by a decrease in the adenylate energy charge corresponding to an increase in energy utilization.

Thus it seems that platelets consume ATP to carry out their functions. It has been suggested that platelet actomyosin was responsible for the degradation of ATP induced by platelet stimulating agents (Holmsen, 1972; Kattlove, 1974).

4. Cyclic AMP.

Several investigators have suggested in recent years that platelet aggregation and the release reaction are controlled by variations in intracellular 3',5'-cyclic adenosine monophosphate (cAMP) levels (Salzman and Weisenberger, 1972; Haslam, 1973, 1975; Mills and Macfarlane, 1976). Cyclic AMP is formed from ATP by the enzyme adenylate cyclase and cAMP is removed by phosphodiesterase.

Many compounds that block platelet aggregation increase platelet cAMP through stimulation of adenylate cyclase (PGE_1) or inhibition of phosphodiesterase (caffeine).

Although some aggregating agents such as thrombin (Brodie et al., 1972), can directly inhibit membrane adenylate cyclase and decrease cAMP levels, there is no convincing evidence that aggregation is linked to a decrease in cAMP and that cAMP can be lowered under basal conditions (Haslam, 1975). However, aggregating agents such as ADP or adrenaline, can lower cAMP if its basal level has been previously elevated (Haslam, 1973).

It is reasonable to suppose that many effects of cAMP are mediated through changes in protein phosphorylation resulting from a cAMP-induced alteration in protein kinase and/or phosphoprotein phosphatase activity. Platelets contain a cAMP-dependent protein kinase and cAMP-independent protein kinase (Booyse et al., 1973, 1976) which can phosphorylate several membrane proteins. Lyons et al. (1975) have shown that, in intact platelets, thrombin induces a rapid increase in phosphorylation of two platelet proteins (M.W. 20,000 and 40,000). Haslam and Lynham (1976) have presented evidence showing that the induction of the release reaction is accompanied by the phosphorylation of these two proteins in the intact platelets, one of which appeared to be a component of actomyosin. These results have been confirmed by Daniel and Adelstein (1976) who found that the 20,000 molecular weight protein is the light chain of platelet myosin. The phosphorylation of the light chains of platelet myosin increases the actin-activated ATPase activity of platelet myosin and thus may control contraction (Adelstein et al., 1975). The phosphorylation of this 20,000 M.W. polypeptide may be dependent on the

activation of the protein kinase by Ca^{2+} ions (Haslam and Lynham, 1976). It is known that cAMP increases the uptake of calcium into the sarcoplasmic reticulum by smooth muscle cells. It is thus possible that cAMP might modify platelet aggregation and the release reaction by controlling the storage and release of calcium from the platelet dense tubular system (White, 1972; George et al., 1976a).

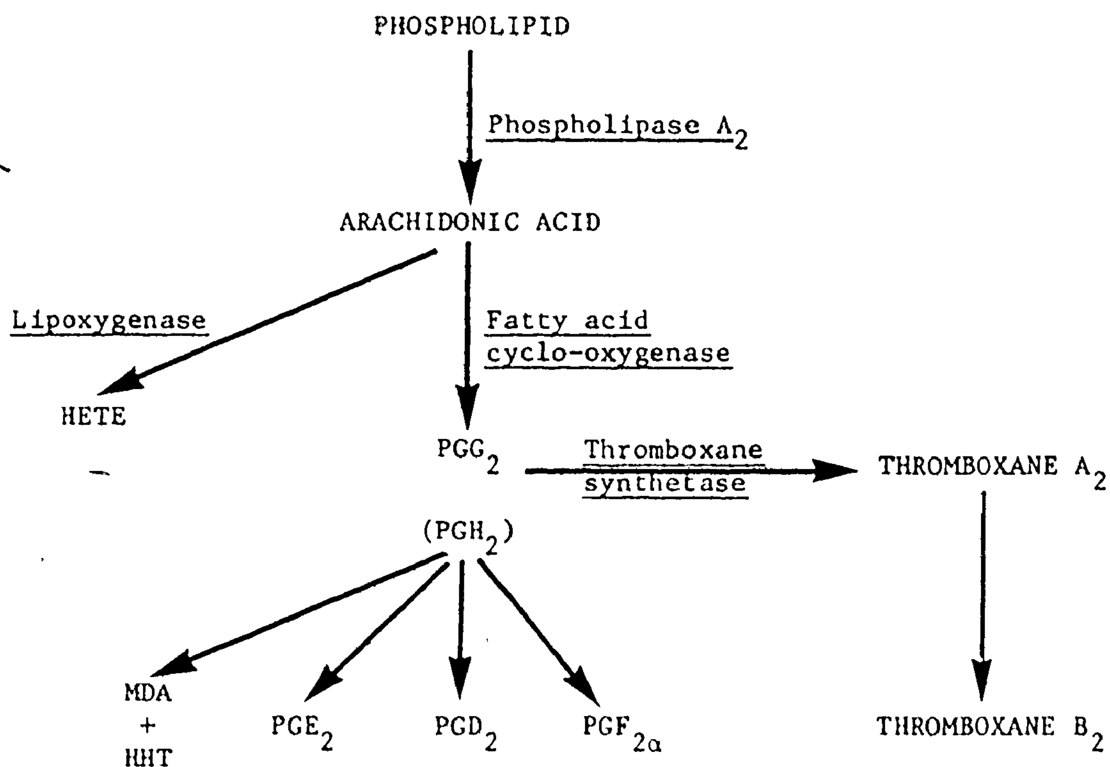
5. Metabolism of arachidonic acid by platelets and its inhibition (Samuelsson et al., 1975; Hamberg et al., 1976; Smith et al., 1975; Smith and Silver, 1976).

Smith and Willis (1970) have shown that when human platelets are stimulated with thrombin they form prostaglandins PGE_2 and $PGF_{2\alpha}$. Subsequently, it was found that aspirin and indomethacin are strong inhibitors of platelet prostaglandin formation (Smith and Willis, 1971).

Arachidonic acid causes platelet aggregation and this is associated with prostaglandin synthesis (Smith and Silver, 1976). Willis and Kuhn (1973) were the first to report that aggregation induced by arachidonic acid could be due to an intermediate in prostaglandin biosynthesis, which appeared to be a prostaglandin endoperoxide (Willis, 1974). Later, two prostaglandin endoperoxides (PGG_2 and PGH_2) were isolated and shown to cause platelet aggregation (Hamberg et al., 1974b). When added to platelets, arachidonic acid (Figure 1) is oxygenated to prostaglandin endoperoxides by a cyclo-oxygenase. Aspirin and indomethacin inhibit the cyclo-oxygenase (Hamberg et al., 1974a; Hamberg and Samuelsson, 1974). More recently it appeared that the endoperoxide intermediate PGG_2 may have to be converted to

FIGURE 1.

Transformations of arachidonic acid in platelets.



HETE	12L-hydroxy-5,8,10,14 eicosatetraenoic acid
PGG_2 and PGH_2	prostaglandin endoperoxides
MDA	malondialdehyde
HHT	hydroxy-5-cis-8,10-transheptadecatrienoic acid

thromboxane A_2 to aggregate platelets (Hamberg et al., 1975) and this was accomplished by an enzyme, thromboxane synthetase, which has been identified in platelet microsomes (Needleman et al., 1976b). In addition, thromboxane A_2 is a potent vasoconstrictor (Needleman et al., 1976a).

It has also been demonstrated that stimulation of platelets with thrombin is accompanied by the formation of prostaglandin endoperoxides from arachidonic acid (Hamberg et al., 1974a; Willis, 1974). Relatively large amounts of esterified arachidonic acid are found in membrane phosphatidylcholine and phosphatidylinositol (Bills et al., 1976). When platelets are aggregated by thrombin, arachidonic acid is released from membrane phosphatidylcholine and phosphatidylinositol (presumably by the action of a phospholipase A_2) and is totally utilized by a cyclo-oxygenase to generate prostaglandin endoperoxides and by a lipoxygenase to generate HETE (Bills et al., 1976; Figure 1).

Prostaglandin synthesis can be inhibited by drugs such as the non-steroidal anti-inflammatory drugs (Flower and Vane, 1974; Smith and Silver, 1976) and this may have potential applications as antithrombotic treatment. Aspirin and indomethacin both inhibit the platelet release reaction (Mustard and Packham, 1975) by inhibiting the enzyme cyclo-oxygenase which converts arachidonic acid to PGG_2 and PGH_2. ASA and indomethacin do not inhibit the formation of thromboxane A_2 from PGG_2 and PGH_2. The effect of ASA is permanent and due to acetylation of the cyclo-oxygenase (Roth and Majerus, 1975). ASA and indomethacin inhibit the synthesis of the short-lived intermediates PGG_2 and PGH_2 which are

potent inducers of aggregation and release, but do not interfere with their biological activity. 5, 8, 11, 14-eicosatetraynoic acid, an analogue of arachidonic acid, inhibits by substrate competition prostaglandin synthesis and arachidonic acid- and collagen-induced aggregation (Smith and Silver, 1976).

The role of PGG_2, PGH_2 and thromboxane A_2 in the mechanism of platelet aggregation induced by various platelet aggregating agents has been reviewed by Packham et al. (1977). ADP-induced platelet shape change and aggregation is independent of the arachidonate pathway. Sodium arachidonate-induced platelet shape change, release and aggregation is inhibited by aspirin and indomethacin and thus totally dependant on PGG_2, PGH_2 and thromboxane A_2 (Kinlough-Rathbone et al., 1976b). Sodium arachidonate can aggregate thrombin-degranulated platelets, independently of ADP and thus can aggregate platelets independently of the release reaction (Kinlough-Rathbone et al., 1976b). The release reaction induced by thrombin is accompanied by the formation of prostaglandin endoperoxides (Hamberg and Samuelsson, 1974). Aspirin or indomethacin inhibits the formation of prostaglandin endoperoxides induced by thrombin, but has very little effect on the release of granule contents (Mustard and Packham, 1975). Evidence has been presented (Packham et al., 1977) that thrombin can aggregate platelets by a mechanism independent of released ADP and of prostaglandin formation. Collagen aggregates platelets by two mechanisms, the released ADP and the formation of PGG_2, PGH_2 and thromboxane A_2 (Packham et al., 1977).

Very recently, Moncada et al. (1976, 1977) have described in the vessel wall a novel metabolic transformation of prostaglandin endoperoxides into an unstable substance called prostacyclin (originally prostaglandin X). Prostacyclin is produced by the cells of the vessel wall from prostaglandin endoperoxides formed by platelets and also from arachidonic acid from the vessel wall (Moncada et al., 1977). Prostacyclin is a potent inhibitor of platelet aggregation and has been postulated by the authors to play an important role in preventing platelets from adhering to the endothelium. Prostacyclin is also a potent vasodilator. However, unless there is a source of prostaglandin endoperoxides in the vessel wall, it is difficult to envision how platelets can generate PGG_2 and PGH_2 unless they have interacted with thrombin or collagen. Moreover, if their theory is correct, one would expect increased adhesion to the surface of the aorta in the presence of drugs that inhibit endoperoxide formation.

III. PLATELET INTERACTION WITH SURFACES.

Platelet adherence to surfaces is one of the main functions of platelets. Interaction of the platelets with subendothelial surfaces is involved in hemostasis and thrombosis (Mustard et al; 1967a; Nossel, 1975; Cooper et al., 1976).

Platelets also interact with artificial surfaces (Berger and Salzman, 1974). The basic mechanisms of platelet adhesion to surfaces are not completely understood but it is clear that collagen is the most thrombogenic component of the subendothelium (Baumgartner and Haudenschild, 1972) and that the interaction of platelets with most surfaces is dependent on the nature of the plasma proteins adsorbed (Vroman et al., 1971).

1. Platelet interaction with the vessel wall.

The vascular intima is composed of the endothelium and the subendothelium. The structure of the intima has been reviewed recently by Stemerman (1974).

(a) Endothelium (Stemerman 1974; Gimbrone, 1976).

The endothelial cell contracts when stimulated by histamine, serotonin, bradykinin or thromboxane A_2 (Needleman et al., 1976a). When the endothelial cell contracts it may widen intercellular gaps, thus exposing subendothelial tissue to which platelets adhere. It is to be noted that some of the substances that contract endothelial cells are released by platelets.

Endothelial cells have a low turnover rate, except at points where there may be unusual stresses or altered flow patterns such as at branches

or bifurcations (Wright, 1972). Progress in the understanding of the biology of the endothelium has been possible with the development of new techniques of culture (Gimbrone, 1976). Endothelial cells can release an activator of plasminogen (Nilsson and Pandolfi, 1970), synthesize tissue factor (Nemerson and Pitlick, 1972) and factor VIII antigen (Hoyer et al., 1973; Jaffe et al., 1973). The endothelium is a blood compatible surface and normally does not activate the blood coagulation system or interact with platelets (see review in Jørgensen, 1971; Gimbrone, 1976).

Platelets will interact with the vessel wall if (1) the endothelium is modified by thrombin generated at minimal sites of injury (Ashford and Frieman, 1967) or by the formation of polymerizing fibrin to which platelets readily adhere (Niewiarowski et al., 1972; (2) the endothelium is damaged.

(b) Subendothelium. (Stemerman 1974; Baumgartner and Muggli, 1976).

Bounameaux (1969) and Hugues (1960) were the first to observe that platelets adhere to subendothelial connective tissue particularly to collagen. The subendothelial structures to which platelets adhere are considered to be collagen, basement membrane and the microfibrils around elastin (Stemerman et al., 1971; Stemerman, 1974; Baumgartner and Muggli, 1976). Using various enzymatic treatments (collagenase will digest collagen and basement membrane but not microfibrils, and α-chymotrypsin will digest microfibrils and basement membrane) it has been

demonstrated that collagen is the most thrombogenic component of the subendothelium of rabbit aortas injured by passage of a balloon catheter (Stemerman et al., 1971; Baumgartner and Haudenschild, 1972; Baumgartner and Muggli, 1976). Platelet adherence to microfibrils or to basement membrane does not induce the release reaction and platelet aggregation (Tranzer and Baumgartner 1967; Huang et al., 1974). In contrast, when platelets interact with collagen, they are induced to release some of their granule contents, including serotonin and adenine nucleotides and to form PGG_2, PGH_2 and thromboxane A_2. The released ADP and the arachidonic metabolites are considered to be responsible for inducing platelets to adhere to each other and to the platelets adherent to collagen (Packham et al., 1977).

2. <u>Platelet adhesion to collagen</u>. (Nossel, 1975; Michaeli and Orloff, 1976).

(a) <u>Biochemistry of collagen.</u>

The precise biochemical mechanism of platelet-collagen interaction is poorly understood, but some features of the structure of collagen appear to be important. Investigators have greatly advanced in our understanding of the chemistry of collagen and have shown that there are several types of collagen differing in amino acid composition and sequence and in carbohydrate content. Most of the review articles have been largely concerned with type I collagen, describing its structure (Traub and Piez, 1971; Gallop et al., 1972; Fietzek and Kuhn, 1976) and biosynthesis (Grant and Prockop, 1972; Bornstein, 1974; Gross, 1974; Miller and Matukas,

1974). The structure and biological significance of type I, II and III collagen (Miller, 1976) and of basement membrane (Kefalides, 1975) have been recently reviewed.

(i) Collagen-structure.

The monomer of collagen, or tropocollagen, consists of 3 polypeptide chains (α chains) coiled about the same axis and held together by cross-links. Each α chain has a molecular weight of 95,000. The tropocollagen molecule measures 3,000 x 15 Å, and contains slightly more than 1,000 amino acids. The triple strand is arranged in a coiled-coil structure: each α chain forming a left-handed polyproline type helix and the 3 α chains forming a right-handed superhelix. Each α chain is formed by the repetition of the following triplet sequence $(GLY\text{-}X\text{-}Y)_n$. So, each third residue is a glycine, X being a proline or an hydroxyproline and Y any amino acid. The triple strand is stabilized by inter α chains hydrogen bonds, 1 per triplet, between glycine on one chain and proline on the other. The tropocollagen molecule is a glycoprotein. Carbohydrate residues are attached to the hydroxylysine by O-glycosidic linkage in form of galactose (incomplete residue) or glucose-galactose (complete residue). In vitro the reconstitution of native collagen proceeds through three states of increasing complexity: monomeric (tropocollagen) microfibrillar and particulate (fibers).

(ii) Collagen types.

Four types of genetically distinct collagen are known (Miller, 1976). Type I ($[\alpha 1(I)]_2$ is encountered in the skin, bone, tendon and

arterial wall. Type II collagen ([α1(II)]$_3$ is found in cartilage. Type III ([α1(III)]$_3$ is found in the arterial wall, the skin and the uterus. Type IV is basement membrane. There is some controversy about the potency of these different collagens as to which is the most potent platelet activator. Measuring ^{14}C-serotonin release, Michaeli and Orloff (1976) found the following order of activation capacity: type I > type II > type III > type IV. Michaeli concluded that the higher the carbohydrate content of collagen, the lower its platelet activation capacity. Carbohydrates are important in controlling fibril formation and it is probable that the degree of fibrillo-genesis is a major determinant of collagen activity with respect to platelets.

Other investigators (Balleisen et al., 1975; Hugues et al., 1976) have found that type III collagen is a more potent platelet aggregating agent than type I. These observations may be important in view of the findings of Gay et al., 1975, that subendothelial microfibrils display immunohistological characters of type III collagen. However, these data are difficult to interpret because Barnes et al. (1976) have shown that type III collagen polymerizes faster than type I in plasma and that type I collagen is as potent an activator as type III when preformed fibrillar collagen is used. Miller (1976) has also pointed out that the surface area of the fine fibrils of type III collagen may not be comparable with that of thick fibrils of type I collagen.

(b) <u>Structural requirements for platelet-collagen interaction.</u>

(i) <u>The tertiary structure of collagen is necessary.</u>

Maintenance of the native conformation of collagen is a requirement for promoting platelet aggregation. This ability is destroyed by digestion with collagenase, or by conversion to the randomly-coiled form upon heat denaturation (Harper et al., 1975; Simmons et al., 1975). Pepsin or pronase (Wojtecka-Lukasik et al., 1967) which cleaves the telopeptides from collagen, preserving the triple helix conformation, has no effect. Finally, rigidly spaced positive or negative polar active sites are important and the ability of insoluble collagen to aggregate platelets can be abolished by chemically blocking or oxidizing the free ε-amino groups (Wilner et al., 1968b; 1971; Balleisen et al., 1976).

More recently, three different groups (1) Muggli and Baumgartner (1973), (2) Brass and Bensusan (1974), (3) Jaffe and Deykin (1974), have shown that if the tertiary structure of the tropocollagen monomer is necessary for collagen induced platelet aggregation and release reaction, it is not sufficient.

(ii) A higher-ordered structure, (quarternary structure), secondary to polymerization or multimerization of tropocollagen is necessary.

Monomeric tropocollagen must first polymerize to microfibrillar collagen to induce platelet release or aggregation. Urea or glucosamine which prevents polymerization inhibits platelet aggregation (Legrand et al., 1968). Guanidination of monomeric collagen increases the rate of polymerization, reducing the lag time in serotonin release (Brass

and Bensusan, 1974).

Using ^{125}I-labeled collagen, Gordon and Dingle (1974) have shown that the amount of platelet-bound collagen paralleled the extent of collagen microfibril formation.

(iii) <u>The role of carbohydrate side chains of collagen in the adherence of platelets to collagen</u>.

Jamieson et al. (1971) and Bosmann (1971) suggested that the carbohydrate side chains of collagen may be involved in the adherence of platelets to collagen because they found that the platelet enzyme, collagen-glucosyl transferase, transfers glucose specifically from UDPG to incomplete heterosaccharide chains of collagen. Jamieson et al. (1971) and Barber and Jamieson (1971) suggested that an enzyme-substrate complex between this enzyme and incomplete carbohydrate chains on collagen may play a major role in platelet:collagen adhesion. This hypothesis appeared to be supported by the experiments of Chesney et al. (1972) showing that oxidation of 6-hydroxymethyl position of the galactosyl residues on collagen to aldehydes abolished its ability to cause platelet aggregation and by the similar experiments of Kang et al. (1974) destroying the carbohydrate moiety of collagen by periodate treatment. However, Muggli and Baumgartner (1973) found that this treatment only delayed "multimerization" of the collagen and did not prevent it from eventually developing full aggregating activity. Thus, the results of Muggli and Baumgartner (1973) indicate that the collagen-bound galactose may not be involved in the interaction of platelets with

collagen. Puett et al. (1973), contrary to Katzman et al. (1973) and Kang et al. (1974) demonstrated that denatured purified α_1 and α_2 chains were several orders of magnitude less potent than collagen and suggested that Kang's data with isolated α_1 isolated chains may be due to partial renaturation.

(iv) Localization of binding sites.

Several investigators have tried to localize platelet-binding sites on the collagen molecule (Michaeli and Orloff, 1976). Kang et al. (1974) have identified a binding on the α_1 chain of chick skin collagen. They have isolated a 37-residue peptide (α_1-CB5) responsible for the binding and performed binding studies (Chiang et al., 1975; Chiang and Kang, 1976).

The glycopeptide glycosylgalactosyl-hydroxylysine has been implicated in binding, but only the chick skin peptide is active (Kang et al., 1974). This is supported by the findings of Brass and Bensusan (1976) indicating that the collagen carbohydrate residue may be either near to or part of the site(s) on the collagen molecule required for platelet adhesion. In contrast, Puett et al. (1973) suggest that the carbohydrate moieties of collagen are not involved in platelet activation.

Michaeli and Swanson (cited in Michaeli and Orloff, 1976) have presented evidence that collagen from rat tendon or human skin has 2 sites for attachment on the platelet membrane (a major activation site of release located on α_2-CB5 and a minor activation site on α_1-CB6) located on the carboxy-terminal end of the peptides. There is also a site near

the amino-terminal end (α_2-CB4) which inhibits the induction of the platelet release reaction by collagen.

3. Platelet interaction with artificial surfaces.

The interest in understanding platelet interaction with synthetic surfaces has been stimulated by the importance of such reactions in thrombus formation on artificial prosthesis in contact with blood (for review see Federation Proceedings, Volume 30, September-October 1971; Berger and Salzman, 1974; Nossel, 1975; Cooper et al., 1976).

The physical and chemical properties of synthetic surfaces responsible for platelet adhesion and activation of the coagulation system are not clear although a number of hypothesis have been proposed. Among the properties of the surface considered to be important are surface smoothness (Neumann et al., 1975), wettability (Baier, 1972) and surface electric charge (Sawyer and Srinivasan, 1972).

Although the properties of the artificial surface are important, a number of investigators have concluded that the primary influence of surfaces on platelets is mediated by the plasma proteins adsorbed to the surface (Packham et al, 1969; Baier et al., 1971; Salzmann 1971a, b, c; Jenkins et al., 1973). Several investigators have also demonstrated the importance of flow conditions and of the presence of red blood cells in determining platelet adhesion to surfaces (Friedman and Leonard, 1971; Leonard et al., 1972; Goldsmith, 1972; Turitto and Baumgartner, 1975).

A number of thromboresistant materials have been designed (Gott and Fukuse, 1971): (1) relatively inert polymers; (2) surfaces with

anionic radicals or imposed negative electrical charges and (3) heparinized surfaces. All these surfaces, although designed to be thromboresistant, still cause clinical thromboembolic complications. A major obstacle for the development of new artificial surfaces compatible with blood has been the lack of satisfactory techniques for prediction of thrombogenicity or thromboresistance (Berger and Salzman, 1974).

4. Methods for measuring platelet adherence to surfaces.

Various methods have been used to measure platelet adherence to surfaces including artificial surfaces, collagen and subendothelium. Several methods have been used to quantitate the number of platelets adhering to a surface after exposure to blood or platelet-rich plasma: (a) measurement of the drop in platelet count after exposure of the surface to platelets by counting the platelets (Hellem, 1960; Salzman, 1963); (b) direct microscopic examination of the surface (Friedman and Leonard, 1971; George, 1972; Baumgartner, 1973); (c) measurement of the amount of platelet-bound radioactivity associated with the surface (Hirsh et al., 1968b; Hovig et al., 1968; Packham et al., 1969; Jenkins et al., 1973; Lagergren et al., 1974).

One of the major problems in measuring platelet adherence to surfaces is to prevent platelet aggregation from occurring and to prevent clotting when blood or plasma is used. This can be achieved by using anticoagulants which chelate calcium, such as EDTA. It should be recognized that this prevents the evaluation of the role of divalent cations in platelet adherence to surfaces.

IV. AIMS OF THE STUDY.

Although it has been recognized for a long time that the first step in hemostasis and thrombosis is adherence of platelets to the constituents of the vessel wall, the factors involved in platelet adherence to surfaces are not understood.

Reproducible methods for studying platelet adherence to surfaces should allow determination of (a) the role of the components of the vessel wall such as collagen; (b) the role of the constituents of the platelet surface; and (c) the role of the composition of the platelet suspending fluid, particularly the concentrations of divalent cations and plasma proteins in the suspending fluid, on platelet adherence to surfaces. This knowledge should enhance our understanding of how this process might be inhibited by drugs or influenced by factors such as diet. Furthermore, it could be of use in developing prosthetic surfaces.

Several investigators have described methods to measure platelet adherence to artificial surfaces, collagen and subendothelium. Some of the problems encountered by these investigators were: (1) use of EDTA to inhibit platelet aggregation, thus preventing an assessment of the role of divalent cations in adhesion; (2) inhibition of coagulation but occurrence of platelet aggregation and thrombus formation when sodium citrate or heparin were used; (3) use of blood or platelet-rich plasma preventing modification of the environment; (4) variable hemodynamic factors; (5) complex methods for quantitating platelet adherence with problems of sampling and reproducibility.

The studies reported in this thesis were directed at:

(1) developing a quantitative method for measuring platelet adherence to collagen-coated surfaces and to the subendothelium in the absence of platelet aggregation and in a suspending medium containing normal concentrations of divalent cations.

(2) studying the effects of flow, divalent cations and hematocrit on platelet adherence to these surfaces.

(3) studying the effects on platelet adherence to these surfaces of enzymatic and chemical treatments of platelets which may modify platelet surface glycoproteins.

(4) studying the effects of modifying the surface of the vessel wall.

(5) studying the effects on platelet adherence of drugs and agents that inhibit platelet function.

(6) developing a reliable assay of drugs inhibiting platelet adherence because these drugs may prove to have potential value as antithrombotic agents.

(7) examining the relationship among the modification of the platelet surface, adherence to surfaces in vitro, and platelet survival and hemostasis in vivo.

CHAPTER TWO

MATERIALS AND METHODS

I. MATERIALS

Unless otherwise noted, all materials to be added to platelet suspensions were dissolved and diluted in modified Tyrode solution (no calcium or magnesium) and adjusted to pH 7.35.

All concentrations given in the text or in the tables are final concentrations in the platelet suspensions.

Acetylsalicylic acid (ASA). For in vivo studies of the effect of ASA, rabbits were given tablets (ground up in water) by stomach tube: For in vitro studies, ASA (British Drug Houses, Toronto (Canada) was prepared in modified Tyrode solution. pH 7.35 immediately before use.

Adenosine diphosphate (ADP) was from Sigma Chemical Company, St. Louis, Missouri.

Bovine Albumin (Pentex, Fraction V) was from Miles Laboratories, Kankakee, Illinois. For the experiments in which the effects of divalent cations were studied, calcium-free bovine albumin (Pentex, Fraction V) was prepared by the following method. Thirty milliliters of a 3.5 percent solution of albumin in distilled water containing 1 mM EDTA was dialyzed against 2 L. of distilled water for 20 hours at 4°C. This concentrated albumin solution was added to the final suspending medium (a modified Tyrode solution containing no calcium, but containing 1 mM magnesium) before it was adjusted to volume so that the final concentration of albumin was 0.35 percent.

Apyrase (E.C. 3.6.1.5) was prepared by the method of Molnar and Lorand (1961). This preparation hydrolysed 1.7 nmol ATP/min/μg protein.

Antimycin (Sigma Chemical Co., St. Louis, Missouri) was prepared as a 5 mM solution in 95 percent ethanol.

Sodium arachidonate was prepared from arachidonic acid (5,8,11,14-eicosatetraenoic acid) [Grade L, 99 percent purity, Sigma Chemical Company, St. Louis, Missouri] as described by Kinlough-Rathbone et al. (1976b).

Sodium borohydride (Fisher Scientific Company) was prepared immediately before each experiment as a 0.1 M solution in distilled water, and the osmolarity was adjusted to 295 mOsm with 30 percent (w/v) NaCl solution. Further dilutions were made with isotonic saline and the solutions were kept at 0°C.

Human Clq, a subcomponent of the first component of complement, was purified by S. Assimeh according to the method of Assimeh et al. (1974). Highly purified Clq was dissolved in 5 x 10^{-5} M EDTA buffer.

Cephalothin (Keflin) was a gift of Dr. R.S. Dolman, Eli Lilly and Company (Canada) Limited, Toronto, Ontario. It was dissolved in modified Tyrode solution and the osmolarity was adjusted to 290 mOsm. The concentrations of the antibiotic are given as millimolarity; 1 mM cephalothin is 0.404 mg/ml.

Alpha-Chymotrypsin (52 units/mg, 3 times recrystallized) was obtained from Worthington Biochemical Corporation, Freehold, New Jersey.

Sodium citrate dihydrate was from British Drug Houses, Toronto, Ontario.

Collagen (Type I from bovine Achilles Tendon, No. C-9879 was obtained from Sigma Chemical Co., St. Louis, Missouri.

Collagen suspensions were prepared as described previously by Packham et al. (1967b). Preparation of acid-soluble collagen: One gram

of bovine tendon collagen was mixed with 12 ml glacial acetic acid in 188 ml of distilled water for 10 minutes at 22°C. The mixture was chilled on ice and homogenized for 10 minutes in the 250 ml container of a Sorvall Omnimix. The entire homogenate was centrifuged at 2,500 x g for 15 minutes at 22°C and the white film on top was discarded. The supernatant fluid and gelatinous precipitate were mixed gently with 200 ml of water and centrifuged at 1,000 x g for 10 minutes at 22°C. The supernatant fluid was stored at 4°C. The collagen concentration was approximately 0.25 percent, pH 2.8, mOSm 540, acetic acid 0.522 M. This preparation retains its activity over at least 3 to 4 months of storage at 4°C.

Collagenase (Type III, Sigma Chemical Co.) was dissolved in modified Tyrode solution containing 5 mM $CaCl_2$ but no magnesium.

Creatine phosphate (CP) and creatine phosphokinase (CPK) were from Sigma Chemical Co., St. Louis, Missouri.

Ethylenebis(oxyethylenenitrilo)-tetraacetic acid (EGTA) was from J.T. Baker Chemical Company (Phillipsburg, New Jersey) and disodium ethylenediaminetetraacetic acid (EDTA) was from Fisher Scientific Company. EGTA (0.1 M) was dissolved in distilled water adjusted to pH 8.2 and EDTA (0.1 M) to pH 8.8 so that the final pH when these solutions had been added to a platelet suspension was 7.35.

Human fibrinogen (Kabi, Grade L, Stockholm, Sweden) was treated with DFP before use to remove procoagulant activity (Mustard et al., 1975b).

Glucosamine hydrochloride (D+) was from Sigma Chemical Co., St. Louis, Missouri.

Heparin sodium (147 units per mg) was from Connaught Laboratories, Toronto, Ontario.

Hepes (N-2-hydroxyethyl-piperazine-N'2-ethanesulfonic acid) was obtained from Sigma Chemical Co., St. Louis, Missouri. It was used to maintain the pH of the suspending medium at 7.35.

Indomethacin (Merck Sharp, and Dohme, West Point, Pennsylvania) was prepared as a stock solution (10^{-2}M) in 95% ethanol and further dilutions were made with modified Tyrode solution.

Monoiodoacetic acid was from British Drug Houses, Toronto, Ontario.

Methylprednisolone. sodium succinate was a gift from the Upjohn Co., Kalamazoo, Michigan. It was dissolved in saline immediately before use.

Neuraminidase (Clostridium perfringens) Sigma type VI was purified by the method of Hatton and Regoeczi (1973) and assayed as described by Greenberg et al. (1975).

Penicillin G, USP-sodium, 1650 units/mg, (General Biochemical, Chagrin Falls, Ohio) was prepared just before use as a stock solution of 88,000 units/ml (0.148M) in distilled water; modified Tyrode's solution was used for dilution. The osmolarity was adjusted to 290 mOsm. The concentration of the antibiotic are given as millimolarity; 1 mM penicillin G is 587 units/ml or 0.352 mg/ml.

Sodium metaperiodate (Fisher Scientific Company, Fairlawn, New Jersey) was prepared immediately before each experiment as a 0.1 M solution in distilled water. The osmolarity was adjusted to 295 mOsm with 30 percent (w/v) NaCl solution. Further dilutions were made with isotonic saline and the solutions were kept at 0°C in the dark.

Plasmin obtained from three s[illegible] was [illegible]zed pig

plasmin (4.42 NE/mg) from Novo Fabrik-Copenhagen, Denmark was dissolved at a concentration of 5 g/ml in unbuffered saline.

Pure rabbit plasmin of specific activity 2.1 μmoles/α-N-benzoyl-L-arginine ethylester (BAEe)/min/mg was prepared by activating pure rabbit plasminogen by urokinase. This preparation was a gift of Drs. M.C.W. Hatton and E. Regoeczi.

Human plasmin (high purity grade, 15 CU per mg protein) was obtained from AB Kabi, Stockholm, Sweden.

Prostaglandin E_1 (PGE_1) was generously supplied by the Upjohn Co., Kalamazoo, Michigan and solutions were prepared as described previously (Kinlough-Rathbone et al., 1970).

Pyrimido-pyrimidine compounds (kindly supplied by Dr. R. Kadatz, Karl Thomae, GmbH, Biberach an der Riss, West Germany) were dissolved in 1 N HCl and diluted with modified Tyrode solution; the pH values of the solutions before addition to the platelet suspension were: RA 8 (persantin, dipyridamole): 4.9; RA 433: 3.2.

Radioactive compounds were obtained from Amersham/Searle Corporation, Arlington Heights, Illinois. ^{14}C-serotonin (^{14}C-5HT, -5-hydroxytryptamine-3'-^{14}C-creatinine sulphate, 55 μCi per micromole, ^{51}Cr (sodium chromate, 1 μCi per microliter, or ^{3}H-DFP (di-isopropyl-(1,3-H^3) phosphofluoridate, 3.3 Ci per micromole.

Reserpine used for the experiments in vitro and in vivo was a commercial product (Serpasil, Ciba). This product is solubilized in a diluent that contains a number of components. To ensure that none of these affected platelet function, control studies were done with the

diluent obtained from Dr. R. Ellis, Ciba Pharmaceuticals, Dorval, Quebec. The diluent was not responsible for the inhibition of collagen-induced platelet aggregation observed with Serpasil.

For studies in vitro, the Serpasil solution was diluted before use with unbuffered 0.85% saline. After addition to the platelet suspension the pH was 7.3. Unbuffered 0.85% saline was added to the control sample. Serpasil was also used for studies in vitro and injected intraperitoneally (5 mg/kg) into rabbits. Control rabbits received intraperitoneally an equal volume of distilled pyrogen-free water. Blood was collected 18 hr later as described below.

Sodium salicylate was obtained from Fisher Scientific Company, Fairlawn, New Jersey.

Soya bean trypsin inhibitor (SBTI) was obtained from Sigma Chemical Co., St. Louis, Missouri.

Sulphinpyrazone (generously supplied by Geigy Pharmaceuticals, Montreal, Quebec) was prepared as described previously by Packham et al. (1967b).

Either topical bovine thrombin (Parke, Davis & Co., Detroit, Michigan), or purified rabbit thrombin (a generous gift of Drs. M.R.C. Hatton and E. Regoeczi, Department of Pathology, McMaster University) were used.

TAMe [p-tosyl-L-arginine methyl ester (HCl)] was obtained from Mann Research Labs, New York, New York.

Uridine 5'diphosphate (UDP) and uridine-5'-diphosphoglucose (UDPG) were obtained from Sigma Chemical Co., St. Louis, Missouri.

II. METHODS

Collection of blood

Venous blood was obtained in the morning from fasting human subjects who had not taken any drugs for at least 10 days.

Blood was collected from 2.5-3.5 kg rabbits, anesthetized with sodium pentobarbital (30 mg/kg), through a polyethylene cannula inserted into the carotid artery.

Pig blood was collected at a slaughterhouse (Canada Packers, Toronto, Ontario) in polyethylene bottles. All glassware used to contain blood or plasma was coated with silicone. The acid-citrate-dextrose solution of Aster and Jandl (1964) was used as anticoagulant (1 part for 6 parts of blood). For pig blood the anticoagulant also contained heparin (final concentration 0.5 U per milliliter of anticoagulated blood).

Preparation of plasma

Platelet-rich plasma (PRP) was prepared from blood collected into 0.1 volume of 3.8 percent sodium citrate dihydrate by centrifugation at 77 g for 15 min at room temperature.

Citrated platelet-free plasma (PFP) (pH 7.35) prepared from blood taken into 0.1 volume of 3.8 percent trisodium citrate dextrose. It was used for some experiments to resuspend the labeled, washed rabbit platelets.

Preparation of suspensions of washed platelets

Suspensions of twice-washed platelets from rabbits (Ardlie et al. 1970, 1971) pigs, or humans (Mustard et al., 1972b) were prepared as described previously except that for pig platelets the osmolarity

of the washing and suspending solutions was adjusted to 340 mOsm with 30 percent NaCl. Platelets from all species were finally resuspended in Tyrode solution or for some experiments, in Eagle's medium (GIBCO, Grand Island, New York) (containing 2.2 g sodium bicarbonate per liter) containing 0.35 or 4% bovine albumin. For some experiments 5 mM Hepes buffer was included to maintain the pH of the medium at 7.35. Apyrase was included in the suspending medium. Platelets were counted by the method of Brecher and Cronkite (1950) and platelet counts were adjusted to 300,000 to 700,000 per mm^3. The platelet suspensions were stored at 37°C.

Platelet labeling

Platelets were labeled in the first washing fluid. Platelets were incubated for 10 minutes at 22°C with 1 μCi of ^{14}C-serotonin (5-hydroxytryptamine-3'-^{14}C-creatinine sulphate). Pig platelets were labeled with ^{51}Cr by incubation at 37°C for 20 minutes with 10 μCi/ml of washing fluid. Labeling of pig platelets with ^{3}H-DFP was done in the same way by incubation for 5 minutes with 0.25 Ci/ml of washing fluid.

Originally, the platelets from two rabbits were labeled in the second washing fluid (10 ml) by incubation for 1 hour at 22°C with 100 μCi ^{51}Cr. Later the platelets from two rabbits were incubated for 30 minutes at 22°C with 200 μCi of ^{51}Cr.

Preparation of washed rabbit red blood cells

The rabbit red blood cells were obtained after removal of the platelet-rich plasma used for the preparation of the platelet suspension. They were washed three times in a calcium-free modified

Tyrode solution at pH 7.35, and finally resuspended in the same medium as the platelets (1 volume red blood cells, 3 volumes suspending medium).

Preparation of collagen-coated glass tubes

New Pyrex glass tubes (10 by 75 mm, Corning Glass Co., Corning, New York, No. 9820) were washed in detergent (Alconox) and then soaked in a solution of alcoholic potassium hydroxide (1N) for 18 hours. The tubes were then washed, soaked for 18 hours in HCl (1N), washed with glass-distilled water, and dried at 100°C. To coat the tubes with collagen, 1 ml of acid-soluble collagen was poured into each tube which was then covered with parafilm and rotated end-over-end at 15 rpm for 10 minutes at 22°C. The solution was decanted and the tubes rinsed by filling them 4 times with a modified Tyrode solution containing no calcium or magnesium; the tubes were inverted to drain for 15 minutes at 22°C before the platelet suspension was added.

The tubes were not used more than 40 times because with aging and repeated washing and soaking, they tended to be less evenly coated with collagen.

Adherence of platelets to collagen-coated tubes

In experiments with inhibitors of platelet function, the platelet suspension (10 parts) was mixed with the inhibitor solution (1 part) at 37°C for 10 minutes before 1 ml of the platelet suspension was transferred to a collagen-coated tube. Solvent solution replaced

the inhibitor solution in control experiments. (All the concentrations of inhibitors are given as the final concentration in the platelet suspensions.) The collagen-coated tubes containing the platelet suspension were covered with parafilm and mechanically rotated end-over-end at 15 rpm for 10 minutes at 22°C. Before and after rotation a sample of the platelet suspension was examined by phase-contrast microscopy to ensure that no aggregates were present. The platelet suspension was decanted, a sample was centrifuged for 1 minute in an Eppendorf centrifuge (Brinkman, Rexdale, Ontario) for measurement of supernatant radioactivity. In the case of ^{14}C-serotonin, it is likely that most of the radioactivity found in the supernatant fluid represented release of granule contents, although loss of ^{14}C-serotonin from the cytoplasm could not be distinguished from release. In the case of ^{51}Cr-labeled platelets, radioactivity in the supernatant fluid is considered to represent loss from the cytoplasm and probably indicates some platelet lysis. The tubes were rinsed 4 times by filling them with a modified Tyrode solution containing no calcium or magnesium; this procedure removes platelets that are not tightly adherent to the collagen-coated surfaee. We showed previously (Cazenave et al., 1973b) that ^{14}C-serotonin or ^{51}Cr that was not associated with platelets did not become adherent to the collagen-coated surface. In the experiments with ^{14}C-serotonin or ^{3}H-DFP the tubes were treated with 0.5 ml of NCS (Nuclear Chicago Solvent, Amersham/Searle, Arlington Heights, Illinois). The NCS was transferred quantitatively to counting vials using 10 ml of toluene-fluor

solution for liquid scintillation counting. In the experiments with ^{51}Cr, the rinsed tubes were transferred directly to a well-type crystal scintillation counter for measurement of radioactivity. The extent of adherence was calculated as a percentage of the total radioactivity in the platelet suspension.

The surface area of a collagen-coated tube was approximately 2,500 mm^2. One milliliter of platelet suspension contained 7 x 10^8 platelets. Therefore, 1 percent adherence of radioactivity to the collagen-coated surface represents approximately 2,800 platelets per square millimeter. In calculating the extent of release or loss of radioactivity from the labeled platelets, the amount of radioactivity present in the suspending fluid of the untreated platelet suspension (always less than 2 percent) was subtracted from the amount of radioactivity in the suspending fluid after rotation of the platelet suspension in the collagen-coated tubes. Then the difference between these values was expressed as a percentage of the total radioactivity in the platelet suspension. There was considerable variation in the extent of adherence from one platelet suspension to another, although with any one suspension the 5 replicate values were very similar for the control samples, as well as for the samples containing the drug under study. Therefore, the results of experiments with different platelet suspensions were not meaned. Results of typical experiments are shown, and the number of experiments is recorded. In each experiment each value represents the mean of 5 replicates and their standard error.

Preparation of collagen-coated segments of glass rods

Glass tubing (external diameter 7 mm) was cut into 1 cm segments, washed as described above for the glass tubes, and 4 or 5 segments were mounted on metal probes. The segments were separated by rubber 0 rings. The assembled segments were immersed in acid soluble collagen for two min and then rinsed in modified Tyrode solution (no calcium, magnesium or glucose) for 30 sec at room temperature. The segments of collagen-coated glass were placed in a humid atmosphere for 15 min before use.

Preparation of damaged segments of aortas

Immediately after exsanguination, the chest and the abdomen of the rabbits were opened to expose the aorta from the arch to below the diaphragm. The abdominal aorta was transected, the aortic arch was incised and the aorta rinsed with 50 ml of modified Tyrode solution. Two forms of injury were used to damage the aorta and expose the subendothelial tissue.

(1) Damaging the aorta by scraping

The aortas were removed from the animals, most of the fat and connective tissue was removed, and the vessels were everted on a piece of polyethylene tubing (external diameter 3.6 mm). The endothelial surface of the vessel wall was damaged by scraping it with a scalpel blade. Approximately 0.5 cm was trimmed and discarded from each end of the everted aorta because this region tended to be extensively damaged by handling. The damaged aortas were kept at 22°C in the Tyrode-albumin medium or in Eagle's medium until they were immersed in the suspension of labeled platelets. The aortas were cut into

1 cm pieces (segments) on the supporting rods before rotation in the platelet suspension. Usually five pieces were obtained but the number varied from 4 to 7. In some experiments, several aortas were used and the segments were exposed in groups (4 to 7 per group) to the platelet suspension; a fresh aliquot of the suspension was used for each group.

(2) Damaging the aorta with a balloon catheter

In each rabbit, a balloon catheter (5F, Edwards Laboratories, Santa Ana, California) was introduced through the incision in the arch of the aorta and pushed down below the diaphragm. The balloon was inflated with 0.7 ml of air (about 450 mm of mercury) and the catheter was withdrawn. This procedure was repeated 5 times to ensure complete removal of the endothelium. The aortas were then rinsed with 50 ml of modified Tyrode solution, removed from the animals and kept at 22°C in Tyrode albumin medium or in Eagle's medium. The "ballooned" aortas were everted, mounted on metal rods and treated in the same way as the scraped aortas.

Adherence of platelets to collagen-coated rods or to damaged aorta segments

Adherence at 37°C over a 10 minute period was studied by rotating the rods in 10 ml of the suspension of ^{51}Cr-labeled platelets. In the early experiments, rotation was done manually at 280 rpm (direction of rotation being changed every 0.6 seconds; in later experiments, rotation was done mechanically in one direction at speeds ranging from 100 to 200 rpm).

Adherence of platelets to the surfaces was measured using a rotating probe device designed in collaboration with Dr. I.A. Feuerstein and built by Mr. J. Newton (Department of Chemical Engineering, McMaster University). The device consisted of two metal probes rotating simultaneously in 2 test tubes of 1.4 cm internal diameter, each containing 10 ml of platelet suspension at 37°C. The speed of rotation was measured by a tachometer and controlled by a rheostat. The probe was fitted with collagen-coated glass segments or an everted segment of rabbit aorta. The fluid dynamic characteristics of the system have been described elsewhere (Feuerstein et al., 1975). Adherence at 37°C over a 10 minute period was studied by rotating the probes at 200 rpm in 10 ml of a suspension of ^{51}Cr-labeled platelets. After rotation in the platelet suspension, the collagen-coated glass segments or the aorta segments were rinsed in fresh modified Tyrode solution by rotation at 50 rpm for 15 seconds, and removed from the probes. In more recent experiments the rinsing procedure was modified; the segments of aorta or collagen-coated glass were rotated in fresh modified Tyrode solution containing 10 mM EDTA, pH 7.35 at 200 rpm for 15 minutes. This was done to ensure that only single platelets and not platelet aggregates were adherent to the surface. When EDTA was used in the rinsing solution, a footnote stating this has been added to the tabulated data.

Calculation of number of adherent platelets

The radioactivity of the collagen-coated glass or aorta

segments was measured in a well-type gamma-scintillation counter. The radioactivity of 0.1 ml samples of the platelet suspension and of the supernatant fluid from the platelet suspension was measured. The radioactivity of the platelets was obtained by subtraction. The number of platelets per cubic millimeter of platelet suspension was counted and the counts per minute per 10^6 platelets were calculated. The surface area of the aorta segments or glass rods was determined from their length and diameter. Using the measured radioactivity of each segment the counts per minute per square millimeter was calculated and the number of platelets which this number of counts per minute represented was determined. Values given in the tables are means ± standard errors. Significance of the difference between experimental and control values was calculated using an unpaired two-tailed t-test.

For some experiments with ballooned aortas, the significance of the effect of drugs on platelet adherence was assessed by Professor M. Gent (Department of Clinical Epidemiology and Biostatistics, McMaster University) using an analysis of variance technique. The number of platelets adherent to different segments (proximal or distal) was examined but no significant difference was found. Likewise, in any one experiment, there was no significant difference in the number of platelets adherent to segments from different rabbits. Any variations in adherence that occurred from experiment to experiment are probably attributable to differences in the reactivity of the platelets

Inhibition studies

The compound to be tested or its appropriate solvent (as a

control) was added to the platelet suspension and incubated for 10 minutes at 37°C before the addition of red cells at a desired hematocrit. Final concentrations of the platelets and of the compounds are given in the tables. The platelet count was expressed as the number per mm^3 of final suspension, whether or not there were red cells present in the suspension.

Platelet suspension

Platelet aggregation was studied by a turbidimetric method (Greenberg et al., 1975) using 1 ml aliquots of platelet suspension. The solution of inhibitor to be tested (0.1 ml) or the appropriate solvent was added to a prewarmed (5 min) sample of suspension and the mixture was incubated for 30 sec - 10 min. It was transferred to the aggregometer, stirred at 37°C and 0.1 ml of the aggregating agent was added. The aggregating agents were: ADP, sodium arachidonate, thrombin or collagen.

Release of labeled serotonin

Labeled serotonin released from prelabeled platelets was measured in the supernatant fluid obtained by centrifuging the platelet suspension in an Eppendorf centrifuge 4 minutes after the addition of an aggregating agent. The release of ^{14}C from [^{14}C]5-HT prelabeled platelets was measured as described previously (Jenkins et al., 1971).

Malondialdehyde formation

Malondialdehyde (MDA) formed by the platelets was assayed by the thiobarbituric acid method of Smith et al. (1976) and the optical

density at 532 nm measured. A standard curve was constructed using malondialdehyde bis-(dimethyl acetal) (Aldrich Chemical Company Inc., Milwaukee, Wisconsin) dissolved in a mixture of equal parts absolute alcohol and 0.2 N HCl. Dilutions were made using platelet suspending fluid and handled in the same way as for the estimate of MDA in platelet suspensions.

Electron microscopy

Samples for electron microscopy were prepared by shadow casting. A tube was coated with collagen, rinsed, exposed to a suspension of platelets, and rinsed in the usual manner. The platelets adherent to collagen were fixed with chilled 1 percent osmium tetroxide in 0.1 M phosphate buffer (pH 7.4), washed, dehydrated in increasing concentrations of alcohol, and air dried. The tube was filled with 5 percent parlodion solution in isoamyl acetate; the solution was removed and the tube drained and dried at room temperature for 18 hours. The thick film of plastic that formed on the tube surface was removed, cut into small squares, and placed in a grid that had been precoated with cellulose acetate dissolved in chloroform. A single evaporating source was used for shadowing and coating; platinum wire was coiled over a carbon tip (1 mm diameter) on the end of a carbon rod (6 mm diameter). The platinum was evaporated onto the grid at an angle of 30° and then the carbon was evaporated at an angle of 90° to form a layer 600 Å thick. The grid was placed on several layers of filter paper and the original plastic film was removed by dissolving it with isoamyl acetate. The specimens were examined by electron microscopy.

Samples for scanning electron microscopy were fixed in 2.5 percent glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2, 340 mOsm.) for 3 hours at 4°C and postfixed in 1 percent osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hour at 4°C. The samples were dehydrated in increasing concentrations of alcohol then in acetone and finally air dried. They were coated with a 3 Å layer of gold-palladium alloy and examined with a AMR 1000 scanning electron microscope.

Modifications of platelet surface by various treatments

(1) Thrombin

In early experiments rabbit platelets were treated with a low concentration of bovine thrombin as described by Reimers et al. (1973b). For the more recent experiments, the preparation of thrombin-treated platelets used was that described by detail by Kinlough-Rathbone et al.

(1975). In the second method, platelet aggregation and release were induced with a high concentration of bovine or purified rabbit thrombin (0.5 units/ml). Upon formation of large aggregates, PGE_1 (10 μM) and plasmin (0.025%) were added to the reagent tube. Plasmin destroys the fibrin which forms around the thrombin-treated platelets so that the platelets can be recovered more easily. PGE_1 has been shown previously to enhance deaggregation after thrombin-induced aggregation (Reimers et al., 1973b). After 30 minute incubation at 37°C, TAMe (1 mM) and SBTI (0.025%) were added as inhibitors of thrombin and plasmin respectively. The platelets were washed once in fresh calcium-free Tyrode-albumin (0.35%) containing heparin and a small amount of rabbit serum as a source of "heparin cofactor" to neutralize any thrombin still remaining with the platelets. These platelets were finally resuspended in Tyrode-albumin containing apyrase and 5 mM Hepes buffer to maintain the pH of the medium at about 7.3. [Thrombin-treated platelets produce about twice as much lactic acid as untreated control platelets (Reimers et al., 1973b)]. Control suspensions were prepared in the same way except that instead of thrombin, Tyrode solution was added to the original platelet suspension.

(2) <u>Plasmin</u>

Rabbit platelets were treated with plasmin, obtained from 3 different sources, in the first washing fluid for 30 minutes at 22°C. The concentrations of the various plasmin used were: 0.05% pig plasmin Novo or 0.1 mg/ml pure rabbit plasmin (obtained by activating plasminogen

with urokinase) or 0.75 CU/ml of pure human Kabi plasmin. The platelets were recovered and resuspended using PGE_1 (10 μM) and SBTI (0.025%) by methods similar to those used with thrombin-treated platelets.

(3) Chymotrypsin

Platelets were treated with purified chymotrypsin as described by Greenberg (personal communication). Platelets were incubated in the second washing fluid (platelet count adjusted to 1,000,000 per mm^3) with chymotrypsin (10 units/ml) for 30 minutes at room temperature. Then, SBTI (0.5 mg/ml) and PGE_1 (100 μM) were added for 10 minutes. Platelets were washed once in calcium-free Tyrode albumin and resuspended finally in Tyrode solution containing 4% albumin, apyrase and 5 mM Hepes buffer to maintain the pH at 7.3. Control suspensions were prepared in the same way except that instead of chymotrypsin, 0.85% saline was added to the platelet suspension.

(4) Neuraminidase

Platelets were treated with neuraminidase as described by Greenberg et al. (1975). In platelet suspensions from rabbits the platelet count was adjusted to 2,000,000 platelets per mm^3 in the second washing fluid and to 700,000 platelets per mm^3 in the final suspension. Glucose was omitted from the second washing fluid to facilitate sialic acid determinations. When the platelets had been resuspended in the second washing fluid, it was divided into two parts: purified neuraminidase (in amounts ranging up to 0.01 unit per ml) was added to one half; an equal volume of 0.1 M acetate buffer (pH 5.5) was added to the other half as a control. Both samples of platelet suspension were incubated for up to 30 minutes. The platelets were

recovered by centrifugation, and the supernatant fluid was saved for sialic acid determinations. The platelets were suspended in Tyrode's solution containing 0.35 percent albumin. Values for total platelet sialic acid (before neuraminidase treatment) and for sialic acid removed by neuraminidase treatment (sialic acid was not released from platelet granules by neuraminidase treatment) were measured after sulfuric acid hydrolysis as free N-acetylneuraminic acid using the thiobarbituric acid assay of Aminoff (1961).

(5) Sodium periodate

Platelets from two rabbits were washed once and resuspended in 40 ml of washing fluid from which glucose was omitted. This suspension was divided into four 10 ml parts: 1 ml of $NaIO_4$ was added to each of the three parts; an equal volume of isotonic saline was added to the fourth part as a control. The final concentrations of $NaIO_4$ ranged from 0.1 to 1 mM; the final pH was 7.1 to 7.3. All four samples of platelet suspension were gently mixed and incubated at 22°C for 10 minutes. The reaction was terminated by adding 0.1 ml of a 1 M glucose solution to quench the $NaIO_4$. The platelets were washed once and resuspended in Tyrode-albumin with apyrase.

Sodium periodate and sodium borohydride

Four 10 ml samples of a platelet suspension were incubated with $NaIO_4$ as described above. The platelets in each sample were recovered by centrifugation and resuspended in 10 ml of washing fluid. One milliliter of $NaBH_4$ was added to each of the three samples of

resuspended platelets, and an equal volume of isotonic saline was added to the fourth sample as a control. The final concentrations of $NaBH_4$ ranged from 0.5 to 5 mM; the final pH was 7.5 to 7.6. All four samples were gently mixed and incubated at 22°C for 30 minutes. The platelets were then centrifuged and resuspended in Tyrode-albumin with apyrase as described above.

Platelet survival

The in vivo survival of washed rabbit platelets modified by various treatments was measured by a modification of the method of Reimers et al. (1973a) using platelet suspensions labeled with ^{51}Cr. Control experiments were done with platelets that had not been treated, but had been subjected to the same washing and resuspending procedures. The radioactivity of samples of whole blood was determined by the method of Aster and Jandl (1964).

Preparation of antiserum to rabbit platelets

The antiserum was raised in sheep and was kindly provided by Dr. J. Gauldie, Department of Pathology, McMaster University. Approximately 200 ml of rabbit blood, pooled from six animals, was collected into 2% EDTA-0.33% saline (9 parts of blood to 1 part anticoagulant). Platelet-rich plasma was centrifuged at 2300 g for 15 min and the platelet pellet was resuspended in 5 ml of 0.85% saline at a platelet count of 5,000,000/mm^3. The platelets were disrupted by sonication and the sonicate was stored at -20°C in 0.1 ml aliquots. For injection, an aliquot was thawed, mixed with 0.1 ml of complete Freund's adjuvant, and injected subcutaneously into a

sheep; this was repeated once a week for 6 weeks. At 7 weeks, the sheep was bled and the serum was prepared, heated for 30 min at 56°C to destroy complement, and stored at -20°C. The presence of antiplatelet antibody was confirmed by the formation of a precipitin line against a sonicated rabbit platelet suspension on an Ouchterlony plate.

Production of thrombocytopenia

Thrombocytopenia in rabbits was produced by whole blood irradiation with a cesium source (930 rad, 465 rad on each side for 14.5 min). During irradiation, both sides of the necks of the rabbits were shielded with a lead ribbon to prevent radiation damage to the blood vessels. The rabbits were given injections of 1 ml penicillin-streptomycin (Derapen-C, with dihydrostreptomycin, Ayerst Laboratories, Montreal Canada) 24 and 48 hr after irradiation. Seven days later the rabbits received an intravenous injection of 0.3 ml of sheep antiserum to rabbit platelets, 12 to 16 hr before the bleeding time studies were to be done.

Platelet counts and bleeding time determinations

One hour before the bleeding time studies, samples of blood were taken from the ear veins of the thrombocytopenic rabbits and the platelets were counted by the method of Brecher and Cronkite (1950). Platelets that had been modified with various treatments (or control platelets) were then injected intravenously into the thrombocytopenic rabbits. Each rabbit received 10^{10} platelets in a volume of 5 ml (the weights of the rabbits were similar (mean ± S.E.M., 2527 ± 47 g).

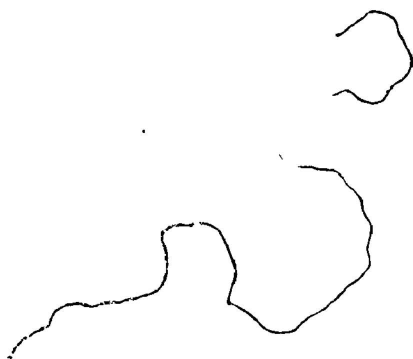
Thirty minutes later the rabbits were anesthetized with sodium pentobarbital, the jugular veins were exposed and each was arranged above a small trough. Blood samples were taken by cardiac puncture for platelet counts and for measurement of the percentage recovery of ^{51}Cr-labeled platelets injected. Then a puncture wound was made in a jugular vein with a 23 gauge needle. The wound site was observed until bleeding ceased and the time was recorded. To prevent accumulation of blood at the wound site, a flow of 0.85% saline was directed into the trough below the exposed jugular vein. The bleeding time was then measured again in the same way by puncturing the other jugular vein.

Statistical considerations. In experiments with thrombocytopenic rabbits the relation between the platelet count and the bleeding time has been found to resemble an exponential function (Hirsh, J., and Senyi, A.F., unpublished observation). Therefore, the bleeding time values were transformed to logarithms before determining the mean for each experiment. The significance between any two of the three means was determined by a two-way analysis of variance. This analysis also provides the best common estimate of the standard deviation. This was then used to develop the 99% confidence intervals for each of the two treatment means and the control mean. The calculated means and confidence intervals from the transformed data were then transformed back to the original scale.

CHAPTER THREE

RESULTS

I. PLATELET ADHERENCE TO COLLAGEN-COATED GLASS SURFACES



A. DEVELOPMENT OF A METHOD FOR MEASURING PLATELET ADHERENCE TO COLLAGEN-COATED GLASS TUBES

The results reported with collagen-coated glass tubes were obtained while I was working in the laboratory of Dr. M.A. Packham at the University of Toronto. This earlier work was continued in the laboratory of Dr. J.F. Mustard at McMaster University where methods of measuring platelet adherence to collagen-coated glass rods and to everted aortas were developed.

1. Adherence of platelets to a collagen-coated glass tube

Previous work (Packham et al., 1969; Jenkins et al., 1973) has shown that it is possible to coat glass tubes with proteins and to measure the number of radiolabeled platelets adherent to these surfaces.

The first experiments with collagen-coated glass surfaces were an extension of these early studies and involved the use of glass tubes coated with acid soluble collagen. Suspensions of ^{51}Cr-labeled platelets, or ^{14}C-serotonin labeled platelets were placed in the tubes and the tubes were rotated for a fixed time, rinsed, and the radioactivity that remained adherent to the tubes was measured (Cazenave et al. 1973b). A number of the conditions required for quantitative measurements of platelet adherence to the surface were established in these early studies. Therefore, a summary of these experiments will be presented as an introduction to the results obtained after the method was modified to measure adherence of platelets to collagen-coated glass cylinders rotated in a platelet suspension. The method was later adapted to the measurement of platelet adherence to the subendothelial structures of the everted rabbit aorta (Cazenave et al. 1975).

(a) Collagen-coated surface

When a glass surface was exposed to acid soluble collagen, and rinsed with Tyrode solution, fibrils of polymerized collagen adhered to the surface and could not be removed by further rinsing. Electron microscopic examination of the fibrils showed typical 640 Å cross-striations. These fibrils were destroyed by collagenase treatment (Cazenave et al. 1973b).

Platelets did not adhere to collagen-coated tubes that had been incubated (30 minutes, 37°C) with collagenase (Type III, Sigma Chemical Co., 60 μg per milliliter in a modified Tyrode solution containing 5 mM calcium, but no magnesium, pH 7.4). These tubes were rinsed twice with a modified Tyrode solution (no calcium or magnesium) before addition of the platelet suspension (mean adherence: uncoated tube - 2,800 per mm^2 collagen-coated tube - 58,800 per mm^2 collagen-coated, collagenase-treated tube - 3,900 per mm^2.

(b) Comparison of adherence of platelets from pigs, rabbits and humans

The adherence to the collagen-coated tube was similar with platelets, labeled with ^{14}C-serotonin from pigs, rabbits, or humans (Table 1). Because pig blood was most readily available in large amounts, most of the earlier experiments were done with pig platelets.

With pig platelets, it was noticed that occasionally some batches of platelet suspension would not adhere to the collagen-coated tubes. This was usually associated with platelets prepared from blood

TABLE 1

Comparison of adherence to a collagen-coated glass tube of washed platelets from pigs, rabbits, and humans.

No. of experiments	Platelet label	Species	Number of platelets per mm^2 (mean ± S.E.)	Percent of total radioactivity released (mean ± S.E.)
70	^{14}C-5HT	Pig	49,300 ± 3,600	1.7 ± 0.2
4	^{14}C-5HT	Rabbit	58,500 ± 18,500	2.4 ± 1.5
9	^{14}C-5HT	Human	50,700 ± 9,800	0.8 ± 0.3

that has been difficult to collect at the slaughterhouse. The results from experiments in which the adherence of the control platelets was less than 5,000 per mm^2 were discarded. This value is about 10% of the adherence of control platelets prepared from most of the blood samples.

(c) Comparison of radioactive labeling methods

Pig platelets were washed and resuspended in Tyrode solution containing 0.35% albumin and apyrase. Samples of platelet suspension were transferred to collagen-coated tubes and the tubes were rotated end-over-end for 10 minutes in most experiments. Apyrase was included in the suspending medium to degrade any ADP lost from the platelets and thus to prevent aggregation and to prevent the contribution that the synergism with ADP would make to the release reaction induced by collagen (Packham et al. 1973).

Single platelets adhered to the collagen fibers. Microscopic observation of collagen-coated glass surfaces that had been exposed to platelets and of the residual platelet suspension after exposure showed that aggregation of the platelets had not occurred on the surfaces or in the platelet suspension (Figure 2). Thus the method made it possible to measure platelet adherence to collagen in the absence of platelet aggregation.

(i) Labeling Pig Platelets with $[^{14}C]$-5HT, ^{51}Cr or $[^{3}H]$-DFP

To ascertain that the labeling procedure had no effect, under the conditions used, on the adherence of platelets to collagen, three

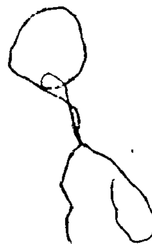


FIGURE 2.

Electron micrograph of washed pig platelets adherent to a collagen-coated glass surface. Shadow casting technique x 4200.

methods of labeling platelets were evaluated. Pig platelets were labeled in suspension with ^{14}C-serotonin, which is transported actively into the amine storage organelles and is releasable with the contents of the amine storage organelles (Holmsen et al., 1973). ^{51}Cr (as sodium chromate) is the label most commonly used in the study of platelet survival. The chromate ion is transported across the platelet membrane possibly by an active mechanism (Tsukada and Steiner, 1970) and probably reduced inside the cell to the chromic state. In human platelets, 20 percent becomes bound to platelet stroma (Aster and Enright, 1969) and 80 percent binds to low molecular weight, dialyzable compounds, possibly nucleotides (Steiner and Baldini, 1970). Kattlove and Spaet (1970) have reported inhibition of platelet function with concentrations of chromate greater than 10 μg/ml. In the present experiments, the final concentration of chromate used for labeling platelets was about 0.1 μg/ml. DFP irreversibly labels the active serine group of serine esterases and inactivates these enzymes. A review of the factors affecting platelet labeling with all these isotopic labels has been given by Aster (1971).

The adherence of radioactivity to the collagen-coated surface was similar when washed pig platelets were labeled with [^{14}C]-5HT or ^{51}Cr or [^{3}H]-DFP. Under these conditions very little radioactivity appeared in the supernatant fluid, indicating very little or no release of the platelet granule contents or loss of cytoplasmic or membrane components (Table 2).

TABLE 2

Comparison of radioactive labeling methods for estimating adherence to a collagen-coated glass tube of washed platelets from pigs.

No. of experiments	Platelet label	Species	Number of platelets per mm^2 (mean ± S.E.)	Percent of total radioactivity released or lost (mean ± S.E.)
70	^{14}C-5HT	Pig	49,300 ± 3,600	1.7 ± 0.2
25	^{51}Cr	Pig	58,200 ± 6,400	3.2 ± 0.2
6	^{3}H-DFP	Pig	51,500 ± 5,600	4.7 ± 0.3

Significance of difference between means:

adherence ^{51}Cr vs. ^{14}C-5HT, $p < 0.15$
^{51}Cr vs. ^{3}H-DFP, $p < 0.3$
^{3}H-DFP vs. ^{14}C-5HT, $p < 0.3$

In an experiment in which platelets were labeled with both ^{14}C-serotonin and ^{51}Cr, adherence to the collagen-coated surface was 57,400 ± 5,600 per mm^2 measured with ^{51}Cr-labeled platelets (radioactivity lost into the suspending fluid being 3.3 ± 0.4 percent) and 53,200 ± 3,900 per mm^2 measured with ^{14}C-serotonin-labeled platelets (radioactivity released being 2.7 ± 0.1 percent). These results and those reported in Table 8 (experiment 2; 22°C) excluded the possibility that the ^{14}C-serotonin-labeled platelet adherent to collagen might release ^{14}C-serotonin which could then be taken up by platelets still in suspension. These results showed that when platelet adherence was measured at 22°C, there was not much release and thus ^{14}C-serotonin could be used as a platelet label.

By this method, adherence of radioactively labeled platelets to collagen-coated surfaces could be measured in the presence of physiologic concentration of divalent cations (2 mM Ca^{2+}; 1 mM Mg^{2+}), in an artificial medium and the composition of the suspending medium varied. Various plasma proteins could be added and anticoagulants were avoided.

The functions and properties of the platelets are similar to those of platelets in plasma (Ardlie et al. 1971; Mustard et al. 1972b); Doery et al. 1973; Kinlough-Rathbone et al. 1977). Washed rabbit platelets survive normally when injected into rabbits (Reimers et al. 1973a).

(ii) Adherence of Free ^{14}C-serotonin and ^{51}Cr to Collagen

The adherence of ^{14}C-serotonin in platelet-free suspending

fluid (32,300 c.p.m. per milliliter) to a collagen-coated surface was 0.8 ± 0.1 percent of the total counts per minute. With ^{51}Cr (as sodium chromate) in platelet-free suspending fluid (5,300 c.p.m. per milliliter), the adherence was 0.6 ± 0.2 percent. In contrast, when a platelet suspension was tested in which the platelets were labeled with ^{14}C-serotonin (29,800 c.p.m./ml of platelet suspension) the percent adherence was 17.6 ± 1.3%; when they were labeled with ^{51}Cr (4,800 c.p.m./ml of platelet suspension) the percent adherence was 20.8 ± 2.3%. It was concluded that these radioactive compounds did not adhere significantly to the collagen-coated surface if they were not contained within platelets.

(iii) Comparison of Changes in Platelet Count and Radioactivity

The number of platelets remaining in the suspension corresponded to the percentage of radioactivity remaining in the platelet suspension after rotation. It was found that the extent of adherence was 12.6 ± 0.7 percent, whereas the decrease in the number of platelets and in the radioactivity of the platelets suspension were 24.9 and 26 percent, respectively. These observations indicate that in this type of experiment about half of the platelets that become adherent to the collagen-coated surface are loosely bound and are dislodged by the standard rinsing procedure. The possibility that the results were due to release of ^{14}C-serotonin from platelets adherent to the collagen and reuptake by platelets in suspension seems unlikely, because in a later experiment (Table 8) in which platelets were doubly labeled with

TABLE 3

Adherence of pig platelets labeled with ^{14}C-serotonin to collagen-coated tubes: effect of repeated transfers of the platelet suspension to fresh tubes.

No. of 10 minute periods of rotation[a]	Radioactivity in platelet suspension before rotation		Radioactivity[b] adherent to collagen-coated tubes	
	c.p.m./ml	Percent of initial radioactivity	c.p.m.	Percent of ^{14}C added to tube
1	23,722	100	1,611 ± 33	6.8 ± 0.1
2	19,550	82.4	1,462 ± 175	7.4 ± 0.7
3	15,940	67.2	1,248 ± 170	7.8 ± 0.7
4[c]	14,070	59.3	870 ± 148	6.2 ± 0.6

a) One milliliter of platelet suspension rotated at 15 r.p.m. in a collagen-coated tube for 10 minutes at 22°C removed to a fresh tube, and rotated again, etc.

b) Radioactivity adherent to collagen-coated tubes after 4 rinsings with Tyrode solution. Loosely adherent platelets are removed because the drop in radioactivity of the platelet suspension during the first rotation is 100 - 82.4 = 17.6 percent, whereas only 6.8 percent of the radioactivity remains adherent after rinsing. (Release less than 1 percent).

c) During the fourth rotation period about 13 percent of the radioactivity was released, probably indicating that after this much manipulation considerable platelet damage has occurred.

^{14}C-serotonin and ^{51}Cr, both labeling methods gave the same estimate of the number of adherent platelets at 22°C.

(iv) Effect of Repeated Transfers to Collagen-Coated Tubes

The possibility that a selected population of pig platelets adhere to the collagen was considered. An experiment was done to investigate whether, after 10 minutes of rotation, any platelets that could adhere to collagen remained in the suspension. The platelet suspension was transferred, after a 10 minute rotation, to a fresh collagen-coated tube and the rotation procedure repeated. This procedure was repeated twice more. The percentage of platelets adhering was approximately the same in each collagen-coated tube (Table 3), although after 4 transfers and rotation procedures, the amount of released radioactivity rose to 13 percent, probably indicating platelet damage.

2. Establishment of optimum conditions for assessment of platelet adherence to collagen-coated-tubes

(a) Effect of Apyrase

The effect of different concentrations of apyrase in the platelet suspending medium was investigated with ^{51}Cr-labeled pig platelets. There was no statistically significant difference in the values for adherence with the different concentrations of apyrase (Table 4). If, however, apyrase were omitted from the suspending medium, aggregation was observed and hence adherence of single platelets could not be measured. An apyrase concentration of 2 µl per milliliter of platelet suspension was chosen for further experiments. This

TABLE 4

Effect of apyrase on the adherence of washed pig platelets labeled with ^{51}Cr to collagen-coated tubes.

Apyrase (μl/ml)	Number of platelets per mm^2 (mean ± S.E.)
2	58,600 ± 5,600
5	42,300 ± 3,400
10	56,600 ± 2,500
20	43,400 ± 4,500
50	37,800 ± 12,900

Each value was the mean of 5 replicates.

concentration of apyrase was chosen because it blocked platelet aggregation induced by 2 μM ADP. Thus the apyrase would degrade any ADP lost from the platelets and prevent any synergism between the ADP and collagen in inducing the release reaction (Packham et al. 1973).

(b) Effect of Platelet Count

The effect of the number of platelets in the suspending medium was examined with ^{14}C-serotonin-labeled pig platelets. The number of platelets adhering to the collagen-coated surface was directly proportional to the concentration of platelets in the suspending medium (Table 5). However, when the platelet count was above 700,000 per cubic millimeter, aggregates were sometimes observed. Therefore, to ensure that adherence of single platelets was being studied, a platelet count of 700,000 per cubic millimeter was chosen for further experiments with the collagen-coated tubes. (The optimum platelet count was also determined for the other procedures used to measure platelet adherence (see section IB)).

(c) Effect of Rate of Rotation

The effect of the rate of end-over-end rotation of the collagen-coated tubes containing platelet suspensions was investigated over a range of 15 to 120 r.p.m. (Table 6). Aggregation was never observed at 15 r.p.m. but at speeds above 15 r.p.m. aggregation was often observed. At speeds at which aggregation occurred, adherence was greater but was undoubtedly the result of combined aggregation and adherence. Little release of radioactivity occurred at 15 r.p.m. but slightly more release was observed at higher rotation speeds. Therefore,

TABLE 5

Effect of platelet count on the adherence of washed pig platelets with ^{14}C-serotonin to collagen-coated tubes.

Number of platelets per mm^3 in suspending medium	Number of platelets per mm^2 (mean ± S.E.)
300,000	6,700 ± 300
500,000	14,600 ± 800
700,000	21,600 ± 300
900,000	30,800 ± 800

Each value was the mean of 5 replicates.
During rotation of the platelet suspension, ^{14}C radioactivity released from the platelets was less than 3 percent at all platelet counts.

TABLE 6

Effect of rate of rotation of washed pig platelets labeled with ^{14}C-serotonin on their adherence to a collagen-coated tube.

Speed of rotation (r.p.m.)	No. of samples examined	No. of samples containing aggregates	Number of platelets per mm^2 (mean ± S.E.)	Percent of total ^{14}C released
15	25	0	32,800 ± 5,600	0.2
30	20	15	67,800 ± 4,500	0.6
60	25	15	59,400 ± 5,900	0.6
120	20	12	40,900 ± 4,500	1.1

a rotation speed of 15 r.p.m. was chosen as the standard method.

(d) Effect of Time of Rotation

The length of time the platelets were rotated at 15 r.p.m. affected the extent of adherence (Table 7). Because of the possibility of loss of ^{14}C-serotonin from the platelets adherent to the collagen-coated surface, during the longer periods of rotation and reuptake of this ^{14}C-serotonin by platelets still in suspension, the experiment was done with ^{51}Cr-labeled platelets. With these platelets, the extent of adherence increased up to 10 minutes, but did not change appreciably with longer times of rotation (Table 7). Therefore, a rotation time of 10 minutes was chosen as the standard method. Microscopic examination of the platelets adhering to the collagen-coated surface after 10 minutes of rotation showed that the surface was far from being completely covered by the platelets. Most of the platelets appeared to be adherent to the collagen fibers rather than to the spaces between them. Platelets adhere to collagen as spiny elements with long pseudopods apparently attached to the collagen fibers (Figure 2). Thus, it is not possible to ascertain the number of platelets that would form a monolayer on the collagen fibers.

(e) Effect of Temperature

The extent of platelet adhesion to the collagen-coated surface was least at 5°C and greatest at 37°C. Because of the possibility that temperature might affect release or reuptake of ^{14}C-serotonin an experiment was done with platelets doubly labeled with ^{14}C-serotonin and ^{51}Cr. The extent of adherence was similar with both isotopes.

TABLE 7

Effect of length of time of rotation on the adherence of washed pig platelets labeled with ^{51}Cr to a collagen-coated tube.

Time of rotation (min)	Number of platelets per mm^2 (mean ± S.E.)
1	11,500 ± 600
2	21,000 ± 800
5	36,700 ± 1,100
10	51,200 ± 2,000
20	48,700 ± 300
30	40,000 ± 11,200

Each value was the mean of 5 replicates.

There was no aggregation at 5°C or 22°C, but aggregates were occasionally observed at 37°C. There was more release from the platelets at 37°C than at the lower temperature (Table 8). Although 37°C is a physiologic temperature and increasing the concentration of apyrase would have avoided platelet aggregation, this temperature was not chosen for the initial experiments reported with collagen-coated glass tubes. This choice was made for convenience because of the lack of a readily available temperature controlled room at 37°C.

(f) Effect of pH

The effect of the pH of the suspending medium on the adherence of ^{14}C-serotonin-labeled pig platelets was studied in 4 separate experiments. At pH values of 5.7 and 6.5, adherence was much lower than at pH 7.35. Tha amount of release was no greater than 2 percent except at pH 8.0 where the extent of release was 4.0 percent and aggregates were observed after rotation (Table 9). Therefore, pH 7.35 was chosen as the standard condition.

(g) Effect of Divalent Cations

When pig platelets were suspended at pH 7.35, in a medium containing magnesium (1 mM) but no calcium, the extent of adherence of the platelets to the collagen-coated surface was less when compared with the adherence in a medium containing 2 mM calcium and 1 mM magnesium (Table 10). When EDTA (pH 7.3) was added to the suspending medium containing calcium and magnesium, the pH fell to 6.8 and very few platelets adhered to the collagen-coated surface. Therefore, experiments

TABLE 8

Effect of temperature on the adherence of washed pig platelets to a collagen-coated tube.

Experiment	Temperature (° C.)	Platelet label	Number of platelets per mm^2 (mean ± S.E.)	Precent of total radioactivity released or lost
1[a]	5	^{14}C-5HT	2,800 ± 300	0.5
	22	^{14}C-5HT	19,000 ± 5,600	3.6
	37	^{14}C-5HT	29,700 ± 3,400	5.3
2[b]	5	^{14}C-5HT	14,800 ± 1,700	0.0
		^{51}Cr	9,800 ± 2,000	0.2
	22	^{14}C-5HT	64,700 ± 1,400	2.4
		^{51}Cr	66,400 ± 3,100	2.3
	37[c]	^{14}C-5HT	70,300 ± 10,100	5.0
		^{51}Cr	93,200 ± 2,500	7.4

a) Each value is the mean of 15 replicates.
b) Each value is the mean of 10 replicates.
c) A few small aggregates were seen by phase-contrast microscopy after rotation of the tubes.

TABLE 9

Effect of pH on the adherence of washed pig platelets labeled with ^{14}C-serotonin to a collagen-coated tube[a].

pH	Number of platelets per mm^2 (mean ± S.E.)	Percent of total ^{14}C-released (mean ± S.E.)
5.7	1,800 ± 100	0.08 ± 0.03
6.5	10,900 ± 600	1.1 ± 0.1
7.35	26,700 ± 1,700	2.1 ± 0.8
8.0[b]	43,100 ± 2,000	4.0 ± 0.7

a) Each value was the mean of 5 replicates. Typical of 4 experiments that gave similar results.

b) Platelet aggregation after rotation.

TABLE 10

Effect of EDTA or EGTA on adherence of washed pig or human platelets to a collagen-coated tube when the final pH of the suspension was adjusted to 7.3.

Species	(n)	Label	Ca^{2+} (mM) in suspending medium	EDTA (mM)	Number of platelets per mm^2 (mean ± S.E.)	Percent of total radioactivity released or lost (mean ± S.E.)
Pig	15	^{14}C-5HT	0	0	21,800 ± 2,500	5.4 ± 0.9
			2	0	55,400 ± 8,100	0.7 ± 0.5
Pig	15	^{14}C-5HT	2	0	61,300 ± 1,700	0.6 ± 0.1
			2	9	27,700 ± 800	0.4 ± 0.04
Pig	10	^{51}Cr	0	0	82,900 ± 3,400	3.7 ± 0.2
			0	9	18,500 ± 1,400	4.1 ± 1.9
			2	0	130,200 ± 4,800	4.0 ± 0.7
			2	9	31,600 ± 4,500	1.1 ± 0.2
Human	4	^{14}C-5HT	0	0	29,100 ± 8,700	0.2 ± 0.1
			0	9	800 ± 100	0 ± 0.1
			2	0	31,600 ± 3,400	0.7 ± 0.2
			2	9	800 ± 600	0.5 ± 0.5
				EGTA (mM)		
Pig	10	^{14}C-5HT	2	0	65,000 ± 5,000	1.2 ± 0.6
			2	9	17,900 ± 1,100	0.1 ± 0.03

The pH of the EDTA or EGTA to be added was adjusted to 8.8 so that after addition of the suspension that contained Ca^{2+} and Mg^{2+}, the final pH was 7.3.

were done in which the pH of the EDTA or EGTA to be added was adjusted so that after the chelating agent was mixed with the platelet suspension, the pH was 7.3 (Table 10). The presence of EDTA or EGTA did not completely prevent adherence of pig platelets to the collagen-coated surface, but the chelating agents greatly reduced adherence. The small amounts of radioactivity released or lost from platelets were also decreased by the presence of EDTA or EGTA. EDTA completely prevented the adherence of human platelets to the collagen-coated surface at pH 7.3 (Table 10).

To establish that EDTA was affecting the platelets rather than the collagen, collagen-coated tubes were filled with 9 mM EDTA solution at pH 7.35, allowed to stand for 10 minutes, and then rinsed 4 times with modified Tyrode solution (no calcium or magnesium). Adherence of ^{14}C-serotonin-labeled pig platelets in the usual medium containing 1 mM magnesium and 2 mM calcium was 41,200 ± 1,100 platelets per mm^2. The similarity of these values indicates that EDTA had little or no effect on the collagen-coated surface.

3. Discussion

The results of these experiments indicate that it is possible to quantitate platelet adherence to collagen-coated glass tubes. The absence of fibrinogen and the addition of apyrase to the suspension of washed platelets prevent aggregation, judged by microscopic examination of the collagen-coated surface and the platelet suspension. Under these conditions, with the use of apyrase, very little release of radioactivity from ^{14}C-serotonin-labeled platelets occurs.

When the platelet suspension was rotated for 10 minutes, increasing the concentration of apyrase in the suspending medium 25-fold did not reduce the extent of adherence. This observation indicates that in the standard medium containing a low concentration of apyrase, adherence of platelets to the collagen-coated surface was not caused or increased by ADP released or lost from the platelets into the medium. Platelets exposed to low concentrations of collagen in the presence of enzymes that convert ADP to ATP or AMP, undergo very little release (Packham et al. 1973). If extensive release is to occur when platelets are exposed to a low concentration of collagen, the synergistic effect of the combination of released ADP and collagen is required. When the contribution of any ADP released or lost from the platelets is prevented by the action of apyrase, low concentrations of collagen cause little release of granule contents (Packham et al. 1973; Kinlough-Rathbone et al. 1977). It appears, therefore, that ^{51}Cr-labeled platelets and ^{3}H-DFP-labeled platelets, the adherence was similar to that observed with ^{14}C-serotonin-labeled platelets, indicating that all these methods of platelet labeling provide a similar measure of the extent of adherence of platelets to collagen.

An experiment was done with platelets doubly labeled with ^{14}C-serotonin and ^{51}Cr to ensure that in the experiments with ^{14}C-serotonin, platelets adherent to the collagen did not release ^{14}C-serotonin which would be taken up by platelets still in suspension. At 22°C, the calculated values for adherence were the same with both

labeling methods. Therefore, it was concluded that release of ^{14}C-serotonin and reuptake had not occurred because if it had occurred, the estimate of adherence with ^{14}C-serotonin would have been lower than with ^{51}Cr, which does not undergo uptake. Furthermore, the decrease in the number of platelets in the platelet suspension after exposure to the collagen-coated surface corresponded to the decrease in the radioactivity in the platelet suspension when the platelets had been labeled with ^{14}C-serotonin.

^{51}Cr is not released from platelets at any temperature, and any that may be lost through platelet lysis is not taken up by other platelets. ^{51}Cr is mainly a cytoplasmic marker and it does not modify platelet function at the concentrations used in these experiments (Kattlove and Spaet, 1970). Loss of ^{51}Cr from platelets into suspending medium parallels loss of the enzyme lactic dehydrogenase and indicates platelet lysis (Kinlough-Rathbone et al. 1976a). Finally ^{51}Cr-labeled platelets can be used for in vivo studies. Thus, it was concluded that ^{51}Cr appears to be the best platelet label for the experiments. However, ^{14}C-serotonin can also be used, particularly when a measure of the amount of release of granule contents is required. The use of ^{3}H-DFP has several disadvantages. DFP is a powerful irreversible inhibitor of serine esterases. Thus, DFP can inhibit enzymes on the platelet surface which are involved in the platelet release reaction (Henson and Oades, 1976). High concentrations of DFP have been shown (Mustard et al. 1967b) to inhibit aggregation induced by thrombin, collagen and antigen-antibody complexes and to prolong primary hemostasis and

platelet survival. However, the concentration of DFP used to label the platelets was much lower than the concentrations that affect platelet functions.

Some of the platelets were removed from the collagen surface by the standard rinsing procedure, whereas others remained and must be assumed to be more tightly adherent. The method that has been developed measures these tightly adherent platelets. The method appears to be applicable to washed platelets prepared from pigs, rabbits, or humans. The conditions chosen were a platelet count of 700,000 per cubic millimeter, a rotation speed of 15 r.p.m. for 10 minutes, room temperature, and a pH of 7.35.

The reason for the very low percentage of the platelets that adhered at 5°C may be either that they were in an unsuitable configuration at that temperature, or that adherence requires an active metabolic process. These findings are in agreement with those of Turitto and Baumgartner (1974), who have demonstrated a marked decrease of platelet adherence to the subendothelium of a rabbit aorta when examined at 4°C. They suggested that the effect could be due to a diminution of platelet diffusivity or to a decrease in the rate of platelet attachment to the surface.

The finding that platelets which were capable of adhering to a surface freshly coated with collagen remained in suspension after 10 minutes of rotation indicates either that the amount of collagen on the surface was insufficient to bind all the platelets readily or

that under the conditions of rotation, only some of the platelets came in contact with the collagen in a manner which resulted in adherence. Although the extent of adherence of platelets to the collagen varied from one experiment to another, within each experiment the replicates were in close agreement with each other and indicated that the tube coating was fairly consistent. Thus the variation appeared to be dependent on the platelet preparations.

Because the collagen does not completely coat the glass surface the question arises whether some of the platelets are adhering to the glass. It was previously shown (Packham et al., 1969; Jenkins et al., 1973) that when washed platelets are exposed to uncoated glass surfaces very little adhesion occurs. If the surfaces are coated with albumin, the extent of adhesion is even less than to uncoated glass surfaces and is about 1,950 platelets per mm^2. Since the platelet suspensions used in the present experiments contained albumin which would probably coat any glass surface that was not coated with collagen, and the extent of adhesion to the collagen-coated surfaces averaged 44,800 to 75,600 platelets per mm^2, it is evident that the technique developed measures the adherence of the platelets to the collagen rather than to the (albumin-coated) glass. Additional evidence that the platelets were adherent to the collagen fibers rather than to the glass surface, was provided by the experiment in which collagenase was used to remove collagen from the surface. This treatment reduced the number of adherent platelets to the same values as observed with uncoated surfaces. Using a different test system and washed human platelets suspended in a

glucose-free medium containing EDTA, Lyman et al. (1971) have reported that albumin (45 to 50 mg per milliliter) supports adhesion of washed platelets in Ringer-EDTA to collagen fibers, whereas at 10 mg per milliliter, albumin had no apparent effect. The platelet-suspending medium contained albumin at a concentration of 3.5 mg per milliliter which would not be expected to enhance adherence of platelets to collagen, although it has a protective effect on the platelets in suspension (Mustard et al., 1972; Rossi, 1972; Doery et al., 1973; Tangen et al., 1973; Kinlough-Rathbone et al, 1977).

The omission of calcium from the platelet-suspending medium usually reduced the extent of adherence of platelets to the collagen-coated surface, but did not inhibit adherence completely. This observation indicates that calcium may have a role in the adherence of platelets to collagen.

The same effect could be achieved with EDTA or EGTA. When the divalent cations in the suspending medium were chelated with EDTA, adherence of platelets to the collagen-coated surface was strongly inhibited but the inhibition was not complete with pig platelets providing the pH was 7.3 in both media (with and without EDTA); with human platelets, EDTA inhibited adherence almost completely. With pig platelets there was a stronger inhibitor effect on the extent of adherence when EDTA was present at a lower pH (6.8). This is in keeping with the finding that less platelets adhere at low pH. This observation emphasizes the need to control the pH when EDTA is used as a chelating agent. Kinlough-Rathbone et al. (1973) have shown

that EDTA inhibits the collagen-induced release reaction of pig platelets; part of this effect could be caused by inhibition of platelet adherence to collagen. Baumgartner et al. (1971) have shown that chelation of calcium causes diminished adherence to the subendothelium. Our findings are in conflict with previous results reported that adherence of platelets to collagen, measured in an EDTA-containing system (Spaet and Lejnieks, 1969), is not calcium dependent. Washed platelets are used and they are suspended in a physiological, isoosmotic medium, at pH 7.35 and containing albumin. The suspending medium contains physiological concentrations of calcium and magnesium. Because apyrase is included in the suspending medium to degrade any ADP that is released or lost from the platelets, adherence occurs without demonstrable platelet aggregates forming in the suspending fluid or on the surface.

The principles of this method of studying platelet adherence to collagen allow the effect of drugs and other compounds to be studied in the absence of anticoagulants, in a medium containing calcium and magnesium that appear to be involved in adherence. In addition it is possible to alter the proteins and other constituents of the medium.

Quantitation of measurement of radioactivity is more rapid and less subject to error than counting the number of platelets, and the extent of release or loss of platelet constituents can be determined as well as adherence. The method has the additional advantage that adherence can be measured in the presence of divalent cations at physiological concentrations, because aggregation is avoided by the use

of apyrase rather than EDTA. In all of the other methods reported, adherence has been measured in the presence of EDTA (Hovig et al., 1968; Spaet and Lejnieks, 1969; Lyman et al., 1971; Gordon and Dingle, 1974; MacKenzie et al., 1974; Brass et al., 1976; Castellan and Steiner, 1976).

4. Summary

An isotopic method has been established to quantitate the adherence of washed platelets to collagen-coated glass surfaces. In these early studies a number of principles have been established and have laid the ground work for the development of new methods of measuring platelet adherence to collagen-coated glass rods or to subendothelium of aorta using a rotating probe device.

Using acid soluble collagen it is possible to coat a glass surface with fibrils of polymerized collagen (Elsdale et Bard, 1972) to which platelets will adhere. The adherence of platelets to the fibrils of collagen is specifically abolished if the collagen is hydrolysed by collagenase.

Adherence of labeled platelets to collagen can be quantitated over a large surface area by counting radioactivity.

The system can be set up so that it measures adherence to collagen. This involves selecting an appropriate platelet concentration, rotating speed and the presence of apyrase to degrade any ADP that is formed.

^{51}Cr is the label of choice and measures adherence to collagen-

coated surfaces without loss of the label into the suspending medium. ^{14}C-serotonin can also be used, but the conditions must be such that there is very little release when the platelets adhere to collagen.

Omission of calcium from the suspending medium or chelation of divalent cations with EDTA or EGTA inhibits strongly platelet adherence to collagen. These results suggest that methods that measure platelet adherence to collagen in the presence of EDTA are opened to question.

The platelet suspending medium can be modified readily. Proteins, drugs and compounds modifying platelet function can be added and tested for their effect on platelet adherence to collagen.

5. Limitations of the Method

Although the adherence of single platelets to a collagen-coated tube could be measured quantitatively and the effect of various drugs and inhibitors were studied (Cazenave et al., 1974b and c), the method has a number of limitations. The principal disadvantages are:

(a) The air interface may lead to denaturation of proteins in the medium or on the platelet surface.

(b) The flow characteristics of the system are unknown and cannot be described or readily controlled.

(c) Although red blood cells could be added to the platelet suspending medium, the air interface and undefined flow conditions would make it difficult to interpret their effects on platelet adherence. It seems desirable to study platelet adherence in the presence of red cells because red cells have

shown to have a major enhancing effect of adherence of platelets to a vessel wall, by increasing physically platelet diffusivity (Goldsmith, 1972; Turitto and Baumgartner, 1975).

(d) The method is technically difficult and time-consuming.

B. DEVELOPMENT OF A METHOD FOR MEASURING PLATELET ADHERENCE TO COLLAGEN-COATED GLASS RODS USING A ROTATING PROBE DEVICE

The rotating probe device was developed to measure platelet adherence to collagen-coated glass rods under controlled conditions. This would avoid some of the problems of the collagen-coated glass tube. It was also our aim to develop a method that could be adapted to measure platelet adherence to subendothelium of an everted rabbit aorta.

1. The rotating probe device.

The rotating probe device consists of 2 metal probes rotating in 2 test tubes containing samples of a platelet suspension. The speed of rotation is controlled (see Methods section and Feuerstein et al., 1975). The metal probe can be fitted with a glass rod or an everted segment of rabbit aorta. The glass rod can be coated with various proteins, including collagen. The test tubes are immersed in a temperature controlled water bath. The number of adherent ^{51}Cr-labeled platelets can be estimated by counting the segments of glass or aorta in a gamma-counter.

The fluid dynamic characteristics have been described by Feuerstein et al. (1975). Flow stream lines in the test tube are circular (Couette flow) and there is no convective transport.

To standardize the technique using the rotating probe device, collagen-coated rods were used for most experiments. Some modifications of the conditions were also tested with everted segments of damaged aortas from rabbits.

2. <u>Establishment of optimum conditions for assessment of platelet adherence with the rotating probe device.</u>

A number of principles were established during the development of the method for measuring quantitatively platelet adherence to collagen-coated tubes (see section I, A) and were used with the rotating probe device. The glass rods were coated with acid soluble collagen. Washed platelets, labeled with ^{51}Cr were suspended in a medium containing physiological concentrations of calcium and magnesium. Apyrase was added to the suspending medium to prevent ADP-induced platelet aggregation by degrading any ADP released in the medium. Although these conditions were not designed to block activation of the arachidonate pathway, it is unlikely that significant production of the aggregation-inducing intermediates of this pathway occurred since platelet aggregate formation on the surface did not occur (see paragraph 4(c) later in this section).

During the development and use of this method, it became evident that the extent of platelet adherence was dependent on several conditions in addition to those that had been investigated in the early studies with the collagen-coated tubes. The main conditions studied are:

(a) Rate of rotation of the probe;

(b) Length of time of rotation of the probe in the platelet suspension;

(c) Platelet count;

(d) albumin concentration;

(e) Hematocrit.

The aim of the development of this method was to establish conditions which gave reproducible measurements of platelet adherence so that the effects of modifications of the platelets, addition of inhibitors to the platelet suspension, or modifications of the suspending medium could be studied. Therefore, the effects of the five conditions listed above were not examined exhaustively, but only insofar as it was necessary for the development of the method.

Because of the principles established previously (see section I, A) the conditions that were kept constant throughout most of these experiments were:

(1) Apyrase concentration

Apyrase (see Materials) was added to the suspending medium at a concentration of 15 μg per ml. This concentration of apyrase was able to inhibit the aggregation of platelets induced by 2 μM ADP.

(2) Temperature of 37°C. This was chosen because it is the normal temperature at which platelets circulate in vivo. It was easy to do the experiments at 37°C with this procedure. The work did not have to be done in a warm room at 37°C since water baths in which the temperature was controlled at 37°C were readily available.

(3) The standard suspending medium. This was Tyrode solution which contains approximately physiological concentrations of Ca^{2+} (2 mM) and Mg^{2+} (1 mM).

(4) pH 7.3. This is approximately the normal pH of the blood.

(5) Albumin. All of the suspending media used contained albumin. The effects of variations in albumin concentration were tested.

(6) Rabbit platelets were used for most experiments because this made it possible to test the effects of platelet modifications in vitro and in vivo.

(7) The platelets were labeled with ^{51}Cr.

In the first experiments, the albumin concentration in the suspending fluid was 0.35% and red blood cells were not included. The probe was rotated for 10 minutes in the platelet suspension and the rotation speed was usually 200 rpm. The platelet count was 700,000 per mm^3. The effects of modifications of all of these conditions were examined, although all possible combinations of variations in the conditions were not explored. During the course of these experiments, it was found that a high albumin concentration reduced the variation in the number of adherent platelets. It also became apparent that the hematocrit had a major influence on the extent of adherence. Since platelets in vivo interact with surfaces in the presence of a high albumin concentration and a 40% hematocrit, it seemed advisable to study adherence under these conditions. Thus many of the later experiments were done with 4% albumin in the suspending medium and a 40% hematocrit. However, some preliminary experiments of each type were usually done with 0.35% albumin and without red cells for reasons of economy and simplicity.

(a) EFFECT OF RATE OF ROTATION OF THE PROBE

The effect of rate of rotation of the probe was tested under several conditions of platelet count, albumin concentration and hematocrit.

When platelets were suspended in a medium containing a low albumin concentration, 0.35% and at a high platelet count of 700,000 per mm^3, increasing the speed of rotation of the probe from 20 to 200 rpm increased the number of platelets adherent to collagen-coated rods (Table 11). At a rotation speed of 325 rpm, platelet aggregates were observed in the suspending fluid and on the surface. As a consequence, adherence could not be estimated. The effects of variations in albumin concentration and hematocrit were studied at a rotation speed of 100 rpm and 200 rpm with a platelet count of 300,000 per mm^3 (Table 12). Albumin concentrations of 0.35% or 4% were used and hematocrits of 0, 10, 20 and 40% were examined. In all cases, adherence was greater when the probes were rotated at 200 rpm and platelet aggregates were not seen on the surfaces when they were examined by scanning electron microscopy. Therefore, a rotation speed of 200 rpm was chosen for most of the experiments. In these conditions, the surface was not saturated with platelets. At the higher albumin concentration, fewer platelets adhered to the surface.

Finally, we examined the effect of increasing the rate of rotation from 50 rpm to 700 rpm on the adherence of platelets suspended in a medium containing 4% albumin and 300,000 platelets per mm^3. In all these experiments the number of platelets adherent after 10 minutes of rotation was measured. Adherence was tested in the absence and also in the presence of red blood cells (Figure 3). In the absence of red blood cells, increasing the speed of rotation of the probe

TABLE 11

Effect of the rate of rotation on the adherence of ^{51}Cr-labeled rabbit platelets to a collagen-coated glass rod[a].

Rate of rotation (r.p.m.)	(n)	Number of platelets per mm^2 (mean ± S.E.)
20	(10)	5,600 ± 500
50	(10)	13,100 ± 1,300
100	(10)	48,800 ± 6,500
150	(10)	122,000 ± 20,800
200	(10)	178,600 ± 36,700
325[b]	(10)	450,300 ± 42,900

a) 0.35% albumin. No red blood cells. Platelet count was 700,000 per mm^3.

b) Presence of platelet aggregates on the surface and in the suspending medium.

FIGURE 3.

Effect of rate of rotation and hematocrit on the adherence of ^{51}Cr-labeled rabbit platelets to a collagen-coated glass rod.

^{51}Cr-labeled platelets were suspended in Tyrode solution containing 4% albumin, apyrase and Hepes buffer. Platelet count was 300,000 per mm^3. Adhesion to collagen-coated glass rods was measured after rotation at speeds ranging from 50 to 700 rpm for 10 minutes at 37°C. Then the collagen-coated rods were rinsed at 200 rpm for 5 minutes at 37°C in a Ca^{2+} free Tyrode solution containing 10 mM EDTA.

●—● experiment performed at 40% hematocrit. Each point is the mean of 8 segments of glass coated with collagen, the vertical bar is one S.E.M. The line was calculated by linear regression analysis ($y = 419\ x + 586$).

○—○ experiment performed at 0% hematocrit. Each point is the mean of 8 segments.

○—○ insert: same experiment as above (abscissa: rpm x 10^2; ordinate: platelets per mm^2 x 10^3).

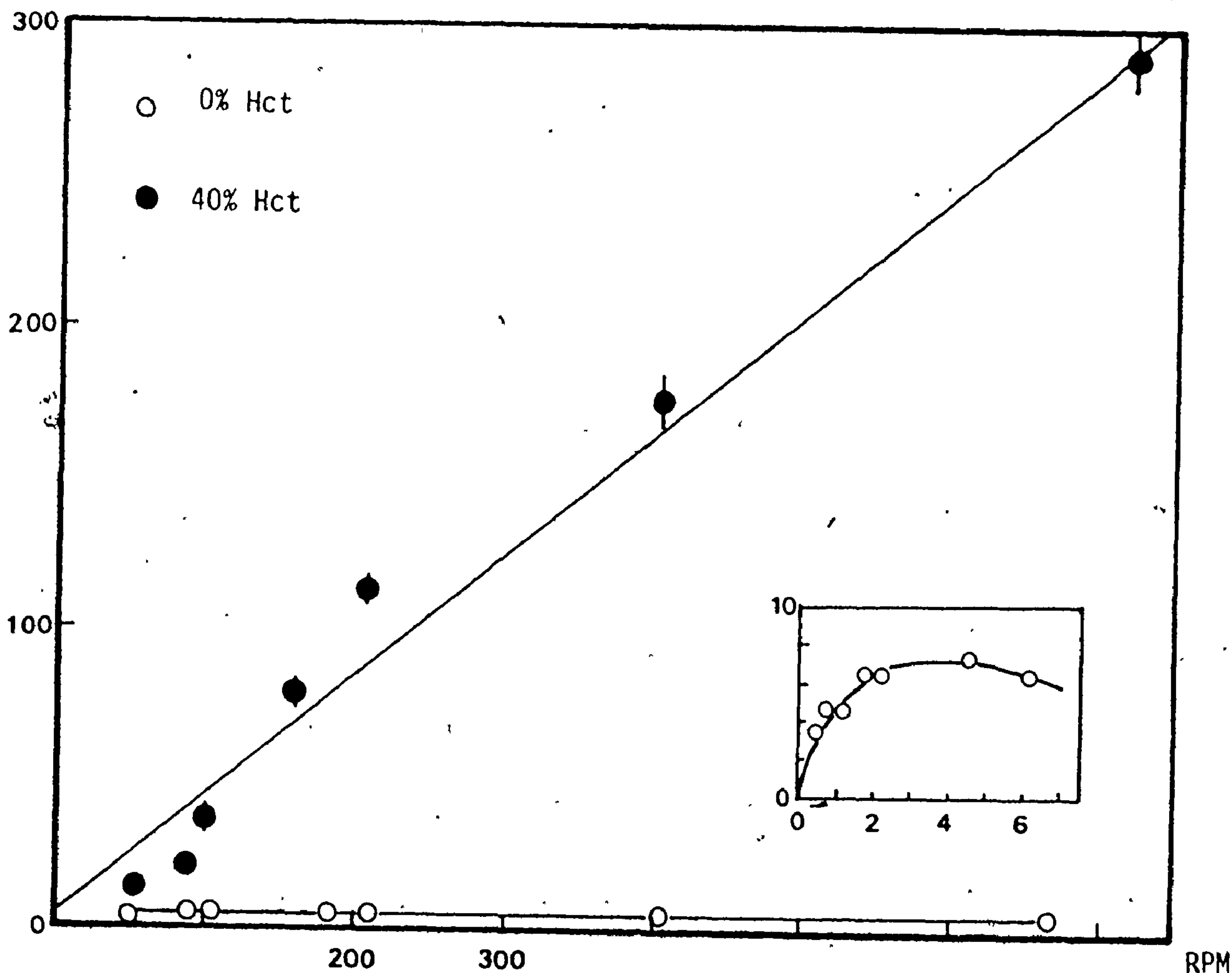


TABLE 12

Effect of rate of rotation, albumin concentration and hematocrit on the adherence of ^{51}Cr-labeled rabbit platelets to a collagen-coated glass rod[a].

Rate of rotation (r.p.m.)	Albumin (%)	Hematocrit (%)	(n)[b]	Number of platelets per mm^2 (mean ± S.E.)
100	0.35	0	(2)	21,600 ± 8,200
		10	(2)	71,000 ± 18,400
		20	(2)	96,700 ± 32,100
		40	(2)	86,400 ± 58,700
100	4	0	(5)	12,500 ± 2,300
		10	(9)	35,000 ± 3,600
		20	(5)	55,000 ± 7,500
		40	(9)	27,400 ± 5,800
200	0.35	0	(5)	60,700 ± 25,100
		10	(5)	146,300 ± 22,800
		20	(5)	164,200 ± 16,600
		40	(5)	160,900 ± 16,100
200	4	0	(16)	6,200 ± 500
		10	(15)	66,900 ± 8,600
		20	(23)	95,900 ± 6,200
		40	(44)	98,400 ± 3,100

a) ^{51}Cr-labeled platelets were suspended in Tyrode solution containing 0.35% or 4% albumin, apyrase and Hepes buffer, as described in materials and methods. Platelet count was 300,000 per mm^3. Adhesion to collagen-coated glass rods was measured after rotation at 100 or 200 rpm for 10 minutes at 37°C. Then the collagen-coated rods were rinsed at 100 or 200 rpm for 5 minutes at 37°C in a Ca^{2+} free Tyrode solution containing 10 mM EDTA.

b) Number of experiments. Each experiment performed with a different suspension on a different day was the mean of 8 segments of glass coated with collagen.

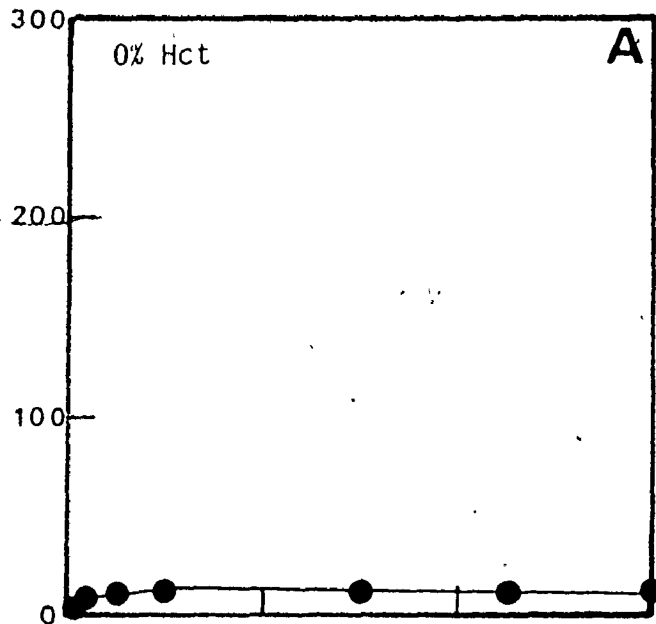
FIGURE 4.

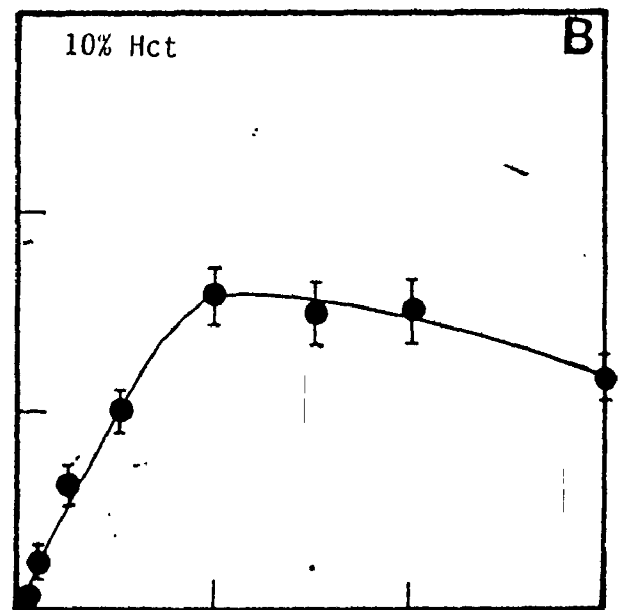
Effect of time of rotation and hematocrit on the adherence of ^{51}Cr-labeled rabbit platelets to a collagen-coated glass rod.

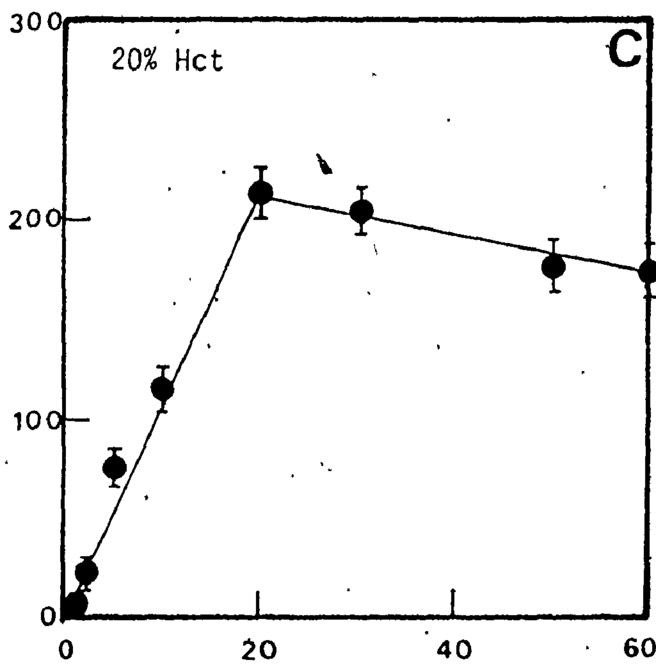
^{51}Cr-labeled platelets were suspended in Tyrode solution containing 4% albumin, apyrase and Hepes buffer. Platelet count was 300,000 per mm^3. Adhesion to collagen-coated glass rods was measured, in presence of various hematocrits, after rotation at 200 rpm for times ranging from 1 to 60 minutes at 37°C. Then the collagen-coated rods were rinsed at 200 rpm for 5 minutes at 37°C in a Ca^{2+} free Tyrode solution containing 10 mM EDTA. Each point and bar is the mean and S.E.M.

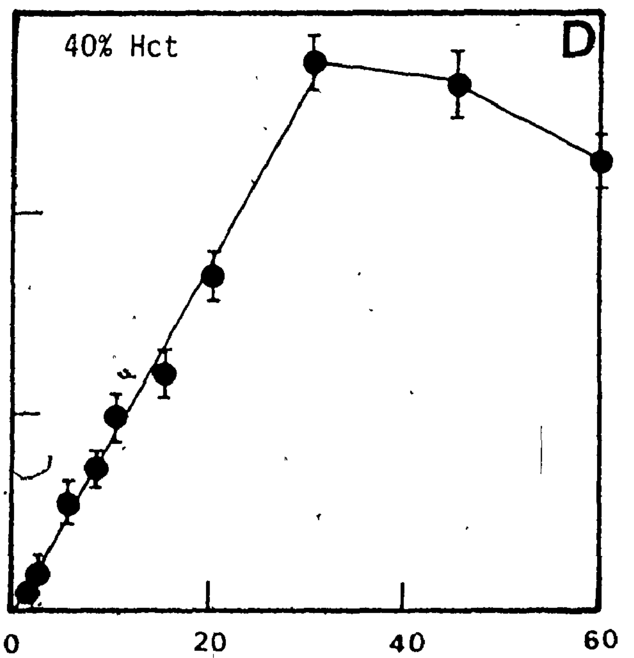
A. 0% hematocrit (number of segments of glass coated with collagen, n = 8)
B. 10% hematocrit (n = 24)
C. 20% hematocrit (n = 24)
D. 40% hematocrit (n = 24)

PLATELETS per mm^2 X 10^3









MINUTES

increased the number of platelets adherent to the surface up to a plateau value of about 6,000 platelets per mm^2 which was reached at a speed of about 200 rpm. The addition of 40% red blood cells increased markedly the number of platelets adherent to the collagen-coated glass rods. The number of adherent platelets increased linearly from 50 to 700 rpm (Figure 3). At a speed of 200 rpm, 112,200 ± 3,800 platelets were deposited per mm^2 of collagen-coated surface in the presence of 40% hematocrit.

(b) EFFECT OF TIME OF ROTATION OF THE PROBE

The effect of time of rotation of the probe on the number of platelets adhering to a collagen-coated rod was determined for rotation times ranging from 1 to 60 minutes. Platelets were suspended at a platelet count of 300,000 per mm^3 in a medium containing apyrase and 4% albumin. The effect of various hematocrits was also examined (Figure 4). In the absence of red cells, there was little platelet adherence and after 10 minutes of rotation a plateau level (about 8,000 platelets per mm^2) was reached. The addition of red blood cells to the platelet suspension greatly increased (by 14 times) the number of platelets adherent at 10 minutes at the 3 levels of hematocrit (10, 20, 40%) examined. The increase in platelet adherence was linear for 20 minutes. Maximum adherence was obtained after 20 minutes at 10 and 20% hematocrit and at 30 minutes at 40% hematocrit. The magnitude of the maximum was dependent on the hematocrit level, being less at 10% hematocrit and higher at 40% hematocrit.

(c) EFFECT OF PLATELET COUNT

Platelet adherence was measured at platelet counts of 300,000 or 700,000 per mm^3 (Table 13). The conditions studied were 0.35% or 4% albumin and 0 and 40% hematocrit. With all combinations of albumin concentration and hematocrit, adherence was much greater at the higher platelet count. For most of the studies without red cells, a platelet count of 700,000 was used whereas at 40% hematocrit, a platelet count of 300,000 per mm^3 was chosen. This latter number is within the normal range of platelet counts in rabbits and platelet aggregates did not form at this lower platelet count whereas in the presence of red cells at a platelet count of 700,000 per mm^3, some aggregates were observed.

(d) EFFECT OF ALBUMIN CONCENTRATION

Effects of albumin concentrations of 0.35% and 4% were studied under various conditions. At both platelet counts tested (300,000 and 700,000 per mm^3) and at 0, 10, 20 and 40% hematocrit, fewer platelets adhered to the collagen-coated rods when the albumin concentration in the medium was 4% than when it was 0.35% (Tables 12 and 13). In the system without red blood cells, the variation among replicate samples was less with 4% albumin than with 0.35% albumin (Tables 12 and 13).

(e) EFFECT OF HEMATOCRIT

The effect of red blood cells on platelet adherence was recognized during the course of these experiments and was investigated in some detail.

TABLE 13

Effect of platelet count, albumin concentration and hematocrit on the adherence of ^{51}Cr-labeled rabbit platelets to a collagen-coated glass rod[a].

Number of platelets per mm^3	Albumin (%)	Hematocrit (%)	(n)[b]	Number of platelets per mm^2 (mean ± S.E.)
300,000	0.35	0	(5)[c]	60,700 ± 25,100
300,000	4	0	(10)[d]	6,400 ± 800
300,000	4	40	(13)	137,100 ± 11,500
700,000	0.35	0	(22)	178,600 ± 36,700
700,000	4	0	(12)	45,100 ± 9,500
700,000	4	40	(3)	475,000 ± 17,200[e]

a) ^{51}Cr labeled platelets were suspended in Tyrode solution containing 0.35% or 4% albumin, apyrase and Hepes buffer as described in material and methods. Adhesion to collagen-coated glass rods was measured after rotation at 200 rpm for 10 minutes at 37°C. Then the rods were rinsed at 50 rpm for 15 seconds at 37°C in Ca^{2+} free Tyrode solution.

b) Number of experiments. Each experiment performed with a different suspension on a different day was the mean of 8 or 10 segments of glass coated with collagen.

c) and d) The rods were rinsed at 200 rpm for 5 minutes in Ca^{2+} free Tyrode solution containing 10 mM EDTA.

e) Presence of platelet aggregates on the surface and in the suspending medium.

At a platelet count of 300,000 per mm^3 and a rotation speed of 200 rpm, adding red blood cells increased the number of platelets that adhered to the collagen-coated rods (Table 12). At an albumin concentration of 0.35% twice as many platelets adhered in the presence of 10% red blood cells as adhered in their absence. Under these conditions and with this concentration of albumin, increasing the hematocrit to 20 or 40% caused only a small increase in the number of adherent platelets. At an albumin concentration of 4%, under these conditions of platelet count and rotation speed, maximum adherence was not observed until the hematocrit was raised to 20% (Table 12 and Figure 4). The numbers of platelets adherent at 20% and 40% hematocrit were similar.

3. <u>Evidence that platelets adhere to the collagen fibers deposited on the glass surface.</u>

When a glass surface was exposed to acid-soluble collagen and then rinsed with modified Tyrode solution at pH 7.35, collagen polymerized in the form of a network of fibers adherent to the glass surface.

Scanning electron microscopic examination of the surface revealed that individual platelets were adherent to the collagen fibers. Platelets which had interacted with collagen had lost their disc shape and had long, thin pseudopods that appeared to be adherent along the length of the collagen fibers. In section I, A, we have demonstrated that platelets did not adhere to a collagen-coated tube that had been incubated with collagenase. A similar experiment was performed with the rotating probe device. Collagen-coated glass rods were incubated

(2 hours, 37°C) with collagenase (50 units/ml in a modified Tyrode solution containing 5 mM $CaCl_2$, but no magnesium (pH 7.4). The rods were rinsed in fresh medium and platelet adherence to them was measured. Platelet adherence to the surface treated with collagenase was virtually abolished (Table 14).

Most, if not all, the adhering platelets were bound to the collagen on a collagen-coated glass surface and very few were adherent to the glass-coated with the albumin which was in between the collagen fiber network. In fact, when a glass surface was coated with albumin, adhesion of platelets to the surface was much less than adhesion to a collagen-coated surface. This was demonstrated in the presence or in the absence of addition of red blood cells and when the platelets were suspended in a medium containing 0.35% or 4% albumin. (Table 15).

4. Evidence that platelet adherence is measured in the absence of aggregate formation.

Three approaches were used to obtain evidence that platelets adhered individually rather than as aggregates.

(a) SCANNING ELECTRON MICROSCOPY (SEM)

Adhesion experiments were done with collagen-coated glass rods in platelet suspensions containing 4% albumin and at 0, 20 or 40% hematocrit. The density of platelets adherent to about 50 mm^2 of surface was examined by SEM. Platelets were seen to be adherent to the collagen fibers by long pseudopods. No platelet thrombi were seen.

TABLE 14

Effect of collagenase treatment of the collagen-coated glass rod on the adherence of ^{51}Cr labeled rabbit platelets.

Surface exposed	(n)	Number of platelets per mm^2 (mean ± S.E.)	P
collagen-coated rod	(10)	159,600 ± 5,400	
collagenase-treated rod	(10)	2,900 ± 100	<0.001

Platelets were resuspended in Eagle's MEM containing 0.35% albumin, apyrase and 10% hematocrit. Platelet count was 500,000 per mm^3. Collagen-coated rods were incubated with 50 U/ml collagenase for 2 hours at 37°C as described in the text. Adherence to the rods was measured after rotation at 200 rpm for 10 minutes at 37°C.

TABLE 15

Effect of hematocrit on the adherence of ^{51}Cr-labeled rabbit platelets to an albumin-coated glass surface.

Experiment No.	Hematocrit (%)	(n)	Number of platelets per mm^2 (mean ± S.E.)
1.	0	(8)	500 ± 400
	10	(8)	4,100 ± 900
	20	(8)	1,400 ± 300
	40	(8)	8,300 ± 300
2.	0	(8)	900 ± 200
	10	(8)	1,400 ± 300
	20	(8)	1,500 ± 500
	40	(8)	5,000 ± 800

Platelets were suspended in Tyrode solution containing 0.35% (Exp. 1.) or 4% albumin (Exp. 2.), apyrase and Hepes buffer. Platelet count was 300,000 per mm^3. Adherence was measured after rotation of the albumin-coated rods at 200 rpm for 10 minutes at 37°C. The rods were rinsed at 200 rpm for 5 minutes in Ca^{2+} free Tyrode solution containing 10 mM EDTA.

(b) REMOVAL OF ADP FROM THE SUSPENDING MEDIUM

In the early experiments with the collagen-coated tubes, it was found that increasing the apyrase concentration did not have a significant effect on the number of adherent platelets (Section I, A). The effect of omitting apyrase from the suspending medium was studied with collagen-coated rods (Table 16). The amount of radioactivity on the surface was significantly greater than in the system in which apyrase was present and aggregation did not occur. Furthermore, in the system without apyrase, if the platelets deposited on the surface were exposed to 10 mM EDTA (which causes deaggregation of platelet aggregates) the number of radioactive platelets on the surface was reduced to the same number as that found when the platelet suspending fluid contained apyrase (Table 16).

(c) EFFECT OF EGTA, EDTA OR PGE_1 ON PLATELETS THAT HAD ADHERED TO COLLAGEN-COATED GLASS RODS

It was reasoned that if platelet aggregates formed on the surfaces under the influence of ADP released from the platelets or PGG_2, PGH_2 and thromboxane A_2 formed by the platelets, it should be possible to break these aggregates by rinsing and rotating the probe in a medium free of platelets, free of calcium, and containing PGE_1 (10 μM) or EDTA (10 mM) or EGTA (10 mM).

Deaggregation experiments

Experiments were done to ensure that EDTA, EGTA or PGE_1 in the concentrations used above for rinsing the collagen-coated rods would

TABLE 16

Effect of apyrase in the suspending medium and of EDTA in the rinsing medium on platelet adherence to a collagen-coated surface.

Apyrase in suspending medium	Rinsing medium	Number of platelets per mm^2 (mean ± S.E.)
No	Tyrode	221,000 ± 12,100
No	Ca^{2+} free Tyrode	116,300 ± 8,000
No	Ca^{2+} free Tyrode + 10 mM EDTA	110,300 ± 9,000
Yes	Tyrode	101,400 ± 13,000
Yes	Ca^{2+} free Tyrode	113,800 ± 7,700
Yes	Ca^{2+} free Tyrode + 10 mM EDTA	109,700 ± 7,500

^{51}Cr-labeled platelets were suspended in Tyrode solution containing 0.35% albumin, and Hepes buffer, as described in Materials and Methods. Platelet count was 700,000 per mm^3. Adhesion to collagen-coated glass rods was measured in the presence or in the absence of apyrase after rotation at 200 rpm for 10 min at 37°C. Then the collagen-coated glass rods were rinsed at 200 rpm for 5 min at 37°C in a Tyrode solution or in a Ca^{2+} free Tyrode solution or in a Ca^{2+} free Tyrode solution containing 10 mM EDTA. Mean and S.E. of 8 segments.

deaggregate platelets that had aggregated on the surface. Collagen is thought to induce platelet aggregation by two mechanisms. One is through the release of ADP, the second by the formation of the prostaglandin endoperoxides PGG_2, PGH_2 and thromboxane A_2 from platelet arachidonate. Therefore, the effect of PGE_1, EDTA or EGTA on platelets aggregated by ADP, sodium arachidonate, or collagen was studied (Figure 5).

Aggregation of washed rabbit platelets induced by 5 μM ADP was followed by deaggregation. However, if 10 μM PGE_1, 10 mM EGTA or 10 mM EDTA were added at the peak of aggregation, deaggregation occurred sooner and the platelets recovered their disc shape sooner. Sodium arachidonate (25 μM) induced rabbit platelet aggregation and the release reaction. At this concentration, aggregation was not followed by deaggregation. But, if PGE_1 (10 μM) or EDTA (10 mM) were added at the maximum of aggregation, rapid deaggregation occurred and the platelets gradually regained their disc shape. In a third series of experiments aggregation was induced by a suspension of collagen or by acid soluble collagen. Addition of PGE_1 (10 μM), EGTA (10 mM) or EDTA (10 mM) 2 minutes after maximum aggregation caused a decrease in light transmission. Addition of modified Tyrode solution to a control suspension was not followed by any change for 10 minutes. Thus, these deaggregation experiments show that PGE_1, EGTA or EDTA are able to deaggregate platelet aggregates formed by ADP, sodium arachidonate or collagen.

If platelet-to-platelet aggregation had occurred on top of

FIGURE 5.

Aggregation of rabbit platelets with ADP, sodium arachidonate, or collagen and deaggregation with EGTA, EDTA or PGE_1.

Aggregation studies with rabbit platelet suspensions were performed as described in the Methods section.

▼ addition of the aggregating agent: ADP 5 μM or SODIUM ARACHIDONATE 25 μM or COLLAGEN suspension.

▽ addition of the deaggregating agent (EGTA 10 mM or EDTA 10 mM or PGE_1 10 μM) or TYRODE solution. The second addition was done 1 minute after the first one in the case of ADP or SODIUM ARACHIDONATE and 2.5 minutes after COLLAGEN.

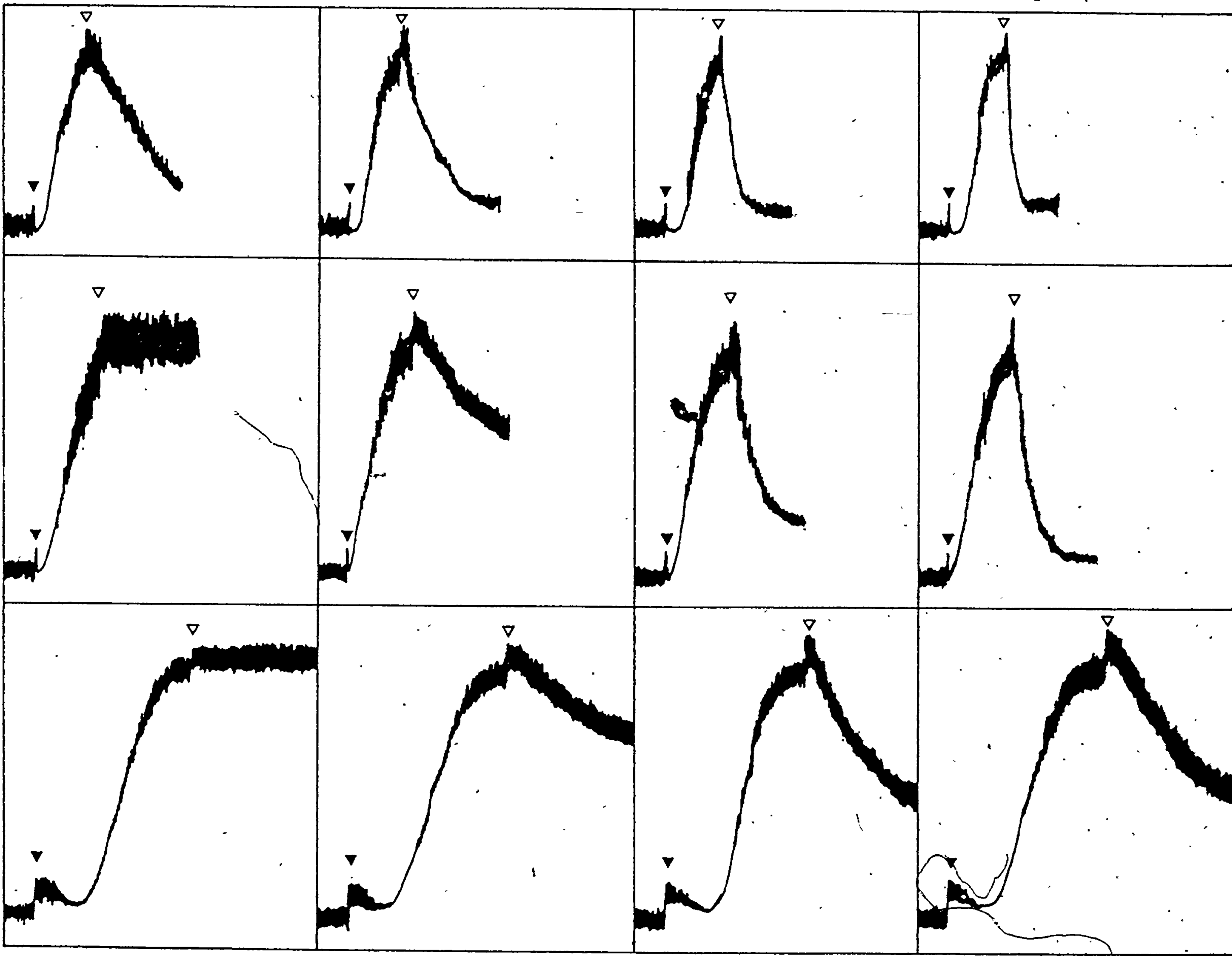

TYRODE
EGTA 10 mM
EDTA 10 mM
PGE1 10 µM
ADP 5 µM
SODIUM ARACHIDONATL 25 µM
COLLAGEN

TABLE 17

Effect of EGTA, EDTA or PGE_1 on ^{51}Cr-labeled rabbit platelets that had adhered to a collagen-coated glass rod[a].

Experiment No.	Resuspending medium	Hematocrit (%)	Rinsing medium[c]	(n)[d]	Number of platelets per mm^2 (mean ± S.E.)	P[c]
1.	Tyrode + 0.35% albumin	0	Control	(16)	169,900 ± 16,800	
		0	EGTA 10 mM	(16)	188,200 ± 15,700	<0.5
2.	Tyrode + 0.35% albumin	20	Control	(8)	91,300 ± 3,700	
		20	EDTA 10 mM	(8)	90,600 ± 6,100	<0.95
		20	PGE_1 10 μM	(8)	81,200 ± 3,200	<0.10
3.	Tyrode + 4% albumin	20	Control	(8)	97,100 ± 2,800	
		20	EDTA 10 mM	(8)	90,800 ± 2,800	<0.20
		20	PGE_1 10 μM	(8)	96,700 ± 4,600	<0.45
4.	Tyrode + 4% albumin	40	Control	(20)	113,300 ± 3,500	
		40	EGTA 10 mM	(20)	104,500 ± 3,500	<0.10
		40	PGE_1 10 μM	(20)	123,700 ± 5,100	<0.10

a) Adhesion to collagen-coated glass rods was measured after rotation at 200 rpm for 10 minutes at 37°C.
b) Platelet count were 700,000 per mm^3 in experiment No. 1; 300,000 per mm^3 in experiments No. 2, 3, and 4.
c) The glass rods were rinsed at 200 rpm for 5 minutes at 37°C in Tyrode solution (control) or in Tyrode solution containing EGTA or EDTA or PGE_1. The rinsing medium was at pH 7.35.
d) Number of segments.
e) P-values calculated with respect to the control suspension.

platelets adherent to the collagen, a drop in [^{51}Cr]platelet-bound radioactivity on the collagen-coated glass surface should have occurred when the rods with adherent platelets were rinsed in EDTA, EGTA or PGE_1. This did not occur (Table 17), indicating that the method measures adherence of individual platelets to collagen. Rinsing for 5 minutes with EGTA, EDTA or PGE_1 did not reduce the number of platelets adherent to collagen when the suspending medium contained 0.35% or 4% albumin and when the hematocrit was 0, 20 or 40%.

The evidence from these three approaches substantiates the conclusion that the rotating probe method developed in this study permits the measurement of the adherence of individual platelets to surfaces without the formation of aggregates on the surface.

5. Summary

Using principles established previously to measure platelet adherence to collagen-coated tubes, a new method was developed to measure platelet adherence to collagen-coated glass rods rotating in a platelet suspension. This new method has many advantages over the previous one while retaining the useful features of the earlier method.

(i) Platelet adherence is measured to a segment of glass, coated with collagen fibers, rotated in a platelet suspension. The probes supporting the glass rods are rotated at a reproducible speed under controlled flow conditions (Couette flow).

(ii) Platelets are labeled with ^{51}Cr. ^{51}Cr is the label of choice

because it is not lost from the platelets when they adhere to collagen. If release of granule contents is to be monitored ^{14}C-serotonin can be used. The use of a radioisotope has advantages over the morphometric technique. The isotopic technique is simpler, faster and more reproducible, platelet adherence over large surface areas is measured, avoiding bias in selection of the area to be examined. The isotopic method does not prevent the use of morphometric evaluation in conjunction with it.

(iii) Platelets are suspended in a physiological medium at pH 7.35, containing albumin and a normal concentration of divalent cations. The presence of divalent cations at normal concentrations is important because divalent cations promote platelet adherence to collagen (see section I, A). Apyrase is included to prevent platelet aggregation due to released ADP and the synergistic effect between released ADP and collagen which results in the release reaction.

(iv) The use of an artificial medium to suspend the platelets has several advantages. The lack of clotting factors prevents generation of thrombin, the composition of the suspending medium can be changed as appropriate, and the effects of drugs can be tested without the effects of anticoagulants and chelating agents.

(v) Increasing the albumin concentration from 0.35% to 4% decreases platelet adherence to collagen and gives less variability

among replicate samples.

Factors governing the extent of platelet adherence

The number of adherent platelets was shown to depend on

(a) The rate of rotation of the probe

(b) The length of time of rotation

(c) The albumin concentration

(d) The hematocrit

(e) The platelet count.

Under the conditions chosen for the experiments, it was demonstrated that ^{51}Cr-labeled platelets were not adherent as aggregates to the surface since agents that cause deaggregation did not reduce the amount of ^{51}Cr that was associated with the surface.

II. PLATELET ADHERENCE TO EVERTED RABBIT AORTA

The response of blood to injury of the vessel wall is a fundamental mechanism in the development of arterial thrombosis and atherosclerosis. Normal endothelium is a non-thrombogenic surface. Platelets will interact with the vessel wall if the endothelium is modified or if it is damaged or removed, thus exposing the subendothelial tissue.

The principles established to measure quantitatively platelet adherence to a collagen-coated glass rod under controlled flow conditions (section I, B) were adapted to measure adherence of platelets to the surface of an everted rabbit aorta.

The interactions of platelets with the constituents of a rabbit thoracic aorta were studied with the rotating probe device. An everted thoracic aorta was mounted on the probe and was rotated at 200 rpm for 10 minutes in a suspension of rabbit platelets labeled with ^{51}Cr. The suspending medium described in the previous section was used.

With an everted aorta on the rotating probe it is possible to test the interaction of platelets with the various constituents of the aortic wall: i.e. with the endothelium and with the subendothelial tissues.

1. ENDOTHELIUM

Rabbit aortas were prepared for study as described in "Methods." A rabbit aorta could be everted on a probe without major damage to the endothelium. Scanning electron micrographs of segments of the aortas showed an apparently intact endothelial layer over most of the surface

although a few damaged areas were visible (Figure 6). When this surface was rotated in a suspension containing ^{51}Cr-labeled platelets, very few platelets become associated with the surface. Microscopic examination showed that the few platelets that interacted with the vessel, did so at points where the endothelium had been damaged (Figure 7). The inclusion of red cells in the platelet suspension caused only a slight enhancement in platelet adhesion (Table 18). These results are in agreement with earlier observations that platelets do not adhere to normal endothelium (Jørgensen, 1971; Gimbrone, 1976).

2. SUBENDOTHELIUM

When the endothelial lining of an artery is damaged, the subendothelial tissue is exposed. Platelets adhere readily to basement membrane, microfibrils and collagen fibers of the subendothelium (reviewed in Stemerman, 1974). We have used two methods of damaging the endothelial lining of the rabbit thoracic aorta. (1) Endothelium was removed by passing an inflated balloon catheter through the aorta in situ. This method did not damage the internal elastic lamina. (2) Alternatively, everted aortas were damaged by scraping their surface with a scalpel blade. With this technique, the damage was deeper than with the balloon catheter. Many more platelets adhered to the damaged aortic surface than to the non-damaged endothelium (Table 18). Treatment of the surface of a ballooned aorta with collagenase almost abolished completely platelet adherence to the surface (Table 19). This observation is similar to the findings that collagen and possibly basement membrane are the principal

FIGURE 6.

Scanning electron micrograph of the undamaged endothelial surface of an everted thoracic aorta of a rabbit. The surface had not been exposed to a platelet suspension. x 1450.

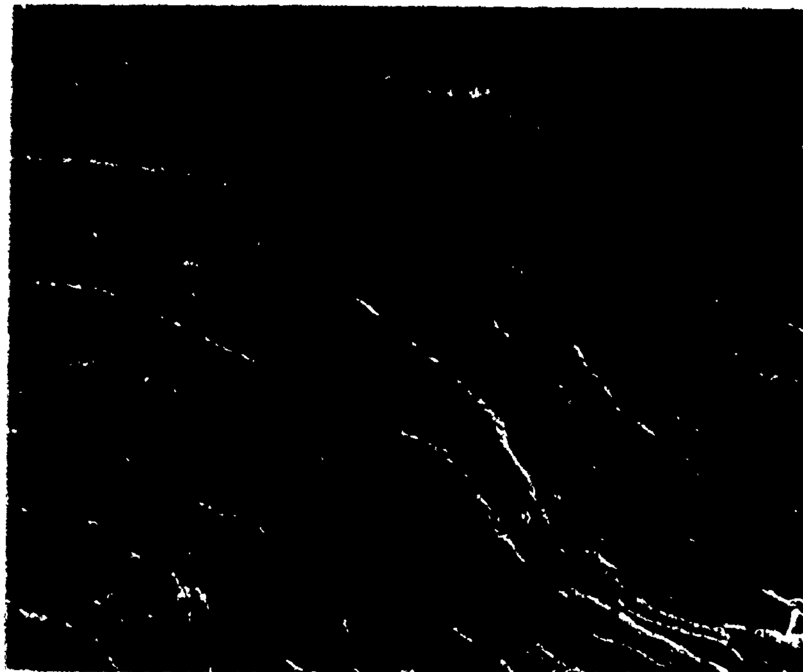


FIGURE 7.

Scanning electron micrograph of the undamaged endothelial surface of an everted aorta of a rabbit. The surface has been rotated at 37°C for 10 minutes at 200 rpm in a platelet suspension containing apyrase, 4% albumin and 40% hematocrit. The platelet count was 300,000 per mm^3. Few platelets adhere to the undamaged surface. x 3,100.

TABLE 18

Effect of hematocrit on the adherence of ^{51}Cr-labeled rabbit platelets to the endothelial surface of everted segments of rabbit aorta.

Experiment No.	Surface exposed	Albumin (%)	Hematocrit (%)	(n)	Number of platelets per mm^2 (mean ± S.E.)	P
1.[a]	non-ballooned	0.35	0	(8)	8,700 ± 1,900	
2.[b]	non-ballooned	0.35	10	(10)	4,200 ± 700	
	ballooned	0.35	10	(10)	59,500 ± 9,800	<0.001
3.[c]	non-ballooned	4	40	(5)	13,500 ± 5,800	
	ballooned	4	40	(5)	91,200 ± 4,800	<0.001

a) and b) Platelet count was 700,000 per mm^3. Aorta segments rotated at 200 rpm for 10 minutes at 37°C.

c) Platelet count was 300,000 per mm^3. Aorta segments rotated at 200 rpm for 10 minutes at 37°C.

TABLE 19

Effect of collagenase treatment of the subendothelial surface exposed by passing a balloon catheter in a rabbit thoracic aorta, on the adherence of ^{51}Cr-labeled rabbit platelets.

Surface exposed	(n)	Number of platelets per mm^2 (mean ± S.E.)	P
ballooned aorta	(10)	110,200 ± 1,100	
collagenase-treated ballooned aorta	(10)	2,000 ± 200	<0.001

Platelets were resuspended in Eagle's MEM containing 0.35% albumin, apyrase and 10% hematocrit. Platelet count was 500,000 per mm^3. Everted segments of ballooned aortas were incubated with 50 U/ml collagenase for 2 hours at 37°C as described in Methods. Adherence to the aorta was measured after rotation at 200 rpm for 10 minutes at 37°C.

structures in the subendothelium with which the platelets interact (Stemerman, 1974). The adherence of platelets to ballooned or scraped aortas was compared at various hematocrit. In the absence of red cells, adherence to an aortic surface damaged by scraping was significantly greater than to a surface damaged with a balloon catheter (Table 20). At 10%, 20% and 40% hematocrit, there was no significant difference between the adherence to the aortic surfaces damaged in the two different ways (Table 20).

An experiment was done to examine the adherence of rabbit platelets, suspended in Tyrode solution with apyrase containing 4% albumin and 40% hematocrit, to the surface of a ballooned everted aorta. After adhesion, the segments of aorta were rinsed in EDTA (10 mM). Scanning electron microscope observation of large surface areas showed that single platelets or a monolayer of platelets were adherent to the subendothelial tissue, but thrombi were not present (Figure 8). Sometimes platelets were present in a cluster, which was not broken up by EDTA and appeared to represent the complex interaction of platelet pseudopods with the collagen fibers in 3-dimensional space (Figure 9).

3. THROMBIN BINDING TO THE VESSEL WALL

In vivo thrombin formation on the surface of endothelial cells could be a factor causing platelet adherence to the vessel wall without loss of endothelial cells. Table 21 shows the results of an experiment in which the surface of a non-damaged aorta was exposed to thrombin and then rinsed. Thrombin-treatment of the aorta caused enhanced adherence

TABLE 20

Effect of hematocrit on the adherence of ^{51}Cr-labeled rabbit platelets to a damaged aorta[a].

Surface exposed	Hematocrit (%)	(n)[b]	Number of platelets per mm^2 (mean ± S.E.)
Scraped aorta	0	(22)	58,500 ± 7,500
	10	(7)	75,600 ± 19,600
	20	(4)	173,600 ± 29,500
	40	(4)	167,100 ± 35,000
Ballooned aorta	0	(4)	12,700 ± 2,800
	10	(13)	61,300 ± 13,500
	20	(5)	186,100 ± 28,300
	40	(9)	142,300 ± 19,200

a) ^{51}Cr-labeled platelets were suspended in Eagle's MEM containing 0.35% albumin, apyrase and Hepes buffer as described in Materials and Methods. Platelet count was 700,000 per mm^3. Adhesion to damaged aorta was measured after rotation at 200 rpm for 10 minutes at 37°C. Then the aortas were rinsed at 50 rpm for 15 seconds at 37°C in Ca^{2+} free Tyrode solution.

b) Number of experiments.

FIGURES 8 and 9.

Scanning electron micrograph of the ballooned surface of an everted thoracic aorta of a rabbit. The surface has been rotated at 37°C for 10 minutes at 200 rpm in a platelet suspension containing apyrase, 4% albumin and 40% hematocrit. Then it was rinsed at 200 rpm for 5 minutes at 37°C in a Ca^{2+} free Tyrode solution containing 10 mM EDTA.

Figure 8. x 3,000.
Figure 9. x 7,600. Cluster of platelets.

8

9

TABLE 21

Effect of thrombin on the adherence of ^{51}Cr-labeled rabbit platelets to a non-damaged aorta.

Treatment of exposed surface	Addition of heparin to platelet suspension (U/ml)	(n)	Number of platelets per mm^2 (mean ± S.E.)
Tyrode	0	(10)	4,200 ± 700
Tyrode	50	(10)	2,900 ± 400
Thrombin (20 U/ml)	0	(10)	53,100 ± 4,600
Thrombin (20 U/ml)	50	(10)	5,200 ± 1,200

Platelets were suspended in Tyrode solution containing 0.35% albumin, apyrase, Hepes buffer and 10% hematocrit. Platelet count was 700,000 per mm^3. Everted segments of aorta were rotated for 1 min in a Tyrode-albumin solution containing 20 U/ml thrombin and rinsed twice in Tyrode solution. Adherence to the aorta was measured after rotation at 200 rpm for 10 minutes at 37°C.

TABLE 22

Effect of thrombin on the adherence of ^{51}Cr-labeled rabbit platelets to an aorta damaged by passing a balloon catheter.

Treatment of exposed surface	Addition of heparin to platelet suspension (U/ml)	(n)	Number of platelets per mm^2 (mean ± S.E.)
1.Tyrode	0	(10)	59,500 ± 7,200
2.Tyrode	50	(10)	54,000 ± 9,800
3.Thrombin (20 U/ml)	0	(10)	471,500 ± 37,500*
4.Thrombin (20 U/ml)	50	(10)	44,200 ± 3,100

Platelets were resuspended in Tyrode solution containing 0.35% albumin, apyrase, Hepes buffer and 10% hematocrit. Platelet count was 700,000 per mm^3. Everted segments of aorta were rotated for 1 min in a Tyrode-albumin solution containing 20 U/ml thrombin and rinsed twice in Tyrode solution. Adherence to the aorta was measured after rotation at 200 rpm for 10 minutes at 37°C. Significance of differences between means:
1 vs 2: $P < 0.7$
3 vs 4: $P < 0.001$

*Formation of platelet aggregates on the surface.

of platelets to the non-damaged endothelium was prevented by heparin. Similarly, exposure of the damaged aortic surface to thrombin increased the adherence of platelets and caused formation of platelet aggregation on the surface; this was also inhibited by high concentrations of heparin (Table 22).

4. SUMMARY

The adherence of rabbit platelets to an everted rabbit thoracic aorta can be measured quantitatively in vitro under controlled flow conditions.

Adherence is measured with a rotating probe device. The method is an adaptation of the method developed to measure adherence of ^{51}Cr-labeled platelets to a collagen-coated glass rod, in a system containing albumin, divalent cations and apyrase. The rabbit thoracic aorta can be everted on a metal probe without major damage to the endothelial lining of the vessel wall. Alternatively, the aorta can be damaged by removing the endothelium and thus exposing the subendothelial connective tissue. Few platelets adhere to the surface of the undamaged aorta even if red cells are included in the suspending medium to increase platelet diffusion to the vessel wall.

Platelet adherence to the aorta is markedly increased if the subendothelium is exposed. Platelet adherence to a scraped aorta is greater than to a ballooned aorta when tested in the absence of red blood cells, but it is similar when red blood cells increases platelet adherence to damaged aorta.

Platelets adhere to the damaged aorta as a single layer of platelets, as demonstrated by scanning electron microscopy. In the experimental conditions used, there was no platelet aggregate formation on the surface.

Exposure of the undamaged or damaged surface of the rabbit aorta to thrombin increases the number of platelets which adhere to the surface. Increased adherence caused by thrombin is abolished by high concentrations of heparin.

III. EFFECTS OF MODIFICATIONS OF THE PLATELET SURFACE ON ADHERENCE

Cell surface proteins and glycoproteins have been implicated in many interactions and events taking place at the cell surface and are of major importance in cell development, for cell function and in the control of cell growth. The adhesion of platelets to collagen and to vessel wall components obviously involves the platelet surface.

It has now been demonstrated that several proteins and glycoproteins are exposed on the platelet surface. Three major platelet surface glycoproteins can be labeled with radioactive iodine using the lactoperoxidase system (Phillips, 1972). Proteolytic enzymes (thrombin, plasmin, chymotrypsin) can be used to treat platelets and they can be recovered as discrete cells. There is evidence that plasmin and chymotrypsin hydrolyse platelet surface glycoproteins and some investigators believe that thrombin may affect membrane glycoproteins. In man, there are at least 2 hereditary diseases (Glanzmann's thrombasthenia and Bernard-Soulier syndrome) where an abnormality of a platelet surface glycoprotein has been correlated with a defect in platelet function (Nurden and Caen, 1976).

Because of the likelihood that platelet surface structures such as glycoproteins may be involved in platelet adherence to collagen the effects of various agents that would be expected to modify the platelet surface were studied.

1. <u>THROMBIN</u>

Thrombin is an enzyme to which platelets may be exposed in vivo. It has both proteolytic and esterase activities but the precise

mechanism by which it causes platelet shape change, aggregation and the release reaction has not been defined. Platelets can be treated with thrombin in vitro so that they release most of the contents of their amine storage granules and after this reaction they can be recovered as discrete platelets. Some of the properties of these thrombin-degranulated platelets are well established (Reimers et al., 1976). They are unresponsive to thrombin but they aggregate in response to ADP, Collagen, sodium arachidonate or the divalent cation ionophore A23,187. They survive normally in vivo but are not as effective as normal platelets in hemostatic plug formation. The effect of thrombin treatment of platelets on their ability to adhere to collagen-coated tubes or to everted scraped aortas was studied (Table 23). Thrombin-treated platelets were less adherent to collagen or to damaged aorta than control platelets. The inhibitory effect on adhesion was present whether platelets had been partially or completely degranulated. Adherence of thrombin-treated platelets was also less than that of the control platelets when the albumin concentration of the suspending medium was 0.35% or 4% and the hematocrit 20%. This inhibition of adherence was not corrected by the addition of fibrinogen to the platelet suspension (Table 23). It seems likely the thrombin-treated platelets are less adherent because thrombin modifies the platelet surface in some way.

2. PLASMIN

Platelets were treated with several types of plasmin (Table 24) using mild conditions in which little release and no aggregation

TABLE 23

Effect of thrombin treatment on adherence of rabbit platelets labeled with ^{51}Cr to collagen-coated glass or everted scraped segments of rabbit aorta.

Exp. No.	Surface Exposed	Resuspending medium	Type of platelets	(n)	Number of platelets per mm^2 (mean ± S.E.)	p[c]
1.[a]	collagen-coated glass tubes	Tyrode + 0.35% albumin	control	(25)	33,700 ± 5,200	
			Thrombin-treated	(25)	13,400 ± 3,000	<0.005
2.[b]	Scraped aorta	Tyrode + 0.35% albumin	control	(10)	239,000 ± 22,000	
			Thrombin-treated	(10)	62,800 ± 3,900	<0.001
		Tyrode + 0.35% albumin + 0.01% fibrinogen	Thrombin-treated	(5)	76,400 ± 4,700	<0.001
3.[b]	Scraped aorta	Tyrode + 4% albumin + 20% hematocrit	control	(10)	70,500 ± 4,800	
			Thrombin-treated	(10)	36,600 ± 3,000	<0.001

a) Experiment 1. Platelets were treated with 0.4 U/ml bovine topical thrombin. Platelet count was 700,000 per mm^3. Tubes were rotated for 10 minutes at 15 r.p.m. at 22°C.

b) Experiment 2, 3. Platelets were treated with 0.5 U/ml purified rabbit thrombin. Platelet count was 700,000 per mm^3 (Exp. 2) and 500,000 per mm^3 (Exp. 3). Adherence was measured after rotation at 200 r.p.m. at 37°C for 10 minutes.

c) P-values calculated with respect to the control suspension.

occurred. Fewer plasmin-treated platelets than control platelets adhered to collagen-coated surfaces or to the damaged aortas (Table 24). This inhibitory effect of plasmin treatment was not corrected by the addition of fibrinogen to the suspending medium. In fact, fibrinogen reduced the extent of adherence in the control experiments.

Plasmin hydrolyses fibrinogen and fibrinogen has been shown to be associated with the surface of platelets. Addition of fibrinogen to plasmin treated platelets restores their ability to aggregate to ADP but not to adhere to collagen. These observations can be used to support the hypothesis that platelet surface fibrinogen is not involved in platelet adhesion to collagen.

High concentrations of plasmin hydrolyse all the platelet glycoproteins that stain with PAS (Greenberg, personal communication).

3. <u>CHYMOTRYPSIN</u>

Rabbit platelets were treated with 10 U/ml chymotrypsin for 30 minutes at room temperature. This treatment did not cause platelet to release nor to aggregate. Chymotrypsin-treated platelets were resuspended in Tyrode solution containing 4% albumin. These platelets adhered significantly less than untreated platelets to collagen-coated rods when the rods were rotated in a platelet suspension containing red blood cells (at 10, 20 or 40% hematocrit) (Table 25).

Under these conditions, chymotrypsin hydrolyses all three major platelet membrane glycoproteins (Greenberg, personal communication).

4. <u>NEURAMINIDASE</u>

The treatment of platelets with purified neuraminidase devoid

TABLE 24

Effect of plasmin treatment on adherence of rabbit platelets labeled with ^{51}Cr to a collagen-coated glass or everted scraped segments of rabbit aorta.

Exp. No.	Surface exposed	Resuspending medium	Type of platelets	(n)	Number of platelets per mm^2 (mean ± S.E.)	P[d]
1.[a]	collagen-coated glass tube	Tyrode + 0.35% albumin	control	(10)	75,900 ± 10,700	
			plasmin	(10)	29,100 ± 6,500	<0.005
		Tyrode + 0.35% albumin + 0.01% fibrinogen	control	(10)	31,000 ± 6,200	<0.001
			plasmin	(10)	11,600 ± 3,000	<0.001
2.[b]	scraped aorta	Tyrode + 0.35% albumin	control	(20)	115,000 ± 14,800	
			plasmin	(20)	27,800 ± 4,100	<0.001
		Tyrode + 0.35% albumin + 0.01% fibrinogen	control	(20)	71,500 ± 14,000	<0.05
			plasmin	(20)	33,100 ± 4,900	<0.005
3.[c]	collagen-coated glass rod	Tyrode + 4% albumin + 40% Hct.	control	(6)	110,000 ± 3,700	
			plasmin	(6)	86,200 ± 2,200	<0.001
		Tyrode + 4% albumin + 0.04% fibrinogen + 40% Hct.	control	(6)	99,500 ± 3,400	<0.1
			plasmin	(6)	78,100 ± 2,200	<0.005

a) Platelets were treated with 0.05% Plasmin Novo for 30 minutes at 22°C. Platelet count was 700,000 per mm^3. Tubes were rotated for 10 minutes at 15 r.p.m. at 22°C.
b) Platelets were treated with 0.1 mg/ml of pure rabbit plasmin (see materials and methods) for 30 minutes at 22°C. Platelet count was 700,000 per mm^3. Adherence was measured after rotation at 200 rpm at 37°C for 10 minutes.
c) Platelets were treated with 0.75 CU/ml of pure human Kabi plasmin for 30 minutes at 22°C. Platelet count was 300,000 per mm^3. Adherence was measured after rotation at 200 rpm at 37°C for 10 minutes. The glass rods were rinsed at 200 rpm for 5 minutes at 37°C in Ca^{2+} free Tyrode solution containing 10 mM EDTA.
d) P-values calculated with respect to the control suspension not containing added fibrinogen.

TABLE 25

Effect of chymotrypsin treatment on adherence of rabbit platelets labeled with ^{51}Cr to collagen-coated glass rods[a].

Type of platelets	Hematocrit (%)	(n)[b]	Number of platelets per mm^2 (mean ± S.E.)	P[c]
control	0	(8)	4,300 ± 700	
chymotrypsin-treated	0	(8)	3,100 ± 500	<0.2
control	10	(8)	19,800 ± 2,000	
chymotrypsin-treated	10	(8)	9,700 ± 900	<0.001
control	20	(16)	91,200 ± 6,600	
chymotrypsin-treated	20	(16)	42,800 ± 1,900	<0.001
control	40	(16)	109,000 ± 4,500	
chymotrypsin-treated	40	(16)	40,100 ± 1,700	<0.001

a) Platelets were treated with 10 U/ml chymotrypsin for 30 minutes at 22°C as described in materials and methods. Platelets were suspended in Tyrode solution containing 4% albumin, apyrase and Hepes buffer. Platelet count was 300,000 per mm^3. Adherence to collagen-coated glass rods was measured after rotation at 200 rpm for 10 minutes at 37°C. Then the collagen-coated glass rods were rinsed at 200 rpm for 5 minutes at 37°C in Ca^{2+} free Tyrode solution containing 10 mM EDTA.

b) Number of segments.

c) P-values calculated with respect to the control suspension.

of proteolytic activity does not cause the platelet release reaction nor aggregation (Greenberg et al., 1975). When platelets were treated with neuraminidase to remove up to 35% of total sialic acid, platelet aggregation induced by ADP, collagen, thrombin, ristocetin, polylysine and serotonin was either not affected or slightly enhanced.

The experiments reported on the effect of neuraminidase treatment of platelets on their adherence to collagen and to damaged aorta were done before the techniques were fully developed and the effect of adding red blood cells to the platelet suspension was not examined.

When up to 35% of total platelet sialic acid was removed, the adherence of platelets to collagen-coated tubes or to damaged aorta was not modified compared to untreated platelets (Table 26). These results indicate that removal of terminal sialic acid, probably from platelet membrane glycoproteins, does not alter platelet adherence to collagen.

5. SODIUM PERIODATE

$NaIO_4$ was used to treat platelets as another means of modifying platelet glycoproteins although it is recognized that this agent may have other effects as well. It seems that under the conditions used, $NaIO_4$ oxidizes the terminal sialic acid of glycoproteins (Presant and Parker, 1976). The effects of $NaIO_4$ pretreatment on platelet responses to aggregating and release-inducing agents were in sharp contrast to the slightly enhancing effects of neuraminidase treatment. $NaIO_4$ pretreatment reduced the response of platelets to ADP, collagen, thrombin, arachidonic acid and ionophore A23,187. $NaBH_4$ partially reversed the

TABLE 26

Effect of neuraminidase treatment on adherence of rabbit platelets labeled with ^{51}Cr to collagen-coated glass or everted scraped segments of rabbit aorta[a].

Experiment No.	Surface exposed	% of total sialic acid removed from platelets	(n)	Number of platelets per mm^2 (mean ± S.E.)	p[d]
1.[b]	collagen-coated glass tubes	0	(5)	100,500 ± 2,200	
		24	(5)	104,400 ± 2,800	<0.4
		24	(5)	95,200 ± 2,800	<0.2
2.[c]	collagen-coated glass rods	0	(5)	37,500 ± 3,600	
		15	(5)	34,600 ± 8,000	<0.9
		17	(5)	28,600 ± 4,700	<0.2
3.[c]	scraped aorta	0	(10)	40,900 ± 7,000	
		35	(10)	50,700 ± 5,000	<0.3
4.[c]	scraped aorta	0	(10)	40,500 ± 3,700	
		8.5	(10)	44,500 ± 3,700	<0.3
		10	(10)	55,600 ± 6,300	<0.05

a) Platelets were treated with purified neuraminidase as described in materials and methods. They were resuspended in Tyrode solution containing 0.35% albumin and apyrase at a platelet count of 700,000 per mm^3.
b) Tubes were rotated at 22°C for 10 minutes at 15 rpm.
c) Adherence to collagen-coated glass rods was measured after rotation at 200 rpm for 10 minutes at 37°C.
d) Compared with untreated control (no sialic acid removed) in each experiment.

TABLE 27

Effect of ten-minute sodium periodate pretreatment on adherence of rabbit platelets[a] labeled with ^{51}Cr to collagen-coated glass or everted scraped segments of rabbit aorta.

Exp. No.	Surface exposed	$NaIO_4$ treatment (mM)	Hematocrit (%)	(n)	Number of platelets per mm^2 (mean ± S.E.)	p^c
1.	collagen-coated glass rods	0	0	(10)	89,700 ± 17,000	
		0.1		(10)	39,400 ± 2,800	<0.005
		0.5		(10)	34,800 ± 3,500	<0.005
2.	collagen-coated glass rods	0	0	(10)	102,600 ± 20,200	
		0.5		(10)	39,900 ± 2,800	<0.005
		1		(10)	36,700 ± 2,600	<0.005
3.	everted scraped aorta	0	0	(10)	50,600 ± 5,300	
		1		(10)	37,000 ± 2,600	<0.005
4.[b]	collagen-coated glass rods	0	0	(8)	4,500 ± 600	
		0.5	0	(8)	2,700 ± 300	<0.025
		0	10	(8)	71,300 ± 2,600	
		0.5	10	(8)	21,400 ± 1,000	<0.001
		0	20	(8)	100,200 ± 3,500	
		0.5	20	(8)	69,500 ± 1,500	<0.001
		0	40	(8)	97,600 ± 7,100	
		0.5	40	(8)	107,600 ± 4,000	<0.001
5.[b]	collagen-coated glass rods	0	0	(8)	4,700 ± 400	
		1	0	(8)	1,900 ± 500	<0.001
		0	10	(8)	60,800 ± 3,000	
		1	10	(8)	19,400 ± 900	<0.001
		0	20	(8)	103,800 ± 4,800	
		1	20	(8)	78,700 ± 2,000	<0.001
		0	40	(8)	102,100 ± 2,500	
		1	40	(8)	104,200 ± 3,900	<0.001

a) The platelets were pretreated with $NaIO_4$ for 10 minutes, washed and suspended in Tyrode solution containing 4% albumin, apyrase and Hepes buffer. Platelet count was 700,000 per mm^3 (Exp. 1, 2, 3) and 300,000 per mm^3 (Exp. 4, 5). Adherence was measured after rotation at 200 rpm for 10 minutes at 37°C.

b) The collagen-coated glass rods were rinsed at 200 rpm for 5 minutes at 37°C in Ca^{2+} free Tyrode solution containing 10 mM EDTA.

c) Compared with untreated control (platelets not exposed to $NaIO_4$) in each experiment.

effect of $NaIO_4$ treatment on platelet aggregation responses (Çazenave et al., 1976c). $NaIO_4$ oxidation of platelets reduced their adherence to collagen and to the subendothelial structures of damaged aorta when adherence was measured at 4% albumin with hematocrits of 0, 10 or 20% (Table 27). This effect was in contrast with the effect of removal of sialic acid from membrane glycoproteins by neuraminidase. However, when adherence to collagen was tested with a 40% hematocrit in the platelet suspension, platelets pretreated with periodate adhered to the same extent as the control platelets. The reason for this finding is not known.

6. SUMMARY

The role of platelet surface membrane glycoproteins in platelet adherence to collagen or to the subendothelium is not understood. Several agents have been used to modify the platelet surface and their effect on platelet adherence to collagen and subendothelium has been examined.

Modifications of platelets by pretreatment with thrombin, plasmin, chymotrypsin or periodate is accompanied by a decrease in platelet adherence to collagen.

Thrombin, plasmin or chymotrypsin hydrolyse membrane glycoproteins exposed at the external surface and cleave glycopeptides from them. Although it is recognized that these enzymes may have additional effects that affect platelet adherence to collagen and the subendothelium. In the case of thrombin or plasmin-treatment of

platelets, the decreased adherence to collagen is not corrected by the addition of fibrinogen. Platelet adherence to collagen and to subendothelium is not changed if the surface sialic acid is removed by purified neuraminidase.

In contrast, if the terminal sialic acid of glycoproteins is oxidized by $NaIO_4$, platelet adherence to collagen and to subendothelium is decreased.

IV. PLATELET RECEPTORS AND PLATELET ADHERENCE TO COLLAGEN

The interaction of platelets with collagen is thought to involve specific properties of the collagen molecules. These have been recently reviewed by Michaeli and Orloff (1976). For example, it is known that the quaternary structure is important in mediating platelet-collagen interaction and that localized binding sites for platelets exist on the polypeptide chains of collagen. The nature of the platelet surface "receptor" sites involved in adhesion to collagen is not established. Jamieson et al. (1971) suggested that platelet:collagen adhesion could be mediated by an enzyme-substrate complex between the platelet membrane enzyme collagen:glucosyl transferase and the incomplete carbohydrate side chain of collagen. We have tested this hypothesis by studying the effects of UDP and UDPG on platelet adherence.

We have also studied the effect of Clq, a subcomponent of the first component (Cl) of complement, on platelet adhesion to collagen. Clq is a collagen-like molecule and was chosen for study because, due to its structural similarity to collagen it was hypothesized that it might interact with a collagen "receptor site" on the platelet surface.

1. Platelet adherence to collagen and the collagen:glucosyltransferase hypothesis.

Jamieson et al. (1971) have suggested that adhesion of platelets to collagen may be mediated by the formation of an enzyme-acceptor complex between collagen;glucosyltransferase present on the platelet surface and incomplete heterosaccharide chains present on the platelet surface

and incomplete heterosaccharide chains present in collagen or basement membrane. This hypothesis was an extension of Roseman's proposal (1970) that glycosyltransferases may play a role in intercellular adhesion. Jamieson et al. (1971) and Bosmann (1971) suggested that the carbohydrate side chains of collagen may be involved in the adherence of platelets to collagen (and subsequent release of granule contents and aggregation) because they found that the platelet enzyme, collagen: glucosyltransferase, transfers glucose specifically from UDPG to incomplete heterosaccharide chains of collagen. To test this hypothesis, we studied the effect of UDP and UDPG on platelet adherence to collagen. Neither of these agents inhibited platelet adherence (Table 28). Glucosamine, added to platelet membranes or to platelet-rich plasma, has been reported to cause parallel inhibition of the collagen:glucosyltransferase of platelet membranes and of platelet adhesion to collagen (Jamieson et al., 1971). These experiments apparently were based on an earlier observation of Legrand et al. (1968) indicating that the presence of glucosamine during the polymerization of tropocollagen interfered with its ability to adhere to platelets. Legrand et al. (1968), however, found that glucosamine added to repolymerized collagen or to platelets was only weakly inhibitory. We found that glucosamine (1 mM) did not inhibit adherence of platelets to a collagen-coated surface when it was present during the reaction of the platelets with the collagen-coated surface, nor when it was present at a concentration of 10 mM during the polymerization of the collagen

TABLE 28

Effect of UDP, UDPG or glucosamine on the adherence of human or pig platelets labeled with ^{14}C-serotonin to a collagen-coated surface[a].

Experiment	Species	Agent tested[b] (final concentration)	Number of platelets per mm^2 (mean ± S.E.)	P[c]
1.	Human	Tyrode (control	85,700 ± 5,900	
		UDP 1 mM	85,400 ± 9,500	<0.95
		UDPG 1 mM	89,600 ± 1,400	<0.60
		glucosamine 1 mM	101,100 ± 6,200	<0.20
2.	Human	Tyrode (control)	73,400 ± 2,000	
		UDP 1 mM	63,000 ± 6,200	<0.20
		UDPG 1 mM	90,400 ± 9,000	<0.20
		glucosamine 1 mM	83,400 ± 5,300	<0.20
3.	Pig	Tyrode (control)	28,300 ± 2,500	
		UDP 1 mM	36,100 ± 2,800	<0.10
		UDPG 1 mM	30,000 ± 2,000	<0.70
4.	Pig	Tyrode (control)	81,200 ± 3,100	
		glucosamine 1 mM	75,300 ± 4,500	<0.40
		glucosamine 10 mM	71,700 ± 2,800	<0.10
5.	Pig	Tyrode (control)	101,600 ± 2,000	
		glucosamine 10 mM	96,300 ± 3,600	<0.30

a) Platelets were suspended in Tyrode solution containing 0.35% albumin and apyrase. In experiments 1, 2 and 3, 1.5 μM $MnCl_2$ was included in the suspending medium. Mean values of 5 replicates.

b) Tubes were exposed to acid soluble collagen for 10 minutes, rinsed 4 times with glucosamine (1 or 10 mM in modified Tyrode solution) and drained before the platelet suspension was added to measurement of adherence.

c) P values calculated with respect to the control.

on the surface (Table 28).

These observations were interpreted as an indication that collagen-glucosyl transferase may not be involved in platelet-collagen adherence. More recently, Jamieson et al. (1975) have suggested that the interaction between collagen and the enzyme may be analogous to the interaction of a lectin with the cell surface, so that donation of glucose from UDPG need not be involved in the process of adhesion.

2. Clq and the collagen receptor of human platelets.

Human Clq, isolated in pure state after affinity chromatography on IgG-Sepharose, inhibited collagen-induced aggregation and release of ^{14}C-serotonin from prelabeled human platelets (Figure 10). Platelet aggregation induced by ADP or thrombin was not inhibited by Clq (Cazenave et al., 1976a; Suba and Csako, 1976). The adherence of platelets to collagen-coated surfaces was inhibited by Clq when tested in a medium containing 0.35% albumin and 20% hematocrit (Table 29). In this experiment Clq inhibited adherence to collagen to some extent. Adherence was 81.7% of the control value and the same batch of Clq reduced platelet aggregation induced by collagen to 37% of the control value. It is thus apparent that the activity of this batch of Clq was much less than the activity of the Clq used in the experiments illustrated in Figure 10. The data are incomplete because no more highly active Clq was available to repeat the adherence experiments. Thus, monomeric Clq in concentration less than those occurring in plasma inhibited specifically the interaction of platelets with collagen. Clq possesses

FIGURE 10.

Inhibition by human Clq of the aggregation of human platelets induced by a collagen suspension. Human platelets labeled with ^{14}C-5HT were resuspended in Tyrode's solution containing 0.35% albumin and apyrase, and the platelet count was adjusted to 200,000/μl. Increased light transmission indicates platelet aggregation. Curve A, control. At the first arrow, 10 μl of EDTA-buffer solution (final concentration 9 μM) was added to the platelet suspension and stirred at 1100 rpm for 1 minute at 37°C; then 100 μl of collagen suspension were added at the second arrow. After a short lag phase, the platelets aggregated extensively and released 68% of their total ^{14}C-serotonin content within 3 minutes. Curve B, At first arrow, 10 μl of human Clq in EDTA-buffer (final concentration of Clq. 18 μg/ml) was added to the platelet suspension and 1 minute later 100 μl of collagen suspension was added. The platelets changed shape, as shown by the reduction of the amplitude of oscillations and the decrease in light transmission, but they did not aggregate; 17% of the total ^{14}C-5HT content was released.

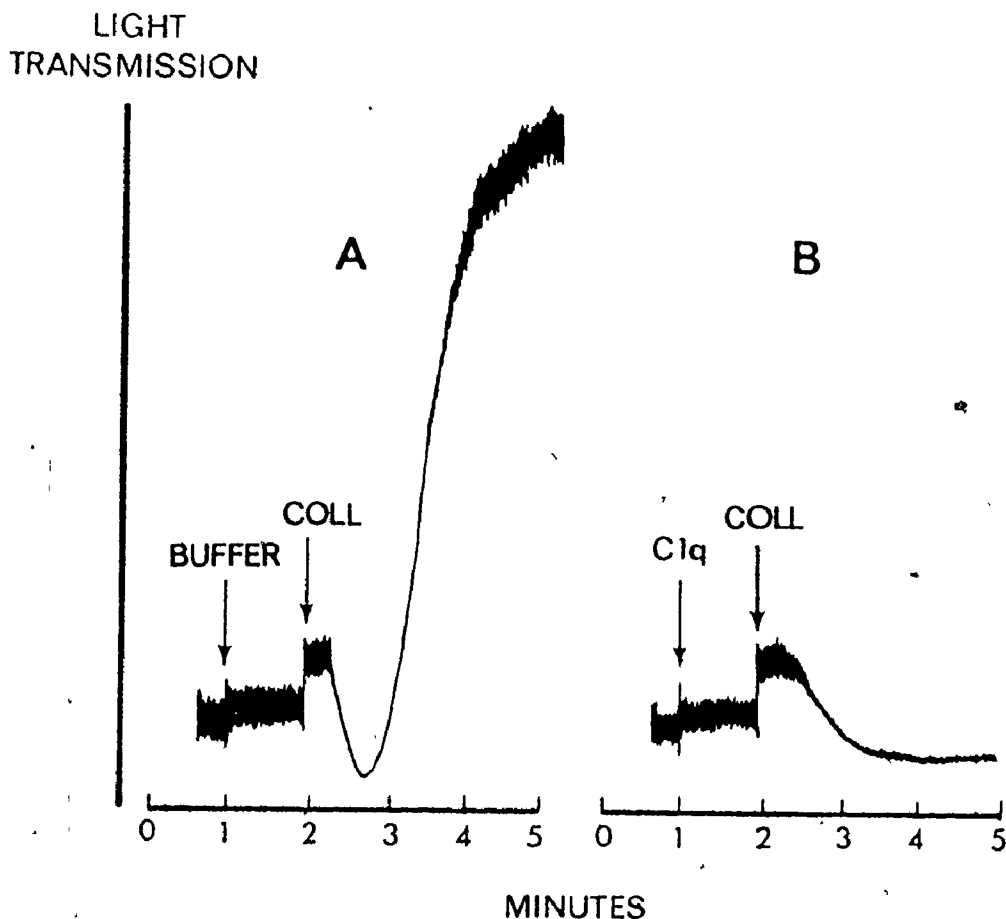


TABLE 29

Effect of human Clq on the adherence of ^{51}Cr-labeled human platelets to a collagen-coated glass surface[a].

Clq µg/ml	(n)	Number of platelets per mm^2 (mean ± S.E.)	2P
0	(10)	116,700 ± 3,900	
100	(10)	95,300 ± 2,900	<0.001

[a]Human platelets were labeled with ^{51}Cr and resuspended in Tyrode's solution containing 0.35% albumin and apyrase. Platelets were incubated at 37°C for 10 minutes with 5×10^{-5} M EDTA-buffer (control) or with 100 µg/ml Clq in EDTA-buffer. Before the experiment was started, homologous human washed red blood cells were added to obtain a 20% hematocrit. The final platelet concentration was 250,000/µl. Glass rods coated with repolymerized acid soluble collagen were rotated at 200 rpm in the platelet suspension for 10 minutes at 37°C. The number of platelets adherent to the collagen-coated surface was determined by measuring the amount of ^{51}Cr which became associated with the collagen-coated glass rods. Practically no ^{51}Cr (0.7%) was lost into the medium, indicating that no lysis had occurred. In this experiment, 100 µg/ml Clq reduced platelet aggregation induced by collagen (1/2200) to 37% of the control value.

structural similarities with collagen and its activity is destroyed by heat denaturation or collagenase. Aggregated Clq mimicked the effect of collagen in causing platelet aggregation and release of serotonin (Cazenave et al., 1976a).

V. MODIFICATION OF PLATELET ADHERENCE BY INHIBITORS OF PLATELET FUNCTION

The adherence of platelets to collagen-containing surfaces is thought to involve several steps. First, platelets have to contact the collagen fibers. It seems likely that platelets have specific collagen receptors on their plasma membrane. The interaction of the collagen receptor with specific parts of the collagen molecule is followed by a number of undefined events which lead to the transmission of a signal across the plasma membrane to the intracellular, metabolic machinery. As a result platelets change shape and emit pseudopods, which may allow more intimate contact with the collagen fibril. The shape change is probably dependent on the contractile system and controlled by calcium ions. The shape change and organization of membrane receptors are also thought to be in some way controlled by the state of microtubules and microfilaments beneath the plasma membrane. Preliminary experiments have been done to examine the effect of a number of agents and drugs on the adherence of pig platelets to collagen-coated glass tubes (Cazenave et al., 1973b; 1974b,c). These experiments have shown that adherence was inhibited by agents that inhibit the platelet release reaction (Cazenave et al., 1974b) or interfere with platelet contractile processes that are responsible for shape change and pseudopod formation (Cazenave et al., 1973b; 1974c).

Using the modifications of the technique that have already been described, we have reinvestigated the effects of a number of these drugs and compounds that are known to affect platelet function. Effects on platelet adherence to collagen-coated glass rods and to the

subendothelium of the rabbit aorta have been studied by the rotating probe method.

We have selected drugs which may have potential clinical applications. The effect of the drugs on adherence to collagen and to the subendothelial surface were compared. The influence of albumin concentration and of hematocrit on the effects of the drugs on platelet adherence were tested. The ultimate goal was to use a system as similar as possible to physiologic conditions to test the effect of drugs on platelet adherence. The measurement of platelet adherence to collagen-coated rods in the presence of 4% albumin and 40% hematocrit seemed to be such a system.

1. Inhibition of platelet adherence by compounds which chelate divalent cations (Ca^{2+}, Mg^{2+}).

(a) EFFECT OF SODIUM CITRATE

When sodium citrate (12.9 or 21.5 mM; final pH 7.35) was added to the suspending medium, platelet adherence to collagen and damaged aorta was reduced. The sodium citrate concentration of 21.5 mM is the concentration in plasma prepared from blood taken into 3.8% sodium citrate dihydrate, 1 part to 9 parts of blood. The inhibitory effect of sodium citrate was present when the albumin concentration was 0.35% or 4% and when the hematocrit was 0 or 40% (Table 30).

(b) EFFECT OF EDTA OR EGTA

When EDTA (9 mM) or EGTA (9 mM) was added to the platelet suspension, platelet adherence to collagen-coated glass rods and to

TABLE 30

Effect of citrate, EDTA, or EGTA on adherence of platelets to a damaged aorta or to a collagen-coated surface.

Experiment No.	Surface exposed	Hematocrit (%)	Addition to platelet suspension	(n)	Number of platelets per mm^2 (mean ± S.E.)	P*
1.	Scraped aorta	0	Tyrode	10	69,100 ± 14,100	
		0	Citrate 12.9 mM	5	29,400 ± 8,600	<0.05
		0	Citrate 21.5 mM	5	23,500 ± 7,800	<0.02
2.	Scraped aorta	0	Tyrode	5	19,700 ± 3,400	
		0	EDTA 9 mM	5	5,900 ± 1,500	<0.01
		0	EGTA 9 mM	5	4,600 ± 700	<0.005
3.	Ballooned aorta	40	Tyrode	4	104,700 ± 5,300	
		40	EGTA 9 mM	4	5,300 ± 300	<0.001
4.	Collagen	20	Tyrode	8	84,100 ± 1,500	
		20	EDTA 9 mM	8	9,400 ± 600	<0.001
5.	Collagen	40	Tyrode	16	82,100 ± 1,500	
		40	Citrate 12.9 mM	8	11,000 ± 500	<0.001
		40	EDTA 9 mM	8	16,400 ± 600	<0.001

^{51}Cr-labeled rabbit platelets were suspended in Tyrode solution containing 4% albumin (except experiment 1 with 0.35% albumin) and apyrase. In experiments 1 and 2 the platelet count was 700,000/mm^3. In experiments 3, 4 and 5, 5 mM Hepes buffer was included in the suspending medium; platelet count was 300,000/mm^3.

*P-values calculated with respect to the control suspension to which Tyrode solution was added.

aortas damaged by scraping or by a balloon catheter, was markedly reduced. The inhibitory effect of EDTA or EGTA was also demonstrated when the albumin concentration of the suspending medium was 4% and when the hematocrit was 20 or 40% (Table 30). In these experiments with chelating agents, the pH was controlled so that the final pH of the platelet suspension plus EDTA or EGTA was 7.35 (see Materials section).

2. Inhibition of platelet adherence by non-steroidal anti-inflammatory drugs.

(a) EFFECT OF ACETYLSALICYLIC ACID (ASA)

Preliminary experiments were performed with collagen-coated glass tubes and have been published (Cazenave et al., 1974b). ASA, in low concentrations, reduced the adherence of platelets to the collagen-coated surface.

However, in an experiment with thrombin-degranulated platelets. in which adherence was decreased compared with that of the control platelets, ASA had no further inhibitory effect on the adherence of the thrombin-degranulated platelets to the subendothelial surface (Table 31).

In another series of experiments, the effects of ASA on platelet adherence to collagen-coated rods and to the damaged surface of a rabbit thoracic aorta were examined with the rotating probe device, under various conditions.

i. Influence of albumin concentration.

The inclusion of ASA in the suspending medium decreased platelet

adherence to collagen-coated surfaces or to damaged aorta whether the platelets were suspended in a medium containing 0.35% or 4% albumin (Table 32).

Increasing the ASA concentration from 0.1 to 10 mM did not increase the inhibitory effect of ASA on platelet adherence to collagen-coated rods when tested in a medium containing 0.35% or 4% albumin and 10 or 20% hematocrit (Table 33).

ii. Influence of sodium citrate and of citrated plasma.

The effect of ASA in media containing 0.35% percent and 4% albumin and in the presence of citrate, was examined (Table 34). When the platelets were resuspended in Tyrode solution containing either concentration of albumin, ASA caused a significant decrease in the number adherent to the damaged aorta segments. ASA was also inhibitory in the presence of citrate, at either concentration of albumin.

In contrast, when the platelets were resuspended in citrated platelet-free plasma, the number of adherent platelets was low and in two out of three experiments ASA did not cause a significant decrease in the number of platelets adherent to the damaged aorta segments (Table 34, experiments 3, 4, 5).

iii. Influence of hematocrit.

The effect of red blood cells on the inhibitory effect of ASA on platelet adherence was tested. ASA inhibited platelet adherence to collagen when platelets were suspended in a medium containing 0.35 or

adherence to collagen-coated surfaces or to damaged aorta whether the platelets were suspended in a medium containing 0.35% or 4% albumin (Table 32).

Increasing the ASA concentration from 0.1 to 10 mM did not increase the inhibitory effect of ASA on platelet adherence to collagen-coated rods when tested in a medium containing 0.35% or 4% albumin and 10 or 20% hematocrit (Table 33).

ii. Influence of sodium citrate and of citrated plasma.

The effect of ASA in media containing 0.35% percent and 4% albumin and in the presence of citrate, was examined (Table 34). When the platelets were resuspended in Tyrode solution containing either concentration of albumin, ASA caused a significant decrease in the number adherent to the damaged aorta segments. ASA was also inhibitory in the presence of citrate, at either concentration of albumin.

In contrast, when the platelets were resuspended in citrated platelet-free plasma, the number of adherent platelets was low and in two out of three experiments ASA did not cause a significant decrease in the number of platelets adherent to the damaged aorta segments (Table 34, experiments 3, 4, 5).

iii. Influence of hematocrit.

The effect of red blood cells on the inhibitory effect of ASA on platelet adherence was tested. ASA inhibited platelet adherence to collagen when platelets were suspended in a medium containing 0.35 or

TABLE 32

Effect of ASA on the adherence of ^{51}Cr-labeled rabbit platelets to a collagen-coated rod when tested in the presence of various albumin and hematocrit concentrations.

ASA (μM)	Albumin (%)	Hematocrit (%)	(n)	Number of platelets per mm^2 (mean ± S.E.)	p*
0	0.35	0	(16)	31,800 ± 8,900	
100		0	(16)	8,200 ± 400	<0.02
0		10	(16)	100,200 ± 4,400	
100		10	(16)	30,000 ± 3,000	<0.001
0		20	(16)	126,400 ± 4,400	
100		20	(16)	104,300 ± 2,600	<0.001
0		40	(16)	132,000 ± 2,700	
100		40	(16)	113,700 ± 3,100	<0.001
0	4	20	(24)	100,300 ± 8,100	
100		20	(24)	61,000 ± 4,500	<0.001
0	4	0	(16)	5,400 ± 600	
250		0	(16)	6,400 ± 500	<0.3
0		10	(16)	25,600 ± 1,300	
250		10	(16)	14,800 ± 1,200	<0.001
0		20	(16)	63,700 ± 2,500	
250		20	(16)	48,200 ± 1,800	<0.001
0		40	(16)	65,300 ± 1,900	
250		40	(16)	60,400 ± 1,700	<0.1

^{51}Cr-labeled platelets were suspended in Tyrode solution containing albumin, apyrase and 5 mM Hepes buffer. They were incubated for 10 minutes with ASA or modified Tyrode solution. Then red blood cells were added. The final platelet count was 300,000 per mm^3. The collagen-coated glass rods were rotated in the platelet suspension at 200 rpm for 10 minutes at 37°C. The rod was then rinsed at 200 rpm for 5 minutes at 37°C in modified Tyrode solution containing 10 mM EDTA.

*Compared with control.

TABLE 33

Effect of increasing concentrations of ASA on the adherence of ^{51}Cr-labeled rabbit platelets to a collagen-coated rod.

ASA (mM)	Albumin (%)	Hematocrit (%)	(n)	Number of platelets per mm^2 (mean ± S.E.)	P
0	0.35	10	(8)	225,000 ± 13,800	
0.1		10	(8)	156,000 ± 14,300	<0.02
1		10	(8)	168,300 ± 17,300	<0.05
10		10	(8)	120,200 ± 19,900	<0.001
0	0.35	20	(16)	168,400 ± 11,200	
0.1		20	(16)	118,800 ± 3,500	<0.001
1		20	(16)	112,700 ± 4,900	<0.001
10		20	(16)	103,200 ± 2,900	<0.001
0	4	20	(16)	71,000 ± 2,200	
0.01		20	(8)	61,500 ± 2,800	<0.02
0.1		20	(8)	51,300 ± 2,300	<0.001
1		20	(8)	43,200 ± 1,700	<0.001
10		20	(8)	40,600 ± 1,100	<0.001

See footnote Table 32.

TABLE 34

Effect of ASA on adherence to scraped thoracic aorta segments of ^{51}Cr-labeled rabbit platelets resuspended in various media.

Experiment	Resuspending medium	ASA in suspending medium (μM)	No. of segments	Number of platelets per mm^3 (mean ± S.E.)	P^a
1.	Tyrode + 0.35% albumin	0	5	321,700 ± 54,200	
		10	5	41,900 ± 13,800	<0.005
		100	5	28,300 ± 4,700	<0.001
	Tyrode + 4% albumin	0	5	94,100 ± 13,500	
		10	5	35,400 ± 5,700	<0.005
		100	5	31,500 ± 7,800	<0.005
2.	Tyrode + 0.35% albumin + 21.5 mM citrate[b]	0	4	15,800 ± 2,200	
		100	4	9,800 ± 400	<0.05
	Tyrode + 4% albumin + 21.5 mM citrate[b]	0	4	20,300 ± 2,700	
		100	4	7,900 ± 800	<0.005
3.	Citrated PFP[c]	0	20	46,700 ± 6,800	
		10	10	22,500 ± 2,800	<0.001
		100	10	31,500 ± 2,800	<0.01
4.	Citrated PFP[c]	0	11	18,500 ± 2,900	
		10	11	15,200 ± 2,000	<0.5
		100	11	12,600 ± 1,300	<0.1
5.	Citrated PFP[c]	0	10	20,500 ± 5,300	
		10	10	30,000 ± 5,200	<0.20
		100	10	12,500 ± 3,800	<0.20

Rotation speed was 190 rpm in Experiments 1 and 2, and 200 rpm in Experiments 3, 4 and 5. No red blood cells added.

a) P values calculated with respect to corresponding control containing no ASA.

b) The pH of the suspending medium was readjusted to 7.35 after the addition of citrate.

c) Citrated PFP contained apyrase at the same concentration as in the artificial suspending media.

4% albumin and in the presence of 10 or 20% red blood cells. When the hematocrit was raised to 40%, the inhibitory effect of ASA was lost at 4% albumin (Table 32). Because of the possibility that the inhibitory effect of the drug was reduced by its binding to red cells and to albumin, platelets in a medium containing 0.35% albumin were exposed to 10 mM ASA for 10 minutes, washed and resuspended in a medium containing 4% albumin and 40% red blood cells. Platelet adherence to the collagen-coated surfaces was not inhibited as had been observed when ASA was not removed from the suspending medium before adherence was tested (Table 35).

In another experiment, a high concentration of ASA (10 mM) was incubated with the platelet suspension containing 4% albumin. At this concentration, platelets treated with ASA (10 mM) did not form malondialdehyde (malondialdehyde formation accompanies prostaglandin endoperoxide formation from platelet arachidonate) when tested with thrombin (1 U/ml) or a high concentration of collagen. At this concentration, ASA did not inhibit platelet adherence to the collagen-coated surface in the presence of a 40% hematocrit (Table 36).

Next, the effect of ASA on platelet adherence to a balloon-damaged aorta was investigated in the presence of red blood cells. ASA inhibited platelet adherence to balloon-damaged aortas in the presence of 10% or 20% hematocrit. However, when the hematocrit was raised to 40%, the inhibitory effect of ASA was not demonstrated (Table 37).

TABLE 35

Effect of pretreatment of platelets with ASA or indomethacin and resuspension in fresh medium on the inhibition of platelet adherence to a collagen-coated surface when tested in the presence of 4% albumin and 40% hematocrit.

Pretreatment	Number of segments	Number of platelets per mm^2 (mean ± S.E.)	P
Control	8	68,300 ± 2,000	
ASA 0.5 mM	8	62,600 ± 2,000	<0.1
Control	8	89,400 ± 2,600	
Indomethacin 100 μM	8	69,100 ± 1,300	<0.001

^{51}Cr-labeled rabbit platelets were incubated in the second washing fluid for 15 minutes with 0.5 mM ASA or 100 μM indomethacin or their solvent. Then the platelets were resuspended in fresh Tyrode solution containing 4% albumin, apyrase, 5 mM Hepes buffer and 40% hematocrit. The final platelet count was 300,000 per mm^3. The collagen-coated glass rods were rotated in the platelet suspension containing 40% hematocrit at 200 rpm for 10 minutes at 37°C. The rod was then rinsed at 200 rpm for 5 minutes at 37°C in modified Tyrode solution containing 10 mM EDTA.

*Compared with control.

TABLE 36

Effect of high concentrations of ASA, sulphinpyrazone or indomethacin, which block the formation of malondialdehyde, on the inhibition of platelet adherence to a collagen-coated surface when tested in the presence of 4% albumin and 40% hematocrit.

Drug	Malondialdehyde* formation (%)	Number of segments	Number of platelets per mm^2 (mean ± S.E.)	P
Control	100	8	71,000 ± 3,400	
ASA 10 mM	0	8	66,500 ± 2,200	<0.3
Sulphinpyrazone 2.5 mM	0	8	70,800 ± 1,400	<0.8
Indomethacin 100 μM	0	8	46,700 ± 1,800	<0.001

^{51}Cr-labeled rabbit platelets were suspended in Tyrode solution containing 4% albumin, apyrase and 5 mM Hepes buffer. They were incubated for 10 minutes with the inhibitor to be tested or its solvent. Then the red blood cells were added to give a final hematocrit of 40%. The final platelet count was 300,000 per mm^3. The rods were then rinsed at 200 rpm for 5 minutes at 37°C in modified Tyrode solution containing 10 mM EDTA.

*Malondialdehyde formation was measured on a sample of platelet suspension after incubation with the inhibitor or its solvent and addition of 1 U per ml thrombin (see materials and methods).

TABLE 37

Effect of hematocrit on the inhibition by ASA of platelet adherence to the subendothelial surface of an aorta damaged by a balloon catheter.

Hematocrit (%)	ASA (μM)	(n)	Number of platelets per mm^2 (mean ± S.E.)	P
0	0	(20)	9,900 ± 2,100	
	100	(20)	5,800 ± 1,500	<0.10
10	0	(30)	57,300 ± 7,900	
	10	(30)	24,200 ± 2,200	<0.001
	100	(30)	22,900 ± 1,200	<0.001
	250	(30)	17,200 ± 1,700	<0.001
20	0	(20)	153,100 ± 16,300	
	100	(20)	81,400 ± 15,100	<0.05
40	0	(80)	112,200 ± 9,400	
	100	(80)	114,800 ± 6,900	<0.10
	250	(80)	100,400 ± 10,600	<0.10

^{51}Cr-labeled rabbit platelets were suspended in Eagle's medium containing 0.35% albumin, 5 mM Hepes buffer and apyrase. ASA or control solution was incubated for 10 minutes with the platelet suspension before addition of the red cells. Platelet count was 700,000 per mm^3. Adherence was measured after rotation at 200 rpm for 10 minutes at 37°C. Statistical significance was assessed by an analysis of variance technique.

iv. Evidence that ASA inhibited adherence in the absence of aggregates formation on the surface.

Further experiments were performed in the presence or absence of red blood cells to demonstrate that ASA inhibited platelet deposition on collagen-coated glass rods by reducing platelet adherence in the absence of the formation of platelet aggregates on the surface.

(a) Another ADP removal enzyme system, CP/CPK was used. Platelet adherence to collagen was measured in the presence of the CP/CPK system, which converted ADP to ATP. In these conditions ASA inhibited platelet adherence to collagen-coated rods, when the suspending medium was containing 0.35 or 4% albumin and in the absence or presence of red blood cells (20% hematocrit) (Table 38). These results confirmed the results obtained when apyrase was used to remove any ADP lost from the platelets and demonstrated that ASA inhibited platelet adherence to collagen under conditions in which platelet aggregate formation was unlikely.

(b) Adherence of platelets suspended in a medium containing 0.35% albumin (at 0 and 20% hematocrit) or in a medium containing 4% albumin (at a 20% hematocrit) was measured in the presence or absence of 100 μM ASA. The probes were rotated for 10 minutes in the platelet suspension and then rinsed for 5 minutes in a platelet-free medium containing EDTA or EGTA. ASA inhibited platelet adherence to collagen-coated glass rods and this effect was not lost when the tubes were rinsed with EDTA after

TABLE 38

Effect of ASA on the adherence of ^{51}Cr-labeled rabbit platelets to collagen-coated rods in the presence of the enzyme converting system CP/CPK[a].

Experiment No.	Resuspending medium	ASA (μM)	Rinsing medium	(n)	Number of platelets per mm^2 (mean ± S.E.)	P
1.[b]	Tyrode + 0.35% albumin	0	EGTA 10 mM	(16)	37,800 ± 5,000	
		100	EGTA 10 mM	(16)	16,800 ± 1,700	<0.001
2.[c]	Tyrode + 4% albumin + Ht 20%	0	EDTA 10 mM	(8)	46,300 ± 1,900	
		100	EDTA 10 mM	(8)	31,600 ± 2,700	<0.01

a) The concentration of CP was 5 mM and that of CPK 10 U/ml. The collagen coated rods were rotated at 200 rpm for 10 minutes at 37°C.

b) Platelet count was 700,000 per mm^3.

c) Platelet count was 300,000 per mm^3.

the 10 minute rotation in the platelet suspension (Table 39). The fact that the inhibitory effect of ASA persisted when the rods were rinsed in a medium containing the chelating agent is evidence that ASA inhibited adherence to collagen and did not exert its effect by preventing aggregate formation on the surface.

v. In vivo effect of ASA is due to a permanent platelet modification.

The inclusion of ASA (10 µM) in the platelet suspending fluid decreased the number of platelets adherent to the scraped endothelial surface (Table 40). Experiments were done to determine whether the ASA was affecting the aorta, the platelets, or both platelets and aorta (Table 40). Scraped aorta segments were incubated at 37°C or 30 minutes in modified Tyrode solution containing 10 µM ASA. The aortic segments were rinsed four times in modified Tyrode solution and rotated in a platelet suspension that had not been exposed to ASA. This ASA treatment of the scraped aorta segments did not diminish the number of platelets adherent to them, compared with segments that had not been exposed to ASA. In contrast, when platelets were incubated for 15 minutes at 37°C in a medium containing 10 µM ASA, washed once in fresh suspending medium, and resuspended without ASA, their ability to adhere to the scraped aorta surface was significantly diminished (Table 40). Thus the inhibitory effect of ASA is exerted on the platelets and not on the damaged vessel wall.

The inhibitory effect of ASA was also apparent following its administration by stomach tubes to rabbits under sodium pentobarbital

TABLE 39

Effect of rinsing with EGTA and EDTA on the adherence of ^{51}Cr-labeled rabbit platelets to collagen-coated rods. Persistence of ASA inhibiting effect in various suspending media.

Experiment No.	Resuspending medium	ASA (μM)	Rinsing medium	(n)	Number of platelets per mm^2 (mean ± S.E.)	P*
1.	Tyrode + 0.35% albumin	0	control	(10)	34,900 ± 6,000	
		0	EGTA 10 mM	(10)	32,300 ± 4,300	<0.80
		100	control	(10)	7,800 ± 600	<0.001
		100	EGTA 10 mM	(10)	13,100 ± 1,800	<0.001
2.	Tyrode + 0.35% albumin + Ht 20%	0	EDTA 10 mM	(8)	96,000 ± 6,200	
		100	EDTA 10 mM	(8)	71,500 ± 1,500	<0.02
3.	Tyrode + 4% albumin + Ht 20%	0	EDTA 10 mM	(8)	94,900 ± 2,500	
		100	EDTA 10 mM	(8)	51,800 ± 1,300	<0.001

*See footnote on Table 17.

Platelet count was 700,000 per mm^3 (Experiment 1) and 300,000 per mm^3 (Experiment 2 and 3).

TABLE 40

Effect of ASA on the adherence of ^{51}Cr-labeled rabbit platelets suspended in Tyrode-albumin solution to scraped thoracic aorta segments.

Type of experiment	No. of segments	ASA (μM)	Number of platelets per mm^2 (mean ± S.E.)	P
ASA in platelet suspension[a]	6	0	260,000 ± 32,200	
	6	10	76,100 ± 12,700	<0.005
ASA-pretreatment of aorta segments[b]	7	0	243,800 ± 47,600	
	7	10	282,000 ± 43,200	<0.6
ASA-pretreatment of platelets[c]	12	0	395,400 ± 44,800	
	12	10	114,000 ± 16,300	<0.001

In all three experiments, the scraped aorta segments were rotated manually in Tyrode solution containing 0.35% albumin and no red blood cells for 10 minutes at 280 rpm at 37°C, and rinsed 4 times in modified Tyrode solution before their radioactivity was determined. No aggregates were observed in the platelet suspension by phase-contrast microscopy. P values calculated for ASA treatment vs. control without ASA.

a) Platelet suspension incubated for 10 minutes at 37°C with modified Tyrode solution or 10 μM ASA before aorta segments were rotated in it.

b) Scraped thoracic aorta incubated for 30 minutes at 37°C with modified Tyrode solution or 10 μM ASA, rinsed 4 times in modified solution, and then rotated in platelet suspension that had not been treated with ASA.

c) Platelets (10^6 per mm^3) were incubated in the first washing fluid for 15 minutes at 37°C with modified Tyrode solution or 10 μM ASA. They were then washed once and resuspended in fresh medium, without ASA, at a platelet count of 700,000 per mm^3.

anaesthesia (20 mg per kilogram) (Table 41). The rabbits were given either 200 mg per kilogram or 25 mg per kilogram of ASA suspended in water at times ranging from 1 hour to 17 hours before the blood was collected and the aortas removed. Control rabbits were given water by stomach tube. Platelets in suspension prepared from blood obtained from rabbits to which ASA had been administered were much less adherent to the scraped aortic segments than platelets from the control rabbits. With both types of platelets, the extent of adherence was unrelated to whether or not the aorta had been exposed to ASA in vivo. There was no significant difference ($p < 0.4$) between the adherence of platelets recovered 1 hour or 15 hours after administration of 25 mg per kilogram of ASA. Washed platelets prepared from these rabbits 1 hour or 15 hours after administration of ASA (25 mg per kilogram) did not aggregate upon the addition of acid-soluble collagen at a dilution of 1/3,000, whereas platelets from the control rabbits aggregated extensively.

(b) EFFECT OF SODIUM SALICYLATE

Sodium salicylate inhibited platelet adherence to collagen-coated glass rods only when used in high concentrations (1 mM). Sodium salicylate did not inhibit platelet adherence to the collagen-coated glass rods when the suspending medium was containing 4% albumin and 20 or 40% hematocrit (Table 42).

(c) EFFECT OF SULFINPYRAZONE

Sulfinpyrazone inhibited platelet adherence to a collagen-coated

TABLE 41

Effect of oral administration of ASA to rabbits on adherence of ^{51}Cr-labeled platelets to scraped thoracic aorta segments.

Exp. No.	Amount of ASA administered (mg/kg)[a]	Time between ASA administration and blood collection (hours)	Source of aorta segments[a]	Source of platelets[a]	Number of segments	Number of platelets per mm^2 (mean ± S.E.)	P[b]
1.	0	1	Control	Control	18	421,000 ± 53,200	
	200	1	Control	ASA	12	147,700 ± 18,100	<0.001
	200	1	ASA	Control	10	385,600 ± 36,900	
	200	1	ASA	ASA	14	143,900 ± 22,300	<0.001
2.	0	17	Control	Control	6	213,000 ± 39,700	
	200	17	Control	ASA	6	91,500 ± 21,600	<0.02
	200	17	ASA	Control	7	176,100 ± 19,000	
	200	17	ASA	ASA	6	99,300 ± 3,800	<0.001
3.	0	15	Control	Control	10	458,100 ± 49,100	
	25	15	Control	ASA	10	120,700 ± 16,900	<0.001
	25	1	Control	ASA	9	100,600 ± 16,600	<0.001

The aorta segments were rotated manually in the platelet suspension. Platelets were suspended in Tyrode solution containing 0.35% albumin, apyrase and no red blood cells. Platelet count was 700,000 per mm^3.

a) Control rabbits were given water by stomach tube before preparation of "control" aorta segments and "control" platelet suspensions.

b) In Experiments 1 and 2, mean values were compared with the "control" platelet values on the line above; in Experiment 3, both values were compared with the value obtained with "control" aorta and "control" platelets.

TABLE 42

Effect of sodium salicylate on the adherence of washed rabbit platelets labeled with ^{51}Cr to a collagen-coated glass rod.

Experiment	Sodium salicylate (mM)	Albumin (%)	Hematocrit (%)	(n)	Number of platelets per mm^2 (mean ± S.E.)	P
1.	0	0.35	0	(16)	135,900 ± 10,000	
	0.1			(8)	114,200 ± 11,000	<0.2
	1			(8)	16,100 ± 1,700	<0.001
2.	0	4	20	(4)	83,800 ± 3,000	
	1	4	20	(8)	76,200 ± 7,700	<0.4
	0	4	40	(8)	110,300 ± 12,800	
	1	4	40	(8)	95,900 ± 6,100	<0.4

Platelets were suspended in Tyrode solution containing albumin, apyrase and Hepes buffer. Platelet count was 700,000 per mm^3 (Experiment 1) or 300,000 per mm^3 (Experiment 2). They were incubated for 10 minutes with sodium salicylate or control solution. The collagen-coated rods were rotated in the platelet suspension at 200 rpm for 10 minutes at 37°C. The rods were rinsed at 200 rpm for 5 minutes in modified Tyrode solution containing 10 mM EDTA.

surface or to an aorta damaged by scraping or by ballooning when measured in a medium containing 0.35% albumin, at 0, 10 or 20% hematocrit (Tables 43 and 44).

In contrast to ASA, sulfinpyrazone (250 μM) inhibited platelet adherence to a balloon-damaged aorta when tested in a medium containing 0.35% albumin and 40% hematocrit (Table 44). However, when the albumin concentration was 4% and the hematocrit 40%, high concentrations of sulfinpyrazone (2.5 mM), enough to block completely malondialdehyde formation in response to 1 U/ml of thrombin, did not inhibit adherence to a collagen-coated rod (Table 36).

Sulfinpyrazone was given orally to rabbits (150 mg/kg) and platelets were harvested 2 hours later. They were labeled with ^{51}Cr, washed and resuspended in a medium containing 0.35% albumin, apyrase and no red blood cells. Scraped everted aortas were rotated for 10 minutes at 200 rpm in the platelet suspension. Platelets from sulfinpyrazone-treated rabbits adhered to the damaged aorta to the same extent as did control platelets, indicating that sulfinpyrazone had to be present to have an inhibitory effect.

(d) EFFECT OF INDOMETHACIN

Indomethacin at low concentrations (10 to 100 μM) inhibited adherence to collagen-coated rods when tested in the presence of CP/CPK in a medium containing albumin (0.35 or 4%) and red blood cells (0, 20, 40% hematocrit) (Table 45). When indomethacin was tested at concentrations ranging from 0.5 to 100 μM, adherence to collagen-coated rods (4% albumin,

TABLE 43

Effect of sulphinpyrazone on adherence of washed rabbit platelets labeled with ^{51}Cr to a collagen-coated rod.

Sulphinpyrazone (μM)	(n)	Number of platelets per mm^2 (mean ± S.E.)	P
0	(10)	120,200 ± 10,100	
1	(10)	95,800 ± 8,900	<0.1
10	(10)	71,100 ± 14,000	<0.01
100	(10)	29,500 ± 3,000	<0.001

^{51}Cr-labeled platelets were suspended in Tyrode solution containing 0.35% albumin and apyrase. Platelet count was 700,000 per mm^3. No red blood cells added. Adherence was measured after rotation at 200 rpm for 10 minutes at 37°C.

TABLE 44

Effect of sulfinpyrazone on adherence of washed rabbit platelets labeled with ^{51}Cr to a damaged segment of rabbit aorta.

Surface exposed	Hematocrit (%)	Sulfinpyrazone (μM)	(n)	Number of platelets per mm^2 (mean ± S.E.)	P
Ballooned aorta	0	0	(20)	8,700 ± 1,600	
		100	(20)	6,900 ± 1,900	<0.10
	10	0	(20)	66,500 ± 8,000	
		10	(20)	53,400 ± 5,500	<0.10
		100	(20)	27,300 ± 6,100	<0.05
		250	(20)	18,300 ± 6,000	<0.05
	20	0	(20)	166,100 ± 17,800	
		100	(20)	69,600 ± 9,800	<0.05
	40	0	(80)	112,200 ± 9,400	
		100	(20)	91,800 ± 8,000	<0.10
		250	(50)	83,000 ± 6,500	<0.05
Scraped aorta	0	0	(20)	7,400 ± 2,000	
		100	(20)	6,900 ± 2,000	<0.10
	10	0	(20)	99,800 ± 7,900	
		10	(20)	105,000 ± 14,500	<0.10
		250	(20)	19,500 ± 1,000	<0.01
	20	0	(20)	131,400 ± 9,700	
		100	(20)	67,900 ± 11,100	<0.05
	40	0	(20)	139,000 17,000	
		100	(20)	105,700 6,100	<0.10

^{51}Cr-labeled platelets were suspended in Eagle's medium containing 0.35% albumin, 5 mM Hepes buffer and apyrase. Sulfinpyrazone or control was incubated for 10 minutes with the platelet suspension before addition of the red cells. Platelet count was 700,000 per mm^3. Adherence was measured after rotation at 200 rpm for 10 minutes at 37°C. Statistical significance was assessed by an analysis of variance technique

TABLE 45

Effect of indomethacin on the adherence of ^{51}Cr-labeled rabbit platelets to a collagen-coated glass rods in the presence of the enzyme converting system CP/CPK*.

Experiment No.	Resuspending medium	Indomethacin (μM)	Rinsing medium	(n)	Number of platelets per mm^2 (mean ± S.E.)	P
1.	Tyrode + 0.35% albumin	0	Ca^{2+} free Tyrode	(4)	20,000 ± 4,500	
		10		(4)	6,300 ± 600	<0.001
2.	Tyrode + 4% albumin + Ht 40%	0	Ca^{2+} free Tyrode	(10)	114,500 ± 2,500	
		10		(10)	105,600 ± 1,300	<0.001
3.	Tyrode + 4% albumin + Ht 20%	0	Ca^{2+} free Tyrode + 10 mM EDTA	(8)	46,300 ± 1,900	
		100		(8)	19,600 ± 1,700	<0.001

*CP (5 mM) and CPK (10 U/ml)

see footnote of Table 33.

20% hematocrit) was inhibited by about 30% in every case. In this range of indomethacin concentrations there was a progressive inhibition of malondialdehyde formation by platelets exposed to thrombin (Table 46).

In another series of experiments the effect of indomethacin on platelet adherence to collagen was tested in a medium containing 4% albumin and 40% hematocrit and compared to the effect of two other anti-inflammatory drugs, ASA and sulfinpyrazone, tested in similar conditions. At 40% hematocrit and 4% albumin, indomethacin inhibited platelet adherence to collagen when low concentrations (1 or 100 μM) were used. In contrast ASA (250 μM) and sulfinpyrazone (250 μM) did not have any inhibitory effect on adherence (Table 47).

Platelets in a medium containing 0.35% albumin were exposed to 100 μM indomethacin for 10 minutes, washed and resuspended in a medium containing 4% albumin and 40% red cells. Platelet adherence to the collagen-coated surface was inhibited to the same extent as had been observed when the drug was not removed from the suspending medium before adherence was tested (Table 35). These results demonstrated that indomethacin inhibited platelet adherence to collagen at a 40% hematocrit and that the effect on platelets persisted despite washing of the platelets and resuspension in fresh medium.

Indomethacin was also tested at a concentration (100 μM) which was shown to inhibit completely malondialdehyde formation in response to 1 U/ml of thrombin. Indomethacin was compared with ASA (10 mM) or sulfinpyrazone (2.5 mM). These are concentrations of these drugs which

TABLE 46

Effect of indomethacin on the inhibition of adherence of washed rabbit platelets labeled with ^{51}Cr to a collagen-coated glass rod and on the inhibition of malondialdehyde (MDA) formation.

Experiment No.	Indomethacin (μM)	MDA* formation (%)	(n)	Number of platelets per mm^2 (mean ± S.E.)	p**
1.	0	100	(32)	60,500 ± 2,300	
2.	0.5	82	(8)	42,200 ± 1,200	<0.001
3.	1	32	(8)	38,400 ± 1,800	<0.001
4.	2	4.5	(8)	40,000 ± 700	<0.001
5.	5	0	(16)	40,200 ± 1,600	<0.001
6.	10	0	(16)	43,200 ± 1,700	<0.001
7.	50	0	(8)	42,800 ± 1,600	<0.001
8.	100	0	(8)	42,900 ± 800	<0.001

^{51}Cr-labeled platelets were suspended in Tyrode solution containing 4% albumin, apyrase and 5 mM Hepes buffer. They were incubated for 10 minutes with indomethacin or control solution. Then the red blood cells were added to give a final hematocrit of 20%. The final platelet count was 300,000 per mm^3. The collagen-coated rods were rotated in the platelet suspension containing 20% hematocrit at 200 rpm for 10 minutes at 37°C. The rod was then rinsed at 200 rpm for 5 minutes in modified Tyrode solution containing 10 mM EDTA.

*MDA formation was measured on a sample of platelet suspension after incubation with the inhibitor or its solvent and addition of 1 U per ml thrombin (see materials and methods).

**Compared with control. 2, 3, 4, 5, 6, 7, 8 were not significantly different from each other $p < 0.30$.

TABLE 47

Effect of non-steroidal anti-inflammatory drugs on the inhibition of platelet adherence to a collagen-coated surface when tested in the presence of 4% albumin and 40% hematocrit.

Drug (μM)	Number of segments	Number of platelets per mm^2 (mean ± S.E.)	p*
Control (0)	8	79,000 ± 800	
Indomethacin (100 μM)	8	54,600 ± 700	<0.001
Control (0)	8	118,900 ± 2,900	
Indomethacin (1μM)	8	81,800 ± 900	<0.001
Control (0)	10	121,600 ± 5,400	
ASA (250μM)	10	115,600 ± 3,300	<0.5
Sulfinpyrazone (250 μM)	10	118,100 ± 2,600	<0.6

^{51}Cr-labeled rabbit platelets were suspended in Tyrode solution containing 4% albumin, apyrase and 5 mM Hepes buffer. They were incubated for 10 minutes with the inhibitor to be tested or its solvent. Then the red blood cells were added to give a final hematocrit of 40%. The final platelet count was 300,000 per mm^3. The collagen-coated glass rods were rotated in the platelet suspension containing 40% hematocrit at 200 rpm for 10 minutes at 37°C. The rod was then rinsed at 200 rpm for 5 minutes at 37°C in modified Tyrode solution containing 10 mM EDTA.

*Compared with control.

also completely inhibit malondialdehyde formation in response to 1 U/ml of thrombin. At these concentrations, only indomethacin inhibited platelet adherence to collagen when tested at 4% albumin and 40% hematocrit (Table 36). These results (Table 36) and the results reported in Table 46 demonstrated that the method of measuring adherence to collagen measured adherence and its inhibition independently of the inhibition of the release reaction and of the formation of endoperoxides and thromboxane A_2 from platelet arachidonate.

3. Inhibition of platelet adherence by metabolic inhibitors.

Iodoacetate markedly reduced platelet adherence to a collagen-coated surface. The addition of antimycin, an inhibitor of oxidative phosphorylation, did not decrease significantly platelet adherence to collagen. A combination of iodoacetate and antimycin decreased adherence to collagen more than iodoacetate alone had and the difference was not significant (Table 48).

4. Inhibition of platelet adherence by agents that increase cyclic AMP levels.

(a) PROSTAGLANDIN E_1

In published experiments (Cazenave et al., 1974b), we have reported that prostaglandin E_1, in concentrations less than micromolar, strongly inhibited the adherence of pig platelets to collagen. Prostaglandin E_1, at low concentrations (1 μM), also inhibited platelet adherence to collagen in the presence of 4% albumin and 40% hematocrit in the rotating probe system (Table 49).

TABLE 48

Effect of metabolic inhibitors on adherence of washed rabbit platelets labeled with ^{51}Cr to a collagen-coated glass rod.

Experiment No.	Addition to platelet suspension	(n)	Number of platelets per mm^2 (mean ± S.E.)	P
1.	Control ethanol	(10)	203,400 ± 28,900	
2.	Iodoacetate 10 μM	(10)	21,300 ± 2,100	<0.001
3.	Antimycin 50 μM	(10)	144,800 ± 9,700	<0.10
4.	Iodoacetate 100 μM + antimycin 50 μM	(10)	16,100 ± 2,000	<0.001

^{51}Cr-labeled platelets were suspended in Tyrode solution containing 0.35% albumin and apyrase. Platelet count was 700,000 per mm^3. No red blood cells added. Adherence was measured after rotation at 200 rpm for 10 minutes at 37°C.

2 vs. 4: $P < 0.10$.

TABLE 49

Effect of PGE_1 on adherence of washed rabbit platelets labeled with ^{51}Cr to a collagen-coated glass rod.

PGE_1 (μM)	Number of segments	Number of platelets per mm^2 (mean ± S.E.)	P*
0	10	153,300 ± 2,700	
1	10	63,000 ± 6,400	<0.001
10	10	78,400 ± 1,300	<0.001

^{51}Cr-labeled rabbit platelets were suspended in Tyrode solution containing 4% albumin, apyrase and 5 mM Hepes buffer. They were incubated for 10 minutes with the inhibitor to be tested or its solvent. Then the red blood cells were added to give a final hematocrit of 40%. The final platelet count was 300,000 per mm^3. The collagen-coated glass rods were rotated in the platelet suspension containing 40% hematocrit at 200 rpm for 10 minutes at 37°C. The rod was then rinsed at 200 rpm for 5 minutes at 37°C in modified Tyrode solution containing 10 mM EDTA.

*Compared with control.

(b) PYRIMIDO-PYRIMIDINE COMPOUNDS

In previous experiments (Cazenave et al., 1974c), 4 pyrimido-pyrimidines (RA 8, RA 233, RA 433 and VK 744) were tested for their effect on the adherence of pig platelets to collagen-coated tubes. Of the 4 compounds tested, RA 433 was the most effective inhibitor of platelet adherence to collagen. RA 8 had the least effect on adherence. The effect of RA 8 and RA 433 on adherence was examined using the rotating probe device (Table 50).

RA 433 and RA 8 were strong inhibitors of adherence to collagen in the presence of 40% red cells. RA 8 effect was less effective when there were no red cells in the medium. RA 8 also inhibited platelet adherence to a ballooned aorta in a medium containing 4% albumin and 20 or 40% hematocrit.

5. Inhibition of platelet adherence by reserpine.

Platelets in suspensions prepared from rabbits given reserpine intraperitoneally were less adherent than control platelets to collagen-coated glass surfaces or to the subendothelium of the rabbit thoracic aorta (Table 51).

The presence of reserpine (0.2 to 10 μM) in the platelet-suspending medium reduced the number of platelets that adhered to collagen-coated glass surfaces or to the subendothelium of the rabbit thoracic aorta (Table 52).

6. Inhibition of platelet adherence by the antibiotics penicillin G and cephalothin.

We have previously (Cazenave et al., 1973a) demonstrated that

TABLE 50

Effect of pyrimido-pyrimidine compounds RA8 and RA433 on adherence of washed rabbit platelets labeled with ^{51}Cr to collagen-coated rods or balloon-injured segments of rabbit aorta.

Exp. No.	Surface exposed	Albumin (%)	Hematocrit (%)	(n)	Inhibitor	(μM)	Number of platelets per mm^2 (mean ± S.E.)	P*
1.	Collagen-coated glass	4	40	(10)	-	0	137,400 ± 3,200	
				(10)	RA 8	100	83,100 ± 1,000	<0.001
				(10)	RA 8	250	79,000 ± 1,300	<0.001
2.	Collagen-coated glass	4	40	(10)	-	0	146,100 ± 5,000	
				(10)	RA 433	100	103,100 ± 1,400	<0.001
				(10)	RA 433	250	67,100 ± 1,700	<0.001
3.	Collagen-coated glass	4	0	(8)	-	0	12,200 ± 600	
			0	(8)	RA 8	100	12,900 ± 1,100	<0.6
			10	(8)	-	0	92,600 ± 6,000	
			10	(8)	RA 8	100	48,800 ± 1,800	<0.001
			20	(8)	-	0	140,100 ± 9,800	
			20	(8)	RA 8	100	98,000 ± 4,400	<0.001
			40	(8)	-	0	124,200 ± 3,900	
			40	(8)	RA 8	100	90,000 ± 2,200	<0.001
4.	Ballooned aorta	0.35	0	(8)	-	0	20,500 ± 2,100	
			0	(8)	RA 8	100	13,000 ± 1,800	<0.02
			20	(8)	-	0	94,700 ± 8,200	
			20	(8)	RA 8	100	41,300 ± 7,400	<0.001
			40	(8)	-	0	127,400 ± 6,100	
			40	(8)	RA 8	100	87,200 ± 3,700	<0.001

Experiments 1, 2, 3. ^{51}Cr-labeled platelets were suspended in Tyrode solution containing 4% albumin, apyrase and 5 mM Hepes buffer. They were incubated for 10 minutes with the inhibitor to be tested or its solvent. For RA 8 the final pH was 6.5 and for RA 433 it was 7.1. Then the red blood cells were added to give the final hematocrit desired. The final platelet count was 300,000 per mm^3. The collagen-coated glass rods were rotated in the platelet suspension containing red cells at 200 rpm for 10 minutes at 37°C. The rod was rinsed at 200 rpm for 5 minutes at 37°C in modified Tyrode solution containing 10 mM EDTA.

Experiment 4. Similar conditions as above except that the albumin concentration was 0.35% and the final platelet count

TABLE 51

Effect of intraperitoneal administration of reserpine on the adherence of rabbit platelets labeled with ^{51}Cr to collagen-coated glass or everted scraped segments of rabbit aorta.

Exp. No.	Surface exposed to platelets	Albumin (%)	(n)	Number of platelets per mm^2 (mean ± S.E.)		P^{**}
				Control	Reserpine*	
1.	Collagen-coated glass tubes	0.35	(5)	70,700 ± 3,700	53,500 ± 4,200	<0.02
2.	Collagen-coated glass rods	4	(5)	30,200 ± 4,400	13,600 ± 1,000	<0.005
3.	Collagen-coated glass rods	4	(9)	28,900 ± 900	25,900 ± 1,000	<0.05
4.	Everted scraped aorta	4	(5)	104,500 ± 13,800	67,400 ± 6,800	<0.05

^{51}Cr-labeled rabbit platelets were suspended in Tyrode solution containing albumin and apyrase. Platelet count was 700,000 per mm^3. No red blood cells added.

* Reserpine (5 mg/kg) was injected intraperitoneally 18 hours prior to collection of blood. Controls were injected similarly with an equal volume of water.

**Compared with control in each experiment.

TABLE 52

Effect of in vitro addition of reserpine on the adherence of rabbit platelets labeled with ^{51}Cr to collagen-coated glass or everted scraped segments of rabbit aorta.

Exp. No.	Surface exposed to platelets	Albumin (%)	(n)	Reserpine (μM)	Number of platelets per mm^2 (mean ± S.E.)	P*
1.	Collagen-coated glass tubes	0.35	(5)	0	67,800 ± 5,900	
			(5)	0.2	18,500 ± 3,100	<0.001
			(5)	2	20,400 ± 1,400	<0.001
			(5)	10	3,600 ± 300	<0.001
2.	Collagen-coated glass rods	4	(5)	0	33,500 ± 1,000	
			(5)	0.2	35,000 ± 3,500	<0.80
			(5)	2	26,600 ± 1,600	<0.01
			(5)	10	19,000 ± 2,800	<0.005
3.	Everted scraped aorta	4	(5)	0	40,300 ± 3,300	
			(5)	0.2	31,500 ± 6,500	<0.30
			(5)	2	16,700 ± 5,100	<0.01
			(5)	10	20,300 ± 5,300	<0.02

^{51}Cr-labeled rabbit platelets were suspended in Tyrode solution containing albumin and apyrase. Platelet count was 700,000 per mm^3. No red blood cells added.

* Compared with control in each experiment.

penicillin G diminished the extent of adherence of washed human, rabbit or pig platelets to a collagen-coated surface.

Cephalothin, at low concentrations almost completely inhibited platelet adherence to collagen-coated glass rods when tested in a medium containing 0.35% albumin and in the absence of red cells (Table 53).

High concentrations of penicillin G (13.6 mM) or cephalothin (13.6 mM) markedly reduced platelet adherence to collagen-coated glass rods in a medium containing 4% albumin and 40% red cells (Table 53).

A similar inhibitory effect of penicillin G or cephalothin was demonstrated with damaged aortas (Table 53).

Both penicillin G and cephalothin at concentrations of 6.8 or 13.6 mM inhibited the adherence of platelets to the surface of everted rabbit aortas that had been damaged with either a balloon catheter or by scraping (Table 53). These inhibitory effects were apparent in medium containing 4% albumin, and 40% red cells, or 0.35% albumin and 10% red cells. At a concentration of 1.36 mM the antibiotics inhibited adherence to the scraped aortic surface when the platelets were suspended in the latter solution (Table 53).

7. Inhibition of platelet adherence by methylprednisolone.

Methylprednisolone, at high concentrations (1.5 mM and 15 mM) was a strong inhibitor of platelet adherence to collagen-coated glass rods rotated in a medium containing 0.35% albumin and no red blood cells (Table 54).

TABLE 53

Effect of cephalothin or penicillin G on adherence of washed rabbit platelets labeled with ^{51}Cr to collagen-coated glass rod or to everted scraped or balloon-injured segments of rabbit aorta.

Exp. No.	Surface exposed	Albumin (%)	Hematocrit (%)	(n)	Antibiotic	(mM)	Number of platelets per mm^2 (mean ± S.E.)	P
1.	Collagen-coated glass	0.35	0	(5)	-	0	23,000 ± 2,400	
				(5)	cephalothin	1.5	10,100 ± 2,600	<0.01
				(5)	cephalothin	7.5	5,000 ± 700	<0.001
				(5)	cephalothin	15.0	4,200 ± 60	<0.001
2.	Collagen-coated glass	4	40	(8)	-	0	89,000 ± 3,300	
				(8)	penicillin G	13.6	33,500 ± 700	<0.001
				(8)	cephalothin	13.6	29,000 ± 700	<0.001
3.	Scraped aorta	0.35	10	(20)	-	0	73,000 ±11,200	
				(10)	penicillin G	1.36	21,100 ± 2,200	
				(10)	penicillin G	6.8	16,100 ± 900	<0.001
				(10)	penicillin G	13.6	13,500 ± 700	<0.001
4.	Scraped aorta	0.35	10	(10)	-	0	40,400 ±11,200	
				(10)	cephalothin	1.36	29,400 ± 3,700	<0.40
				(10)	cephalothin	6.8	8,600 ± 900	<0.02
				(10)	cephalothin	13.6	9,000 ± 1,100	<0.02
5.	Ballooned aorta	4	40	(20)	-	0	118,200 ± 7,600	
				(10)	penicillin G	13.6	25,900 ± 2,100	<0.001
				(10)	cephalothin	13.6	49,900 ± 6,100	<0.001
6.	Ballooned aorta	4	40	(20)	-	0	107,700 ±11,700	
				(20)	penicillin G	6.8	52,400 ± 5,500	<0.001
				(20)	cephalothin	6.8	74,200 ± 9,400	<0.05
7.	Ballooned aorta	4	40	(10)	-	0	108,100 ± 6,300	
				(10)	penicillin G	13.6	30,500 ± 2,300	<0.001
				(10)	penicillin G	1.36	73,200 ± 9,700	<0.02
				(10)	cephalothin	1.36	121,100 ± 6,100	<0.20

^{51}Cr-labeled platelets were suspended in Tyrode solution containing albumin, apyrase and red blood cells. In experiments 1, 3, 4, 5, 6, 7 platelet count was 700,000 per mm^3. In experiment 2, it was 300,000 per mm^3. Adherence was measured at 200 rpm for 10 minutes at 37°C.

TABLE 54

Effect of methylprednisolone on the adherence of rabbit platelets labeled with ^{51}Cr to collagen-coated glass rods.

Methylprednisolone (mM)	(n)	Number of platelets per mm^2 (mean ± S.E.)	P
0	(10)	173,400 ± 29,000	
1.5	(10)	12,400 ± 1,200	<0.001
15	(10)	9,900 ± 2,600	<0.001

^{51}Cr-labeled platelets were resuspended in Tyrode solution containing 0.35% albumin and apyrase. The final platelet count was 700,000 per mm^3. No red blood cells added. Adherence was measured at 200 rpm for 10 minutes at 37°C.

TABLE 55

Effect of methylprednisolone on the adherence of rabbit platelets labeled with ^{51}Cr to a ballooned aorta.

Methylprednisolone (mM)	(n)	Number of platelets per mm^2 (mean ± S.E.)	P
0	(12)	117,900 ± 9,900	
0.025	(12)	42,100 ± 8,100	<0.001
0.25	(12)	29,700 ± 10,900	<0.001

^{51}Cr-labeled platelets were resuspended in Tyrode solution containing 0.35% albumin, apyrase and 40% hematocrit. The final platelet count was 700,000 per mm^3. Adherence was measured at 200 rpm for 10 minutes at 37°C.

TABLE 56

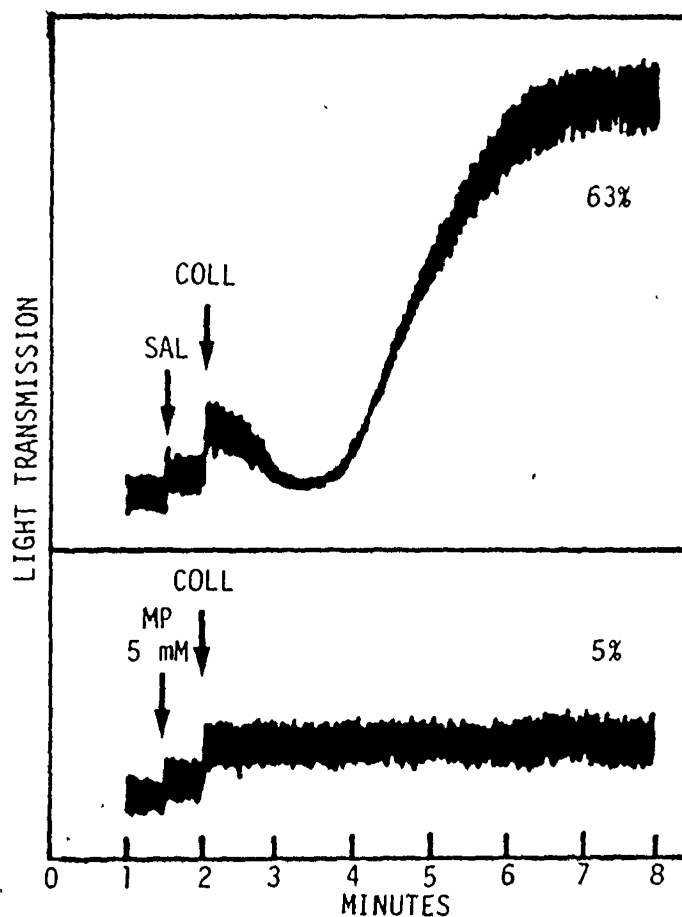
Effect of methylprednisolone on the adherence of rabbit platelets with ^{51}Cr to a collagen-coated glass rod.

Methylprednisolone (mM)	(n)	Number of platelets per mm^2 (mean ± S.E.)	P
0	(8)	118,800 ± 5,100	
0.25	(8)	100,100 ± 3,900	<0.02
1	(8)	94,000 ± 2,400	<0.001
2.5	(8)	77,800 ± 1,700	<0.001
5	(8)	49,400 ± 1,000	<0.001
10	(8)	19,600 ± 3,000	<0.001
15	(8)	11,400 ± 600	<0.001

^{51}Cr-labeled platelets were resuspended in Tyrode solution containing 4% albumin, 5 mM Hepes buffer, apyrase and 40% hematocrit. The final platelet count was 300,000 per mm^3. The collagen-coated glass rods were rotated at 200 rpm for 10 minutes at 37°C. The rods were rinsed at 200 rpm for 5 minutes at 37°C in modified Tyrode solution containing 10 mM EDTA.

FIGURE 11.

Methylprednisolone (MP) inhibition of the effects of collagen on washed rabbit platelets labeled with ^{14}C-serotonin. Platelet count was 500,000 per mm^3. MP or saline (SAL) was added 30 seconds before the addition of a collagen suspension (COLL). MP 5 mM inhibited shape change, aggregation and the release of ^{14}C-serotonin (shown on the right of the curves as % of total ^{14}C in the platelets) induced by collagen.



Similarly, methylprednisolone at a lower concentration (0.025 mM and 0.25 mM), strongly inhibited adherence to the surface of an aorta damaged by a balloon catheter. This inhibitory effect was demonstrated in a medium containing 0.35% albumin and a 40% hematocrit (Table 55).

High doses of methylprednisolone inhibited platelet adherence to collagen at 40% hematocrit. The degree of inhibition increased with the dose of methylprednisolone (Table 56). Methylprednisolone in high doses (5 mM) inhibited collagen-induced shape change (Figure 11). This dose-related effect on adherence was in contrast with that of indomethacin. Increasing indomethacin concentration did not increase the degree of inhibition of platelet adherence (Table 46).

8. Summary.

A number of drugs, which may have clinical relevance, has been examined for their effect on platelet adherence to collagen or to subendothelium. The rotating probe device was used and measurements of the adherence of ^{61}Cr-labeled rabbit platelets were performed under controlled flow conditions. The most rigorous test of the effect of a drug was to measure adherence in the presence of 4% albumin and 40% hematocrit. However, this system is costly for a screening procedure and can be replaced by a low albumin concentration (0.35%) and no red cells. Using such conditions, no drug would be missed due to false negative results.

The results obtained with collagen-coated glass rods or everted damaged aortas were in agreement, indicating that a collagen-

coated surface can be used in screening procedures. This is hardly surprising since collagen and collagen-like material appears to be the principal material in the vessel wall with which platelets interact.

Chelation of calcium ions by addition of EGTA, EDTA or sodium citrate markedly reduced platelet adherence to the surfaces. This indicates that there is a major problem in studies of platelet adherence in plasma containing anticoagulants.

Platelet adherence to collagen and subendothelium was inhibited by non-steroidal anti-inflammatory drugs (ASA, indomethacin, sulfinpyrazone), metabolic inhibitors, pyrimido-pyrimidines (RA 8, RA 433), inhibitors of shape change (PGE_1, methylprednisolone), reserpine and antibiotics (penicillin G, cephalothin).

The inhibitory effect on platelet adherence was influenced by the hematocrit and the albumin concentration, for example, the inhibitory effect of ASA on platelet adherence was lost when the hematocrit was raised to 40%.

Sulfinpyrazone inhibited platelet adherence to a ballooned aorta with 0.35% albumin and 40% hematocrit. However, when 4% albumin and 40% hematocrit were used, sulfinpyrazone did not inhibit adherence to a collagen-coated rod. Using these later conditions (4% albumin, 40% hematocrit), low concentrations of indomethacin inhibited adherence to collagen.

The degree of inhibition of adherence to collagen by ASA, sulfinpyrazone and indomethacin varied whereas all these drugs

completely inhibited malondialdehyde formation.

The pyrimido-pyrimidines (RA 8, RA 433), PGE_1, methylprednisolone and the antibiotics (penicillin G, cephalothin) were effective inhibitors of adherence to collagen when tested with 4% albumin and 40% hematocrit. RA 8, RA 433 and PGE_1 increase platelet cyclic AMP levels, PGE_1 and methylprednisolone inhibit platelet shape change and the antibiotics are thought to coat the platelet surface. Thus a variety of treatments inhibit the ability of platelets to adhere to collagen and the subendothelium.

VI. PLATELET ADHERENCE, BLEEDING TIME AND PLATELET SURVIVAL

In previous sections (III and V) it was shown that a number of platelet modifications by various treatments affected platelet adherence to collagen or to subendothelium, tested in vitro.

There is no simple relationship between platelet function in vivo and in vitro. Also, the factors that control platelet survival, primary hemostasis as measured by the bleeding time, and platelet adherence to collagen and to subendothelium are poorly understood. Several compounds which inhibit platelet adherence to surfaces have been shown to prolong platelet survival. Harker et al. (1974) have shown that endothelial damage due to experimental homocystinemia cause shortened platelet survival. Administration of dipyridamole to the animals corrects the shortened survival, probably by inhibiting platelet interaction with the vessel wall. ASA is a potent inhibitor of platelet release and aggregation induced in vitro by collagen (Zucker and Peterson, 1970); prolongs the bleeding time (Mustard and Packham, 1975) and does not prolong platelet survival in man (Genton et al., 1975). In vitro, the inhibitory effect of ASA on platelet adherence to collagen and subendothelium is lost at 40% hematocrit (Section V). In contrast to ASA, sulfinpyrazone inhibits platelet adherence to the subendothelium (Section V) and prolongs shortened platelet survival in man (Steele et al., 1973b; Smythe et al., 1965).

One of the reasons rabbit platelets were chosen for the adherence studies was the possibility of correlating the in vitro adherence results with in vivo tests of platelet function. We have

studied the effects of a number of platelet modifications by drugs and of modifications of the platelet surface on platelet survival and on the bleeding time from the jugular vein of rabbits. These studies have been done in collaboration with H.-J. Reimers, J.P. Greenberg, J. Hirsh and A. Senyi.

1. Modifications of platelets and platelet survival.

We have studied the effect of several treatments of platelets, which were shown in previous sections (III and V) to affect platelet adherence to collagen and subendothelium on platelet survival in rabbits. Platelets were modified in vitro by treatment with thrombin, neuraminidase or sodium periodate. The platelets were also treated in vitro with penicillin G and methylprednisolone.

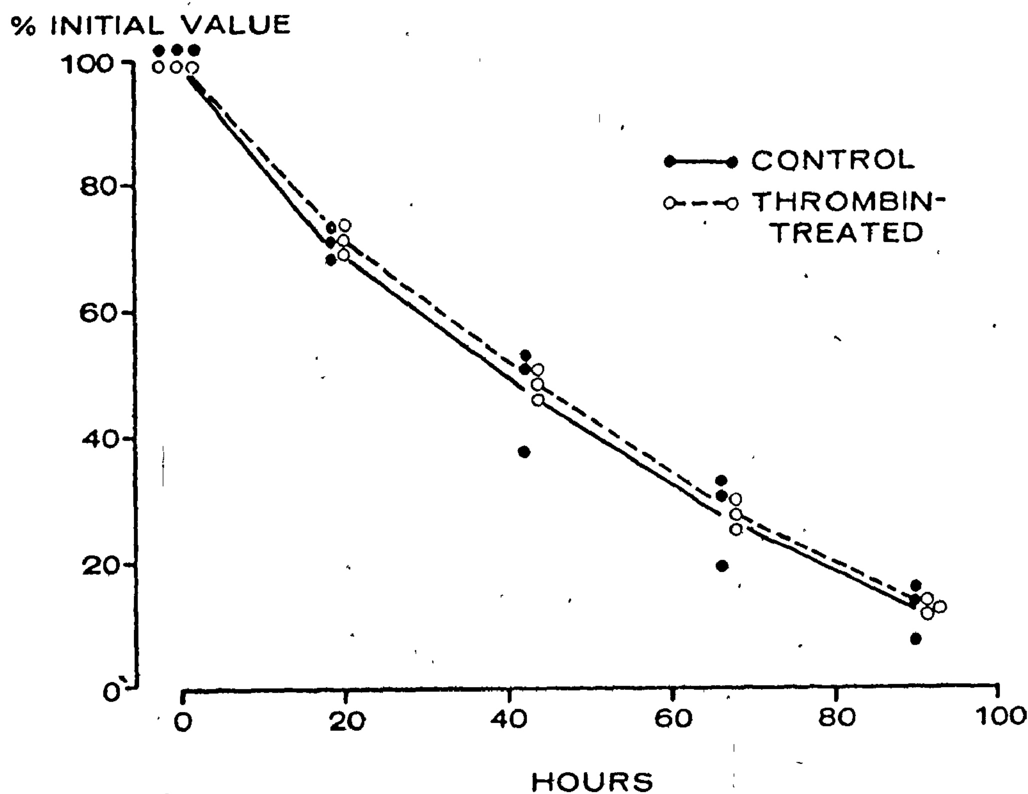
(a) Modifications of platelet membranes.

(i) Survival of thrombin-degranulated platelets (Reimers et al., 1976).

When thrombin-degranulated platelets were injected into normal rabbits, 46 ± 6% (mean ± S.E.; n = 9) were recovered in the circulation two hours after their infusion. In contrast, 87 ± 4% (mean ± S.E., n = 9) of the control platelets were recovered within the circulation two hours after their injection into rabbits. The difference is significant ($P < 0.001$). The thrombin-degranulated platelets that remained within the circulation at two hours showed the same pattern of disappearance as the control platelets (Figure 12). It should be pointed out that it may be the plasmin treatment used to recover the thrombin-degranulated

FIGURE 12.

Survival of thrombin-treated platelets in the rabbit circulation. One of three similar experiments in which thrombin-treated platelets or control platelets labeled with ^{51}Cr were injected into 3 rabbits in each group. Whole blood samples for radioactivity determinations were taken 2 hours (initial value), 19 hours, 42 hours, 66 hours and 90 hours after the injection of the radioactive platelets. Radioactivity at these times in whole blood samples was expressed as percentage of the radioactivity measured in the first sample (taken at 2 hours).



platelets that is responsible for the low percentage of these platelets that circulate in vivo (Reimers and Greenberg, personal communications).

(ii) Survival of neuraminidase-treated platelets (Greenberg et al., 1975).

Neuraminidase-treated rabbit platelets, injected intravenously into rabbits were rapidly cleared from the rabbits' circulation. If 8 or 10 percent of the total platelet sialic acid were removed, the reinjected platelets were not cleared immediately but were removed at an accelerated rate over a longer period of time. Provided that 15 percent or more of the total platelet sialic acid was removed, the reinjected platelets were cleared in less than 1 hour (Figure 13).

(iii) Survival of periodate-treated platelets.

^{51}Cr-labeled rabbit platelets which had been pretreated with $NaIO_4$ were infused into the circulation of rabbits. Platelets pretreated with 0.05 or 0.1 mM $NaIO_4$ survived normally, but platelets pretreated with 0.5 or 1 mM $NaIO_4$ for 10 minutes were rapidly removed from the circulation (Figure 14).

Platelets that had been pretreated with 1 mM $NaIO_4$ and then with 5 mM $NaBH_4$ remained in the circulation almost as long as platelets that had been treated with 5 mM $NaBH_4$ only, or the control platelets (Figure 15). The control platelets had not been treated with $NaIO_4$ or $NaBH_4$ but had been subjected to the same washing and resuspending procedures.

FIGURE 13.

Effect of neuraminidase treatment on survival of ^{51}Cr-labeled rabbit platelets injected intravenously into rabbits. The percentage of labeled platelets remaining in the circulation at times up to 90 hours after injection is shown.

Each line represents the mean values obtained with one group of rabbits. The eight control rabbits (●———●) received ^{51}Cr-labeled platelets that had been subjected to all the washing and resuspending procedures but had been incubated with acetate buffer instead of neuraminidase. Two rabbits received platelets from which 8% of the total sialic acid had been removed by neuraminidase treatment (○- - - -○), and two rabbits received platelets from which 10% of the total sialic acid had been removed (▲- - - -▲).

Four rabbits that received platelets from which 35% of the total sialic acid had been removed by neuraminidase treatment (△- - - -△) cleared all of the labeled platelets from their circulations before the first blood samples were taken at one hour.

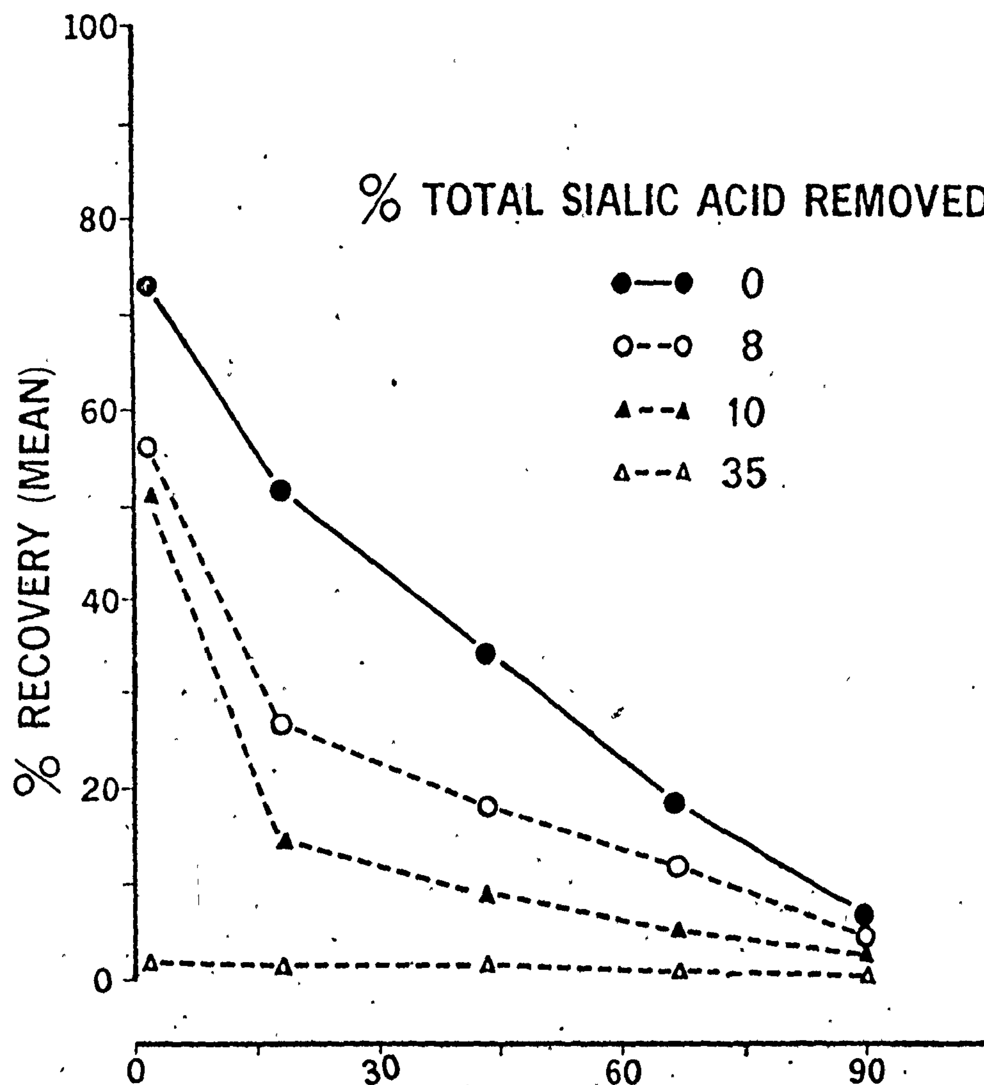


FIGURE 14.

Effect of $NaIO_4$ pretreatment on survival of ^{51}Cr-labeled platelets injected intravenously into rabbits. The platelets were treated with the concentrations of $NaIO_4$ shown on the figure for 10 minutes. The percentages of labeled platelets remaining in the circulation at times up to 90 hours after reinjection are shown. Each line represents the mean value of separate measurements. (Eight rabbits received control platelets, two rabbits received platelets treated with 0.05, 0.1, or 0.5 mM $NaIO_4$ and six rabbits received platelets treated with 1 mM $NaIO_4$).

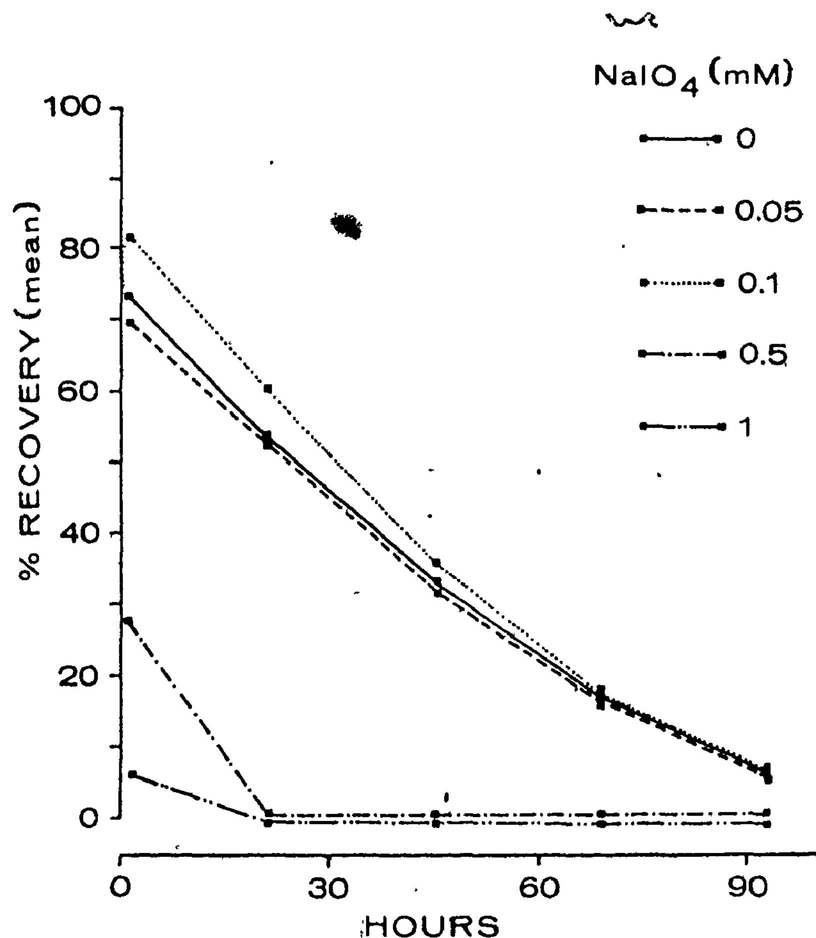
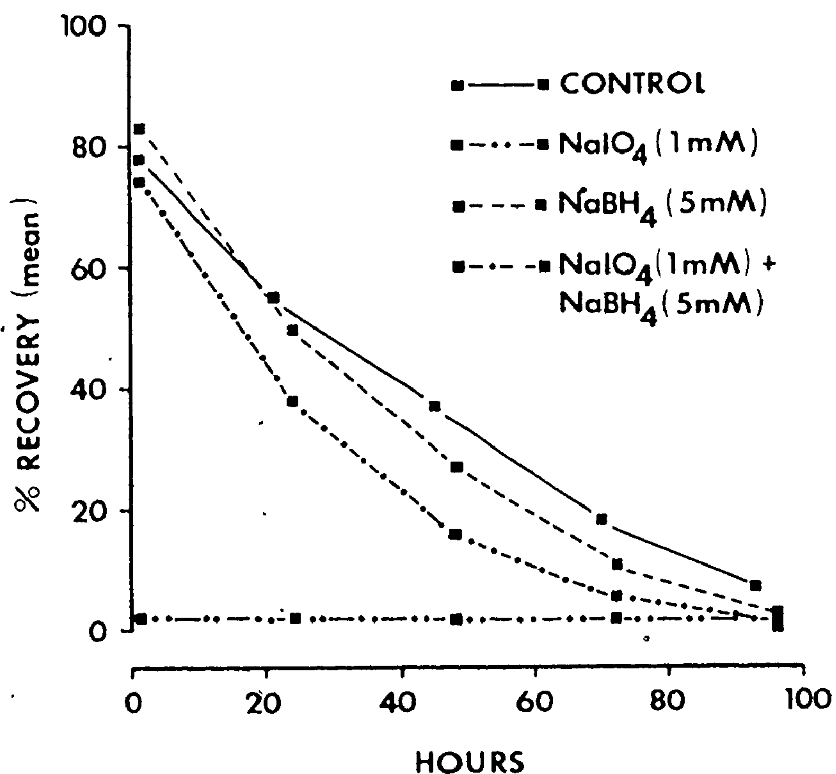


FIGURE 15.

Effect of $NaBH_4$ treatment of $NaIO_4$-pretreated platelets on their survival in the circulation of rabbits. The platelets were incubated with 1 mM $NaIO_4$ or 5 mM $NaBH_4$ or both. They were prelabeled with ^{51}Cr and the percentage of labeled platelets remaining in the circulation at times up to 96 hours after reinjection are shown. Each line represents the mean values of separate measurements (two rabbits for each treatment, except the control values which are the mean values from 10 rabbits). (This is one of three similar experiments).



(b) Modifications of platelets by drugs.

Rabbit platelets, labeled with ^{51}Cr, were pretreated with drugs, washed and resuspended in fresh medium. They were then reinjected intravenously into rabbits and their survival determined.

(i) Survival of penicillin G-treated platelets.

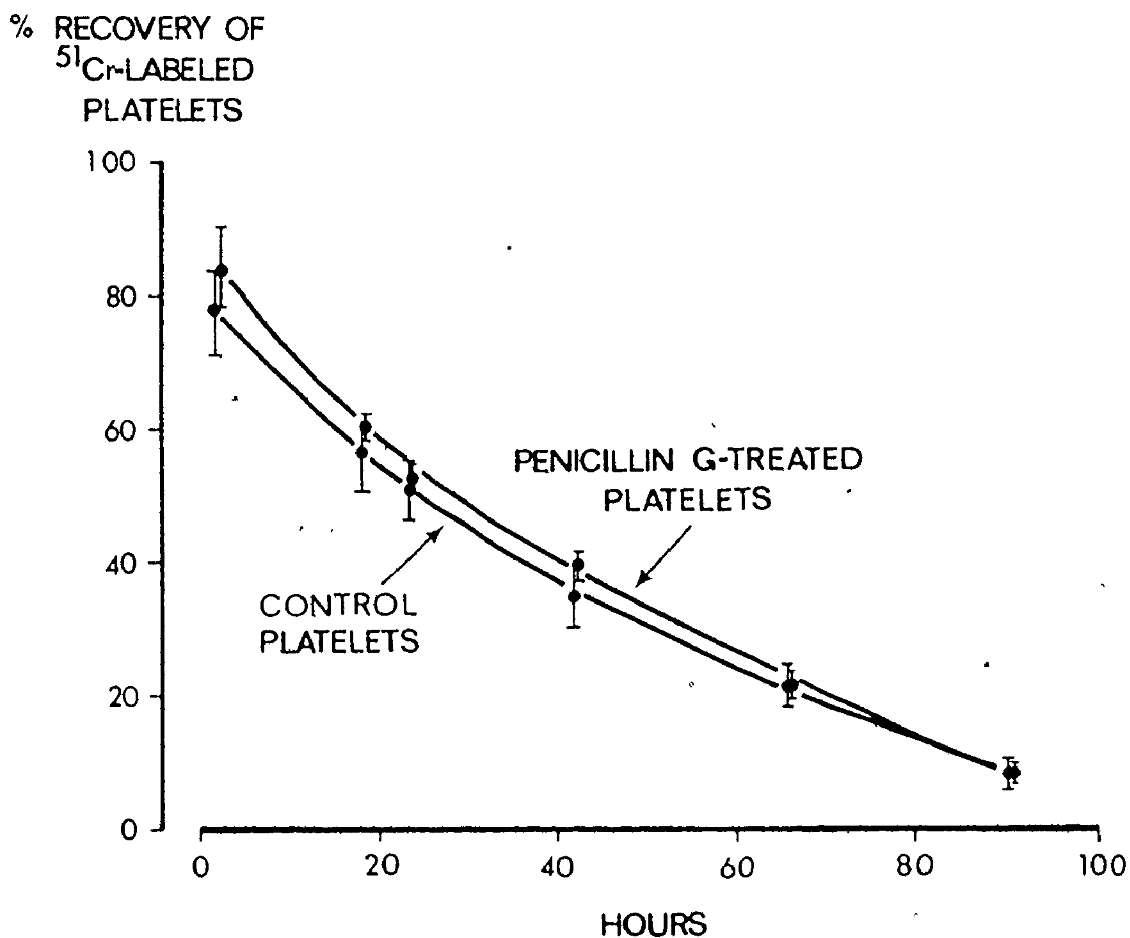
Pretreatment of platelets with penicillin G had no apparent effect on their survival in vivo (Figure 16). It has been demonstrated that the inhibitory effect of penicillin G in vitro was not removed by washing and resuspending the platelets (Cazenave et al., 1976d, 1977a), indicating that some penicillin probably remained associated with the platelets in vivo. Experiments by Brown et al. (1974) have shown that the effects of carbenicillin, a derivative of penicillin, on platelets persist for the life of the platelets in the circulation.

(ii) Survival of methylprednisolone-treated platelets.

Suspensions of washed rabbit platelets were labeled with ^{51}Cr, incubated with 0.9 or 4.5 mM methylprednisolone for 30 minutes, washed, resuspended and injected into normal rabbits. Tests with other samples of these methylprednisolone-treated platelets showed that the washing procedure did not remove the inhibitory effect of methylprednisolone on ADP, collagen or thrombin-induced aggregation (Cazenave et al., 1976). Methylprednisolone treatment had no significant effect on the recovery of injected platelets at 30 minutes [control (n = 9): 87 ± 3%; 0.9 mM methylprednisolone (n = 6): 84 ± 4%; 4.5 mM methylprednisolone (n = 6): 88 ± 9% (mean ± S.E.)], nor on their

FIGURE 16.

The effect of penicillin G on survival of control washed rabbit platelets and platelets that had been incubated in vitro with penicillin G (15 mM for 20 minutes at 37°C) and resuspended in fresh medium before infusion into the rabbits. Platelets were labeled in vitro with ^{51}Cr before penicillin G treatment. Means and standard errors of the means are shown. Four rabbits in each group.



survival time (Figure 17) [control: 81.6 ± 1.6 hours; 0.9 mM methylprednisolone: 85.2 ± 3.7 hours ($p < 0.7$) 4.5 mM methylprednisolone: 83.5 ± 4.4 hours ($p < 0.3$)].

2. Modifications of platelets and venous bleeding time.

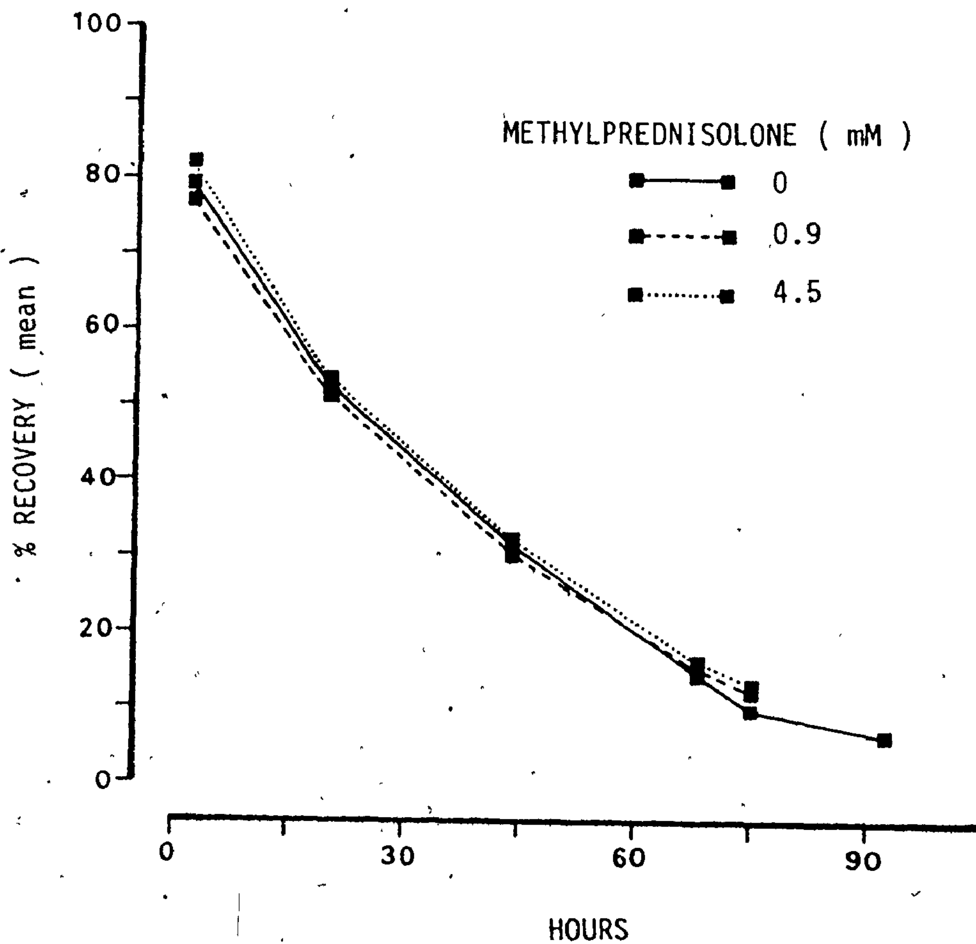
Rabbits were made thrombocytopenic by a combination of irradiation and administration of platelet antibody. In these results the bleeding time of a puncture wound in the jugular vein was markedly prolonged (764 to 1220 sec). The prolonged bleeding time was corrected to normal by infusion of normal rabbit platelets. We investigated the effect of various modifications of platelets on their ability to correct the prolonged jugular vein bleeding time of thrombocytopenic rabbits.

(a) Venous bleeding time upon infusion of thrombin-degranulated platelets into thrombocytopenic rabbits.

The combination of irradiation and platelet antibody caused the platelet counts in rabbits to fall consistently to less than 10,000/mm^3. The platelet count remained depressed after injection of the antibody for at least 48 hours. Two hours after the infusion of thrombin-degranulated platelets into thrombocytopenic rabbits 38 ± 5% (mean ± S.E.; n = 18) of the platelets were recovered in the circulation. In contrast, 71 ± 5% (mean ± S.E.; n = 17) of the control platelets could be recovered 2 hours after the infusion. To obtain the same peripheral platelet count, therefore, the thrombocytopenic animals which received thrombin-degranulated platelets were given twice as many platelets as the thrombocytopenic animals which received normal control platelets.

FIGURE 17.

Effect of methylprednisolone pretreatment of platelets on their survival in the rabbit circulation. ^{51}Cr-labeled platelets were incubated with 0, 0.9 and 4.5 mM methylprednisolone, washed and resuspended in fresh medium. Each line represents mean values of separate measurements (9 rabbits for the control group and 6 rabbits in each treatment group).



The infusion of 15 x 10^9 control platelets or 30 x 10^9 thrombin-degranulated platelets raised the platelet count in the thrombocytopenic animals from less than 10,000/mm^3 to 76,000 ± 7,000 (n = 7) when thrombin-treated platelets were infused, and to 81,000 ± 5,000 when untreated control platelets were infused. Control platelets shortened the bleeding time from a jugular vein puncture would from more than 900 sec to 124 ± 23 sec. Thrombin-degranulated platelets shortened the bleeding time from more than 900 sec to 463 ± 58 sec. The difference between the mean value for the normal and thrombin-treated platelets was statistically significant ($p < 0.001$) (Table 57).

(b) Modifications of platelets by drugs.

(i) Venous bleeding time upon infusion of penicillin G- or cephalothin-treated platelets into thrombocytopenic rabbits.

The range of bleeding times of 41 untreated rabbits (mean platelet count ± S.E.M., 414,000 ± 12,900/mm^3) was 60 to 180 sec [geometric mean and (99% confidence intervals). 100 (73 - 136)sec]. Bleeding time from puncture wounds in the jugular veins of thrombocytopenic rabbits with platelet counts of 4,000 to 16,000/mm^3 (mean ± S.E.M., 8,000 ± 130/mm^3) ranged from 764 to 1220 sec [geometric mean and (99% confidence intervals), 1012 (943-1098)sec]; these values were determined in a separate group of 12 rabbits that did not receive injections of platelets. Platelets which had not been treated with an antibiotic shortened the bleeding time of thrombocytopenic rabbits to

TABLE 57

Bleeding times[a] of thrombocytopenic rabbits given in vitro thrombin-treated (0.5 U/ml) platelets or control platelets.

Type of platelets	Number of determinations	Mean platelet count[b] (number per mm^3)		Bleeding time[c] (sec) mean
		Before infusion	Before bleeding time determinations	
Control	7	<10,000	81,500 ± 5,000	124
Thrombin-treated	7	<10,000	76,000 ± 7,000	463

a) Bleeding times of rabbits that had been made thrombocytopenic in this way and were not given infusions were greater than 900 seconds.

b) Mean ± S.E.M.

c) Geometric mean
Difference between means $P < 0.001$

within the range of bleeding times of untreated rabbits with normal platelet counts (Table 58). Although the platelets that had been pretreated with penicillin G or cephalothin did shorten the bleeding time from the puncture wounds in the jugular veins, they were much less effective than the control platelets which had not been treated with an antibiotic (Table 58). Injection of platelets pretreated with penicillin G shortened the bleeding time more than injection of cephalothin pretreated platelets. The observed difference between any two of the three means was statistically highly significant ($p < 0.001$).

The mean platelet counts of the 21 thrombocytopenic rabbits used for studies with the antibiotic-treated platelets are shown in Table 58. The mean platelet counts and the percentage recovery of ^{51}Cr-labeled platelets before the bleeding time determinations are also shown. In no instance was there a statistically significant difference ($p < 0.5$) between the three groups of animals; thus, the effect of the antibiotics on the bleeding time cannot be attributed to differences in platelet counts or platelet recovery.

(ii) Venous bleeding time upon infusion of methylprednisolone-treated platelets into thrombocytopenic rabbits.

When platelets that had been treated with 1 mM methylprednisolone were injected into thrombocytopenic rabbits, they did not correct the bleeding time which remained prolonged to 617 seconds. In contrast, the injection of control platelets did correct the bleeding time to normal, 104 seconds (Table 59).

TABLE 58.

Bleeding times[a] of thrombocytopenic rabbits given platelets preincubated in vitro with penicillin G, cephalothin, or neither antibiotic.

Antibiotic used for pretreatment of platelets	Number of determinations	Mean platelet count[b] (number per mm^3)		Percent of recovery of ^{51}Cr-labeled platelets[b]	Bleeding time (sec) mean (confidence interval)
		Before infusion	Before bleeding time determinations		
None (control)	14	4,600 ± 600	62,700 ± 4,700	68 ± 9	119 (98-144)
Penicillin G 1.5 mM	14	4,000 ± 500	56,600 ± 6,900	69 ± 8	375 (309-455)
Cephalothin 1.5 mM	14	4,600 ± 300	55,100 ± 6,200	65 ± 4	616 (507-748)

a) Bleeding times of rabbits that had been made thrombocytopenic in this way and were not given platelet infusions ranged from 764 to 1220 sec.

b) Mean ± S.E.M.

c) Geometric mean and 99% confidence intervals. Difference between any two of the three treatment means ($p < 0.001$, two-way analysis of variance).

TABLE 59

Bleeding times[a] of thrombocytopenic rabbits given platelets preincubated in vitro with methylprednisolone.

Methylprednisolone pretreatment of platelets	Number of determinations	Mean platelet count[b] (number per mm^3)		Percent of recovery of ^{51}Cr-labeled platelets[b]	Bleeding time[c] (sec) mean
		Before infusion	Before bleeding time determinations		
None (control)	10	13,400 ± 3,750	61,200 ± 9,200	69 ± 8	104
Methylprednisolone 1 mM	10	9,600 ± 3,200	64,600 ± 3,200	60 ± 10	617

a) Bleeding time of rabbits that had been made thrombocytopenic in this way and were not given platelet infusions ranged from 764 to 1220 sec.

b) Mean ± S.E.M.

c) Geometric mean. Difference between means $p < 0.001$.

In a second series of experiments, methylprednisolone (77 mg/kg) was injected intravenously to thrombocytopenic rabbits, 90 minutes before the jugular bleeding was measured. Control thrombocytopenic rabbits received saline. In control rabbits the bleeding remained > 900 seconds. The intravenous injection of methylprednisolone shortened the bleeding time to 173 seconds (Table 60).

3. Summary.

Rabbit platelets have been modified in vitro by various treatments with enzymes (thrombin, neuraminidase), chemicals (sodium periodate) and drugs (penicillin G, cephalothin and methylprednisolone) and reinjected into rabbits to study their ability to survive in the circulation and to participate in hemostasis. The effects of these platelet modifications were compared with their effects on platelet adherence to collagen-coated glass and to the subendothelium.

There was no apparent correlation between the factors which were found to influence platelet survival and platelet adherence to collagen and to the subendothelium. Thrombin, penicillin G or methylprednisolone treatment of platelets decreased their ability to adhere to collagen but did not influence their survival. Removal of platelet surface sialic acid by neuraminidase or modification of it by sodium periodate caused the platelets to be cleared from the circulation. Thus, platelet surface sialic acid may be an important determinant of platelet survival.

The measurement of platelet adherence to collagen in vitro seems

TABLE 60

Effect of intravenous injection of methylprednisolone on the jugular bleeding time of thrombocytopenic[a] rabbits.

Treatment	Number of determinations	Bleeding time[b] (sec) mean
Control	6	>900
Methylprednisolone (77 mg/kg)	6	173

a) Platelet count was 5,000 per mm^3

b) Geometric mean. Difference between means $P < 0.001$.

to bear some relationship to hemostasis as measured in the bleeding time studies. Treatment of platelets with thrombin, penicillin G or methylprednisolone decreased platelet adherence to collagen and to the subendothelium and depressed the ability of platelets to participate in hemostatic plug formation.

CHAPTER FOUR

DISCUSSION

The interaction of platelets with the components of the vessel wall is of key importance in hemostasis, in the initiation of thrombosis and in the development of atherosclerosis and its complications. The first step in the interaction of platelets with the vessel wall is platelet adherence to exposed components of the subendothelium, of which collagen fibers are the most thrombogenic (Stemerman, 1974; Baumgartner et al., 1976). Two principal methods, morphometric and isotopic, have been used to study the adherence of platelets to collagen, other protein-coated surfaces or subendothelium. These methods have been applied in vivo and in vitro. Some of these previous attempts to quantitate platelet adherence have met with several difficulties. Among some of the difficulties encountered were the need to use agents, such as EDTA or sodium citrate, to chelate calcium and thus prevent thrombin generation in the system. In addition, although EDTA made it possible to measure adhesion in the absence of platelet aggregation, it prevented an assessment of the role of divalent cations in platelet adhesion to collagen. The morphometric techniques involving preparation of histologic sections are time consuming and are associated with problems of selection of the appropriate sections. This technique gives a semi-quantitative evaluation of the interaction of platelets with the vessel wall.

For the above reasons, we have looked for an in vitro test for measuring the adherence of platelets to collagen-containing surfaces when no platelet aggregates are formed on the surface or in the suspending

medium. Platelets are labeled with radioisotopes and the adherence of platelets to the surfaces is quantitatively estimated by counting the platelet-bound radioactivity associated with the surface.

I. DEVELOPMENT OF THE ROTATING PROBE DEVICE TO MEASURE PLATELET ADHERENCE TO COLLAGEN-COATED GLASS AND TO EVERTED AORTA

The development of quantitative isotopic methods to measure platelet adherence to collagen-containing surfaces has culminated in the rotating probe device. This was possible because of previous work done when I was working in the laboratory of Dr. M.A. Packham at the University of Toronto (Cazenave et al., 1973a, b; 1974 a, b, c). During these early studies an isotopic method was established to quantitate the adherence of washed platelets to a collagen-coated glass tube. The method had advantages and disadvantages which have been already discussed (Results, Section I, A). It was demonstrated that, using radioactively labeled washed platelets, platelet adherence to a collagen-coated surface could be measured in the absence of platelet aggregation in a medium containing albumin, apyrase and physiological concentrations of divalent cations. This early method was useful because it established some of the conditions required to measure quantitatively platelet adherence to collagen-coated surfaces.

The rotating probe device was developed to measure, under controlled conditions, the adherence of platelets to collagen-coated glass rods (Results, section I, B) or to segments of everted thoracic aortas of rabbits (Results, section II). In addition to the advantages described for the collagen-coated tubes (Results, section I, A), the rotating probe device has advantages in the measurement of platelet adherence, which solve some of the limitations of the previous method. The effect of various factors on platelet adherence could be studied with the rotating probe device. Although all the problems were

not solved, some principles have been established. Only a few variables were studied in detail (albumin concentration, presence of red blood cells and nature of the surface used).

1. Evidence that platelet adherence is measured in the absence of platelet aggregation.

The fundamental difference between the methods developed in this study and other techniques for measuring platelet adherence (Hirsh et al., 1968b;Hovig et al., 1968; Spaet and Lejnieks, 1969; Lyman et al., 1971; Blaisdell et al., 1974; Gordon and Dingle, 1974; Baumgartner and Muggli, 1976; Brass et al., 1976; Castellan and Steiner, 1976; Dosne et al., 1976) is, as indicated above, that adherence studies are carried out in the presence of divalent cations. With collagen, there appear to be two mechanisms (Packham et al., 1977) whereby platelet aggregation can be induced: (1) the release of ADP; (2) the formation of the intermediates of arachidonic acid metabolism, such as the prostaglandin endoperoxides PGG_2 and PGH_2 and thromboxane A_2. In these experiments, the use of apyrase or CP/CPK removed the small quantities of ADP that might be released or lost from the platelets and thus prevented ADP-induced aggregation. However, since compounds which inhibit cyclo-oxygenase also appeared to inhibit platelet accumulation on the surface, it might be thought that some platelet aggregates could be formed through the action of the prostaglandin endoperoxides and thromboxane A_2. However, studies of the suspending medium and of the surface of the collagen-coated glass and of the subendothelium did not show the

presence of platelet aggregates. Moreover, with thrombin-degranulated platelets that can interact with collagen leading to the formation of platelet aggregates through the prostaglandin endoperoxides and thromboxane A_2 (Reimers et al., 1976) there was no effect of aspirin on the adherence of these platelets to the surface of the damaged aorta. In the presence of aspirin, collagen-induced aggregation of thrombin-degranulated platelets is completely inhibited. Thus it appears that under the conditions of these studies, the extent of the platelet-collagen interaction is not sufficient to generate quantities of prostaglandin endoperoxides and thromboxane A_2 that are adequate to induce platelet aggregation. Finally, it was not possible to decrease the extent of platelet adhesion to collagen-coated surfaces or to the subendothelium after the initial interaction by using agents which cause deaggregation of platelets previously aggregated by ADP, arachidonic acid or collagen. All this evidence shows that the method that has been developed makes it possible to study platelet interaction with collagen or the subendothelium of the vessel wall under conditions described in this thesis in which the principal measurement is platelet adherence to the collagen-containing surface in the absence of significant platelet aggregation.

2. Effect of plasma proteins.

(a) Albumin.

The effect of increasing the albumin concentration from 0.35% to 4% was studied under various conditions of platelet count,

speed of rotation and hematocrit. In any condition the number of platelets adherent to a collagen-coated glass rod or to a damaged aorta was less when the albumin concentration was 4% compared to the adherence measured with 0.35% albumin. The reason for the inhibitory effect of albumin on adherence of platelets to collagen-containing surfaces is not readily evident. Lyman et al. (1971) have reported that albumin (45 - 50 mg/ml) enhanced platelet adherence to collagen. It should be noted that their test system was different and that the platelet suspending medium contained EDTA and no glucose. Several investigators (Mustard et al., 1972; Rossi, 1972; Doery et al., 1973; Tangen et al., 1973; Kinlough-Rathbone et al., 1977) have demonstrated that albumin has a protective effect on platelets in suspension. It is also possible that albumin interacts with collagen fibers, masking sites that react with platelets and thus reducing platelet adherence to collagen. Upon exposure of glass and various polymer surfaces to solutions containing albumin, they become coated with a thin film of albumin (Brash and Lyman, 1969; Vroman et al., 1971). Several authors have demonstrated that albumin decreases adhesion to surfaces by coating the surface (Packham et al., 1969; Lyman et al., 1971; Jenkins et al., 1973; Mason, 1972; Mohammad et al., 1976). Albumin has been covalently bonded to polystyrene and the resulting surface has demonstrated thromboresistance (Baier et al., 1970). More recently, Mohammad et al. (1976), have isolated a component from plasma, serum or albumin (fraction V) which inhibited platelet adherence to glass.

The component appeared to be a low-density lipoprotein and was suggested to be the factor responsible for the adhesion-inhibiting effect of albumin of low purity such as fraction V Pentex.

Nossel et al. (1971) have described in normal plasma a component that inhibited platelet aggregation induced by collagen. The plasma component was destroyed at 56°C, migrated with the alpha globulins and had a MW in the range of 330,000. It has not been further characterized.

(b) Fibrinogen.

The effect of adding fibrinogen to the suspending medium was not investigated extensively. Addition of fibrinogen did not increase platelet adherence to a collagen-coated rod, but actually slightly decreased it. The reason for this effect is not clear, but it is possible that fibrinogen interacts with collagen, masking collagen sites involved in the platelet-collagen interaction. It is now well established that fibrinogen coats glass surfaces as a thin film (Vroman et al., 1971) and that platelet adhesion to glass is enhanced by fibrinogen (Packham et al., 1969; Zucker and Vroman, 1969; Mason et al, 1971; Vroman et al., 1971; George, 1972; Jenkins et al., 1973; Mohammad et al., 1974).

(c) Von Willebrand factor.

Weiss et al. (1975) have observed that platelets from citrated blood of patients with von Willebrand disease adhere in smaller numbers than normal to the subendothelium of a rabbit aorta. Recently, these authors (Weiss et al., 1976) have shown that the defect in adhesion to subendothelium could be corrected by addition of factor VIII to the test system. Although we have not investigated the role of factor VIII in human platelet adherence to subendothelium in our system, human platelets were able to adhere to collagen and to subendothelium in the

absence of factor VIII. This important question of the role of von Willebrand factor in platelet adhesion to the subendothelium deserves to be further investigated using the rotating probe device.

3. Effect of hematocrit.

One of the disadvantages of the use of the collagen-coated tubes to measure adhesion of platelets was the existence of an air-interface associated with uncontrolled flow characteristics of the system. There is no air-interface with the rotating probe system (Feuerstein et al., 1975).

The fluid dynamic characteristics of the rotating probe device have been described by Feuerstein et al. (1975). The flow is laminar with circular streamlines (Couette flow). The platelets are uniformly transported to the probe surface by diffusion alone without convection. At the speeds used (50 to 200 rpm, corresponding to a shear rate of 10 to 40 sec^{-1}) there were no flow instabilities similar to Taylor vortices.

When the hematocrit was raised, more platelets adhered to the collagen-coated surfaces or the subendothelial structures. This finding is in keeping with observations in other types of flow systems in which the effect of red blood cells in causing increased diffusion of platelets to surfaces has been well demonstrated (Goldsmith, 1972; Leonard et al., 1972; Turitto and Baumgartner, 1975). Baumgartner and Haudenschild (1972) have reported that platelet deposition was significantly lower when the subendothelium was exposed in a perfusion chamber to platelet-rich

plasma as compared to whole blood; the addition of ADP to platelet-rich plasma did not increase the deposition of platelets. We also found that ADP did not increase platelet adherence to collagen-coated tubes (Cazenave et al., 1974c). Goldsmith (1972, 1974) has established, that in flow systems, red blood cells increase platelet interaction with the vessel wall by physical forces. The rotational motion and the continuous sideways motions of the red cells result in collision and displacement of other blood corpuscles, including platelets. These collisions tend to carry platelets to the wall.

When the hematocrit level was kept constant, it was found that platelet adherence to collagen was directly related to the concentration of platelets in the suspending medium and to the speed of rotation when the number of platelets adherent to collagen was examined in the absence of red cells and at 10, 20 or 40% hematocrit. After prolonged periods of rotation (30 to 60 minutes), the number of platelets adherent had reached a plateau level and rotation for longer times did not increase the number of adherent platelets. The maximum number of platelets adherent was related to the hematocrit. For example, compared to the determinations without red cells, there were about 36 times more platelets adherent to collagen when the hematocrit was 40%; 26 times at 20% hematocrit and 16 times at 10% hematocrit. With the rotating probe device, Feuerstein et al. (1975) and Brash et al. (1976) have analyzed the reaction of pig platelets with collagen-coated glass rods using a kinetic model in which the diffusion and reaction rates of platelets at the

surface are considered equal. This treatment has enabled them to estimate a platelet diffusion coefficient and a reaction rate constant. They have concluded that the effective platelet diffusion coefficient (D) is dependent on the presence of red cells, and reaches a maximum at about 35% hematocrit (D, about 1.0×10^{-7} cm^2/sec, compared with the Brownian motion diffusion coefficient in the absence of red cells of 10^{-9} cm^2/sec). They estimated a surface reaction rate constant ($k = 8.4 \times 10^{-5}$ cm/sec), which is related to the energy of the collision of a platelet with the surface and is important in determining the effectiveness of the platelet-surface interaction. They showed that the surface reaction rate constant was dependent on the hematocrit level between 15 and 45%, but was not dependent on the shear rate. They also found that the level of surface saturation by platelets, after a long time of rotation of the probe, increased with increasing red cell concentration; this effect is probably due to an increase in surface interaction energies.

4. Surfaces used in studies of platelet adherence.

(a) Collagen-coated glass rods.

Segments of glass-rods were coated with fibrils of polymerized collagen as previously described for the glass tubes and then exposed to a suspension of platelets labeled with ^{51}Cr. The inclusion of apyrase in the system prevented ADP-induced platelet aggregation and thus, the adherence of individual platelets to collagen could be measured. Although,

the possibility exists that the arachidonate pathway may be activated in this system, it seems reasonable to conclude that the extent of this activation must be very small since aggregate formation was not observed and rinsing the probes in PGE_1 or EDTA did not decrease the number of adherent platelets. The system measured mainly adherence to collagen fibers on the surface because denaturation of the collagen by hydrolysis with collagenase abolished platelet adherence. Another possibility was that the system would measure adherence to the parts of the glass surface coated with the albumin from the medium, which were not covered by the collagen fibrils. In fact, scanning electron microscopic examination of the collagen-coated surface showed a loose network of collagen fibers. The surface area covered by the collagen fibrils was much smaller than the surface not covered by collagen and presumably coated with albumin from the platelet suspending medium. Most, if not all the platelets adherent to the collagen-coated rod were associated with the fibrils of collagen as observed by microscopic examination. Scanning electron microscopy (Figure 9) revealed that the adherent platelets had changed shape; their long pseudopods were tightly bound to the collagen. Experiments were also performed in which the glass rod was coated with 4% albumin. Adhesion of platelets to this surface was very low and not increased appreciably by the addition of red blood cells to the platelet suspension. This demonstrated that albumin prevented adhesion to glass. These observations are in agreement with earlier findings by Packham et al. (1969) and Jenkins et al. (1973).

(b) Everted rabbit aorta.

Platelets do not normally adhere to the undamaged endothelial surface. In the present experiments, very little platelet adherence was observed on everted aorta surfaces that were not deliberately damaged. However, there was some morphological evidence of endothelial injury in a few areas on these aortic surfaces.

Interaction of platelets with damaged vessel walls is of importance in hemostasis and thrombosis. Platelets will adhere to the vessel wall if the endothelium is altered or removed, exposing the subendothelial surface. The components of the subendothelium involved are basement membrane, microfibrils and collagen fibers (reviewed in Stemerman, 1974; Baumgartner et al., 1976; Baumgartner and Muggli, 1976).

(i) Subendothelium.

We have examined the interaction of platelets with everted damaged aortas with the rotating probe device. Two types of injury of the endothelial lining were used. In earlier experiments, rabbit thoracic aortas were everted on the probe so as to expose the luminal surface to the outside. The endothelium was then removed by scraping it with a scalpel blade. This procedure severely damaged the aorta, and exposed connective tissue below the internal elastic lamina. The exposed surface contained numerous collagen fibers with their typical cross-striation. In more recent experiments, the endothelium was removed in situ by passing an inflated balloon catheter, using a technique similar to the one described originally by Baumgartner (1963).

This procedure was more gentle than scraping, gave a more homogenous exposed surface and thus more reproducible results. Ballooning removed only the endothelium, exposing the subendothelial tissue and left intact the internal elastic lamina. Platelet adherence to scraped-aortas was significantly greater than to the ballooned-aorta surface. However, the increased reactivity of the scraped aorta compared to the ballooned-aorta was not apparent when tested in the presence of red blood cells. Microscopic observation of the two types of exposed surfaces demonstrated more collagen fibers exposed at the surface in the case of aortas damaged by scraping. This might explain the increased adherence of platelets to scraped aortas. Baumgartner and Haudenschild (1972) have presented evidence that collagen is the most thrombogenic component of the subendothelial tissue, but their method measured thrombus formation rather than the adherence of single platelets. The effect of collagenase treatment of everted segments of aorta damaged by ballooning was studied on platelet adherence. Platelet adherence to the ballooned-aorta was greatly reduced after the subendothelial surface had been exposed to collagenase for two hours (Stemerman et al. (1971). These observations indicate that collagen and or basement membrane are the main component of the vessel wall to which platelets adhere. Baumgartner et Haudenschild (1972) and Baumgartner et al. (1976) also observed that collagenase digestion of the subendothelium for 24 hours markedly reduced platelet adherence using a morphometric technique. They observed more adherence after this time than was seen in the

present studies but the differences in time of incubation and the type of medium (citrated platelet-rich plasma) may account for the quantitative differences in adhesion. They have demonstrated that collagen fibers are the most thrombogenic material, but are rarely encountered at the subendothelial surface of the rabbit aorta. The collagen fibers are destroyed by collagenase but are resistant to trypsin. Quantitatively, the most abundant component of subendothelium to which platelets can adhere is basement membrane. The basement membrane is digestible by collagenase, trypsin and α-chymotrypsin. The microfibrils of the subendothelium are resistant to collagenase, but are digested by trypsin or α-chymotrypsin. These microfibrils are similar to the microfibrils around elastin described by Ross and Bornstein (1969). Platelets can adhere to the microfibrils of the subendothelium (Ts'ao and Glagov, 1970), although it is not a good substrate for platelet adhesion (Baumgartner and Haudenschild, 1972; Suresh et al., 1973).

(ii) Endothelium.

The interaction of platelets with endothelial cells is still a matter of dispute (Jørgensen, 1971; Gimbrone, 1976). Our results demonstrated that a rabbit aorta could be everted on a probe, exposing the endothelium to the outside with minimal damage. Few platelets adhered to the endothelial surface when the segment of aorta was rotated in a suspension of platelets. These few adhering platelets may adhere to subendothelial tissue exposed by damage of the endothelium, when the vessel is everted. These results support the view that platelets do

not adhere to normal endothelium (Spaet and Erichson, 1966; Ashford and Freiman, 1967; Brånemark and Ekholm, 1968; Warren and de Bono, 1970; Ts'ao, 1970; Baumgartner, 1972). However, in many published electron micrographs platelets appeared to be in contact with endothelial cells and in many instances there was no evidence of endothelial damage. It is possible that platelets were adhering to subendothelial tissue exposed through a gap not demonstrated on the tissue section examined. Tranzer and Baumgartner (1967) have demonstrated that ability of platelets to fill small endothelial gaps in the vessels in the iris of cats.

It is also possible that the endothelium becomes modified if thrombin is formed on its surface and bound to it. Our results indicated that thrombin may bind to endothelial cells or to the subendothelial surface exposed by a balloon catheter. Platelets will adhere to endothelium that has been exposed to thrombin and this adherence can be blocked by heparin. These findings are in agreement with previous observations which demonstrated that platelets adhere to the surface of endothelial cells in culture that have been exposed to thrombin (Rafelson et al., 1973; Awbrey et al., 1975) and that adherence is blocked by heparin (Awbrey et al., 1975).

In vivo, thrombin formation on the surface of endothelial cells could be a factor causing platelet adherence to the vessel wall without loss of endothelial cells. The data from Ashford and Freiman's (1967) experiments are compatible with the hypothesis. They found

that platelets adhered to the damaged endothelial surface of a vessel wall when there was no morphological evidence of loss of endothelial cells providing fibrin had formed at the site. Gregorius and Rand (1976) have studied with the scanning electron microscope the effect of the administration of high systemic doses of heparin on sutured common carotid arteries in rats. They observed that the endothelial surfaces showed a decrease in platelet adhesion and a reduction in the deposition of red blood cells, leukocytes and fibrin.

There are at least two ways in which thrombin can be generated in vivo: (1) minimal endothelial damage resulting in tissue factor generation and activation of the extrinsic pathway of coagulation (Nemerson and Pitlick, 1972) leading to thrombin generation and fibrin deposition (Ashford and Freiman, 1967); (2) during disseminated intravascular coagulation.

Local formation of thrombin and its binding to endothelial cells could promote platelet adhesion and then the release of platelet constituents, leading to the formation of a thrombus. Generation of thrombin and thrombus formation could cause endothelial damage (Lough and Moore, 1975), exposing the subendothelial tissue and causing more platelets to adhere and to release their constituents.

There are several other mechanisms that could modify the non-thrombogenic endothelium so as to render it attractive to circulating platelets. Local generation of thrombin will also lead to fibrin formation on the surface of endothelial cells (Ashford and Freiman, 1967).

Platelets can adhere to polymerizing fibrin, but not to fully polymerized fibrin (Niewiarowski et al., 1972). Humoral antibodies (O'Connel and Mowbray, 1973, Friedman et al., 1975), circulating immune complexes or specific anti-endothelial antibodies could also bind to the endothelium and thus promote platelet adherence. Hirschberg et al. (1975) have shown in vitro damage to human endothelial cells by non-immune allogeneic lymphocytes in the presence of anti-HLA antibodies. Endothelial injury has also been demonstrated after prolonged ischemia (Gertz et al., 1975) and in rabbits following acute lipid mobilization after ACTH injection (Maca and Hoak, 1974). These mechanisms could play an important role in inducing platelet-mediated vascular damage. Endothelial damage, exposure of subendothelial tissue and platelet adherence to these structures are the first steps in thrombosis.

5. The rotating probe device as a screening system for antiplatelet drugs.

Platelets are a major component of arterial thrombi (Mustard et al., 1974). Many drugs affect platelet function in vitro (Mustard and Packham, 1970; Mustard and Packham, 1975). Only a few drugs have been considered for use in clinical situations in man (Harker et al., 1975; Mustard and Packham, 1975; Jobin, 1976; Weiss, 1976). It is difficult to determine in in vitro tests the usefulness in thromboembolic disorders of a drug that inhibits platelet function. Furthermore, there is little correlation between the effects of drugs on platelet function in vitro and on platelet survival in vivo or on the effects on the

clinical manifestations. The rotating probe device provides a technique for testing the effects of antiplatelet drugs on platelet adherence to collagen and to the subendothelium which may be related to the initial events of thrombus formation.

In an attempt to approach more closely the in vivo situation, some experiments were done with 4% albumin, 40% hematocrit and a platelet count of 300,000 per mm^3. Since many drugs are bound to albumin in the circulation, the use of 4% albumin makes it possible to estimate the concentration of such drugs which might be expected to inhibit platelet adherence in vivo. A hematocrit of 40% and a platelet count of 300,000 per mm^3 are in the physiological ranges. It seemed desirable to examine the effect of inhibitory conditions in the presence of the physical forces exerted on the platelets by the red blood cells.

A number of drugs were inhibitory when their effect on adherence was examined at 40% hematocrit. Dipyridamole, RA 433, indomethacin, penicillin G, cephalothin or methylprednisolone were good inhibitors of adherence to collagen or to subendothelium when tested with 40% hematocrit and 4% albumin. In contrast, ASA or sulfinpyrazone did not inhibit adherence to collagen under these conditions (40% hematocrit, 4% albumin). Sulfinpyrazone, but not ASA, inhibited platelet adhesion to a ballooned aorta at 40% hematocrit and 0.35% albumin.

The results obtained with these drugs at 40% hematocrit seem to parallel some in vivo effects on platelets. For example, dipyridamole has been shown to prolong a shortened platelet survival to normal in man

and to be effective in cases of arterial thrombosis (Genton et al., 1975). Our results provide evidence that dipyridamole and RA 433 are good inhibitors of platelet adherence to collagen in the presence of 40% hematocrit. The inhibitory effect of dipyridamole on adherence to collagen was greater when tested in the presence of red blood cells than in their absence (Cazenave et al., 1974b).

In vitro, ASA is a potent inhibitor of the release reaction induced by collagen (O'Brien, 1968; Fleming et al., 1970; Zucker and Peterson, 1970). However, it does not prolong platelet survival in man and conflicting results have been reported about its effectiveness in thromboembolic disorders (Genton et al., 1975). Sulfinpyrazone, a weak inhibitor of aggregation and release (Packham et al., 1967), prolongs survival (Smythe et al., 1965; Steele et al., 1973b) and clinical studies have shown benefit (Blakely and Gent, 1975; Kaegi et al., 1975). The apparent discrepancy in performance of ASA and sulfinpyrazone in vivo and in vitro led to an examination of their effects on platelet adhesion to subendothelial structures of a rabbit aorta in a perfusion system (Davies et al., 1975 and personal communication). The effects of ASA and sulfinpyrazone on platelet adherence to a balloon-damaged aorta were examined in a perfusion system at a 10% hematocrit. Rabbit platelets suspended in 0.35% albumin were circulated at 150 ml per minute at 50 pulses per minute using a perfusion pump. Perfusion was carried out for 10 minutes at a pressure of 80 mm of mercury. In these conditions, sulfinpyrazone (250 μM) reduced adhesion by 61%, but ASA (1.2 mM) was ineffective.

Baumgartner and Muggli (1974) did not observe an inhibitory effect of aspirin on platelet adherence but the conditions in their experiments were different than in the present studies. They were measuring platelet adhesion in whole citrated blood to an everted rabbit aorta using a perfusion chamber. In addition to the differences in flow conditions and hematocrit, their system contained plasmin proteins and sodium citrate. We have demonstrated (Cazenave et al., 1975; and Results, Section V) that in the presence of citrate and plasma proteins ASA has little effect. Although the flow conditions in the perfusion system used by Davies et al. (1975) might be closer to those of Baumgartner's perfusion chamber rather than to the hemodynamic conditions with the rotating probe device, the main differences from Baumgartner's system are the presence of divalent cations and the absence of plasma proteins.

The contradictory effects of ASA in our experiments could be due to differences in the test systems used (rotating probe device and perfused aorta) and related to flow conditions, presence of red cells, and the nature of the damaged aortic surface exposed (ballooned or scraped).

Experiments with ASA and sulfinpyrazone were repeated with the rotating probe system to elicit the reason for the difference in the results obtained.

Initial experiments were designed to test the hypothesis that the difference in the aspirin effect observed was due to the

difference in subendothelial structures exposed by passage of a balloon catheter or by scraping. However, both ASA and sulfinpyrazone were found to be equally effective in inhibiting adherence to balloon-damaged or scraped surfaces. We next investigated the effect of adding red blood cells to the platelet suspension containing 0.35% albumin. ASA (100 μM) or sulfinpyrazone (100 μM) inhibited platelet adherence to balloon-damaged aortas in the presence of a 10% or 20% hematocrit. However, when the hematocrit was raised to 40%, the inhibitory effect of ASA or sulfinpyrazone on platelet adherence was not demonstrable.

In a final experiment, high concentrations of ASA (250 μM) and sulfinpyrazone (250 μM) were tested at 40% hematocrit. Sulfinpyrazone, but not ASA, significantly decreased platelet adherence to a balloon-damaged aorta. These results imply that the conditions of the test system were important to test the effects of drugs on platelet adherence to a damaged aorta. This may explain, at least partly, the discrepant results obtained by several authors on the effect of ASA on adhesion to collagen with the conditions used in the adherence studies. ASA inhibited collagen-induced aggregation, but not platelet adherence when measured at a 40% hematocrit. This was further evidence that formation of platelet aggregates on the surface was not a major factor. Increasing the red cell concentration in the system, increased the forces driving platelets to the wall. In these conditions sulfinpyrazone was a better inhibitor of adherence than ASA.

There are at least two possible explanations for the influence

of the hematocrit on the effect of drugs on platelet adherence to collagen and to the subendothelium. One explanation is that the red blood cells are interacting in some way with the drug, so that it is not available to the platelets. This is unlikely because the drugs were incubated for 10 minutes with the platelets before the red blood cells were added and in addition, the effect of ASA or indomethacin on platelets is irreversible and persists despite washing of the platelets. The other explanation is that the presence of red cells increases the diffusion of platelets to the wall and the energy at which platelets bind to it. Feuerstein et al. (1975) have obtained some evidence, using a rotating probe system, that increasing the hematocrit increases the platelet reaction rate constant. It may thus be possible that some drugs, ASA for example, are poor inhibitors of adherence when platelets are colliding with the surface at high energy.

There is probably more than one mechanism of platelet adherence to collagen. One of them (which is increased by increasing hematocrit) is not blocked by ASA. There may be another which is inhibited by PGE_1, which would indicate that platelet shape change may be important in platelet adherence to collagen.

The preceding experiments have shown that the effect of red blood cells makes the testing of drugs difficult. Furthermore, the presence of albumin or citrate lessens the apparent effect of ASA on platelet adherence to surfaces (Cazenave et al., 1975). Since the results of studies of platelet adherence to subendothelial surfaces

were similar to those with collagen-coated surfaces, the latter technique is useful for exploratory studies of the effects of agents or conditions that modify platelet adherence.

The conditions which appeared to be most suitable for testing the effect of drugs on platelet adherence to collagen-coated rods were 4% albumin, 40% hematocrit, a rotation speed of 200 rpm for 10 minutes and a platelet count of 300,000 per mm^3.

For a simple screening system, the conditions used in most experiments have been 0.35% albumin, no red blood cells, a rotation speed of 200 rpm for 10 minutes and a platelet count of 700,000 per mm^3. Under these conditions, the extent of platelet adherence to the surfaces is sensitive to the presence of inhibitors of platelet reactions, to modification of the platelet reactions and to modification of the platelet surface. In no case have inhibitory effects been demonstrable at 4% albumin, 40% hematocrit and a platelet count of 300,000 per mm^3 that were not apparent with the simpler system (0.35% albumin, no red blood cells, 700,000 platelets per mm^3). There are several advantages to the simpler system: (1) the procedure does not require the preparation of washed red blood cells; (2) the method is less expensive because of the lower concentration of albumin used. The low concentration of albumin lessens the drug binding effect of albumin.

6. Unique aspects of the rotating probe method.

The methods developed in the present study differ from those used by other investigators in a number of ways:

(a) Nearly all the other studies have examined the adherence of platelets in plasma or whole blood (Hirsh et al., 1968b;Hovig et al., 1968; Spaet and Lejnieks, 1969; Stemerman et al., 1971; Baumgartner and Haudenschild, 1972; Lyman et al., 1971; Baumgartner, 1973; Baumgartner, 1974; Blaisdell et al., 1974; Gordon and Dingle, 1974; MacKenzie et al., 1974; Muggli and Baumgartner, 1975; Baumgartner et al., 1976; Castellan and Steiner, 1976; Dosne et al., 1976). This has necessitated the use of anticoagulants which we found in our preliminary experiments to decrease platelet adherence to collagen-coated tubes and to the subendothelium (Cazenave et al., 1975; Cazenave et al., 1977b). Baumgartner et al. (1971) have also observed that chelating agents diminish platelet adherence to subendothelium. The use of suspensions of washed platelets in the present study has made it possible to study adherence in the presence of physiological concentrations of calcium and magnesium without the complications introduced by the formation of thrombin.

(b) The most extensive studies of platelet adherence to the subendothelium have been done by Baumgartner and his associates (1973; 1976). Baumgartner and Muggli (1976) have recently reviewed in detail their technique of evaluating platelet interaction with subendothelium. This technique is different from the rotating probe device in the following ways: (1) blood is perfused under controlled laminar flow conditions through an annular perfusion chamber on which everted segments of ballooned rabbit aorta are mounted; (2) blood is

anticoagulated with 14 mM sodium citrate in plasma and perfused for 10 minutes at 37°C at various flow velocities; (3) platelet deposition on the surface is evaluated by a morphometric technique on semi-thin sections of the vessel. Four types of platelet-surface interactions are recognized: (a) surface devoid of platelets, (b) surface covered with platelets which are not spread out (contact), (c) surface covered with spread out platelets (adhesion), and (d) surface covered with aggregates of more than 5 μM in height (thrombi); (4) Plasma proteins and red blood cells are present; and (5) platelet aggregation is not prevented.

The conditions of the present experiments, particularly the inclusion of apyrase in the suspending medium, have made it possible to measure the adhesion of individual platelets without the formation of platelet aggregates or appreciable release of platelet granule contents. This has facilitated the investigation of inhibitors of platelet adherence without the complications of inhibition of platelet aggregation and the release reaction.

(c) The use of labeled platelets to quantitate platelet adherence has some advantages over the morphometric technique:

(1) It is much simpler and less time-consuming so many more samples can be examined.

(2) Adherence to relatively large surfaces is measured. With the morphometric technique the area examined is small and may not be representative of the entire surface.

(e) The morphometric technique is limited to tissues or soft materials which can be fixed and sectioned for microscopic examination. The method developed in the present study is also applicable to solid surfaces coated with collagen or other proteins. This has made it possible to use collagen-coated rods as a simple surface for screening inhibitors of platelet adherence.

Morphometric techniques can be used in addition to platelet labeling if it seems desirable.

II. MODIFICATIONS OF THE PLATELET SURFACE, PLATELET RECEPTORS AND PLATELET ADHERENCE TO COLLAGEN.

The glycoproteins of the platelet surface appear to be involved in platelet adherence to surfaces. Three major glycoproteins with apparent molecular weights of 155,000 (glycoprotein I), 135,000 (glycoprotein II), and 100,000 (glycoprotein III) are exposed on the surface of the human platelet (Nachman and Ferris, 1972; Phillips, 1972). Similarly the rabbit platelet plasma membrane has three major glycoproteins (George et al., 1976b; Greenberg, personal communication).

1. Modifications of the platelet surface.

We have examined the effect of enzymatic or chemical modifications of platelet surface membrane glycoproteins in order to gain some insight in the membrane components possibly involved in platelet adhesion to collagen or subendothelium. The use of rabbit platelets allowed the study of the effect of these surface modifications on platelet function in vivo.

(a) THROMBIN.

The properties of thrombin-degranulated platelets have been described (Reimers et al., 1973b;1976). After treatment with high concentrations of thrombin (0.5 u/ml), single rabbit platelets can be recovered that have lost most of their releasable serotonin and adenine nucleotides. These platelets do not change shape or aggregate upon a second exposure to thrombin. They aggregate in response to the calcium ionophore A23,187 in the absence of added fibrinogen (Kinlough-Rathbone et al., 1975), but aggregation to ADP or to sodium arachidonate requires the addition of fibrinogen (Kinlough-Rathbone

et al., 1976b). Thrombin-treated platelets can aggregate in response to collagen. The aggregation response is not inhibited by CP/CPK but is blocked by ASA or indomethacin (Reimers et al., 1976). From this it has been concluded that thrombin-degranulated platelets are still capable of forming metabolites from arachidonate and that these metabolites can cause aggregation independently of released ADP.

Thrombin-treated platelets were less adherent to collagen-coated surfaces and to the subendothelium than normal platelets. The inhibitory effect was demonstrable in the presence of 4% albumin and 20% hematocrit. The addition of fibrinogen to the suspending medium did not correct the inhibition. The addition of ASA did not decrease further the ability of thrombin-treated platelets to adhere to a damaged aorta. From these experiments a number of conclusions can be drawn. (1) Thrombin-treated platelets adhere less readily to collagen. This effect of thrombin is demonstrable when low concentrations of thrombin (that induce only a partial release reaction) or high concentrations (that induce practically complete degranulation) are used. (2) Thrombin-treated platelets may be less adherent because thrombin modifies the platelet surface. (3) The effect of ASA on adherence of platelets that have not been treated with thrombin is not mediated in this system by inhibition of the cyclo-oxygenase (Roth and Majerus, 1975) and inhibition of the formation of endoperoxides (Malmsten et al., 1975) and thromboxane A_2 (Hamberg et al., 1975) because thrombin-treated platelets are still capable of generating prostaglandin

endoperoxides or thromboxane A_2 from membrane arachidonate, but ASA does not decrease their adherence to collagen. A possible interpretation is that in this system endoperoxides and thromboxane A_2 do not contribute much to platelet adherence of either normal or thrombin-treated platelets. Thus, ASA would decrease platelet adherence by modifying a surface component which is thrombin sensitive. ASA has been shown to acetylate a variety of proteins from plasma and platelets (Pinckhard et al., 1968; Al-Mondhiry et al., 1970; Roth and Majerus, 1975; Rome et al., 1976). The available evidence suggests that both proteolytic and binding sites for thrombin are present on the platelet plasma membrane (Phillips, 1974). Phillips and Agin (1974) have demonstrated proteolysis of glycoprotein II by thrombin by lacto-peroxidase iodination of the platelet membrane. Binding occurs at specific sites on the platelet membrane (Ganguly, 1974; Tollefsen et al., 1974) and more recently Okumura and Jamieson (1976a) have suggested that the surface receptor for thrombin or ristocetin is a glycoprotein loosely bound to the membrane; they named it glycocalicin. Glycocalicin has structural similarities with glycoprotein I, but different accessibility to membrane labeling. (Okumura and Jamieson, 1976b, Okumura et al., 1976c). The precise relationship between glycocalicin and glycoprotein I is not known. Glycoprotein I is markedly reduced in the Bernard-Soulier syndrome (Nurden and Caen, 1975; Caen et al., 1976; Jenkins et al., 1976). Platelets from patients with the Bernard-Soulier syndrome show decreased adhesion to the subendothelium and do not aggregate with ristocetin

in the presence of plasma von Willebrand factor (Howard et al., 1973; Weiss et al., 1974). It has been suggested that this surface membrane glycoprotein I may be a potential receptor for a component or components of the subendothelium (Nurden and Caen, 1975). It is not known if ASA can acetylate glycoprotein I or glycocalicin and thus modify its interaction with the subendothelium.

(b) PLASMIN.

The treatment of washed rabbit platelets with purified urokinase activated rabbit plasminogen or purified human plasmin did not cause the platelets to release or to aggregate. This is in contrast with the results obtained by Niewiarowski et al. (1973) using trypsin-activated pig plasmin. Fewer plasmin-treated platelets adhered to the subendothelium or to collagen than normal platelets. Platelet fibrinogen undergoes almost complete degradation during the action of plasmin on platelets (Niewiarowski et al., 1973). The addition of fibrinogen to the platelet suspending medium did not reverse the adhesion defect. Niewiarowski et al. (1973) have shown that platelets exposed to plasmin in vitro are less sensitive to collagen. Similarly, platelets obtained from rabbits injected with streptokinase, an activator of the fibrinolytic system, are less reactive to collagen and it has been suggested that this effect may contribute to the prolongation of the bleeding time that was observed. Hirsh et al. (1968a) have also demonstrated that streptokinase induces an hemostatic defect in vivo. These effects of plasmin seem to be related mainly to an effect on the platelets. In addition to its effect on platelet

fibrinogen plasmin has been shown to affect platelet membrane glycoproteins. Matsuda et al. (1975) have shown that plasmin hydrolyses a 100,000 molecular weight glycoprotein component of intact human plasma membranes. Greenberg (personal communication) has demonstrated that plasmin cleaves the three major surface glycoproteins from intact rabbit platelets. Thus, it is possible that the decrease in platelet adherence to collagen and to the subendothelium is related to a modification of glycoprotein I by plasmin.

Other possibilities are that plasmin modifies other proteins associated with the platelet surface besides glycoprotein I. Plasmin undoubtedly hydrolyses platelet fibrinogen which can be detected on the platelet surface (Tollefsen and Majerus, 1975) and in platelet granules. The fact that addition of fibrinogen to plasmin-treated platelets which restores platelet aggregation to ADP (Niewiarowski, 1973) does not restore the ability of the platelets to adhere normally to collagen-coated glass or to the subendothelium is not in favor of a role of platelet surface fibrinogen in adherence. Plasmin also hydrolyses factor VIII (Pasquini and Herschgold, 1973) which has been shown to be associated with platelets (Howard et al., 1974). Von Willebrand's disease is characterized by a decrease in factor VIII and an impaired adhesion of platelets to the subendothelium. Although human platelets do adhere to collagen and the subendothelium in our system in the absence of plasma, the role of factor VIII in platelet adherence deserves to be studied.

(c) CHYMOTRYPSIN.

Chymotrypsin has been shown to cleave platelet membrane glycoproteins, but in contrast to trypsin does not induce the platelet release reaction and platelet aggregation (Davey and Lüscher, 1967). Chymotrypsin-treated platelets (10 U/ml or 192 μg/ml, for 30 minutes at 22°C) do not aggregate to ADP, collagen or thrombin (Greenberg et al., 1976). Platelets treated with chymotrypsin in this way show reduced adherence to collagen. Platelets treated with chymotrypsin and analyzed by polyacrylamide gel electrophoresis show a reduced concentration of glycoproteins I, II and III (Jenkins et al., 1976; Greenberg, personal communication). In addition, chymotrypsin-treated human platelets do not aggregate to ristocetin in the presence of human plasma (Jenkins et al., 1976; Nachman et al., 1977). These results are similar to those obtained with platelets from patients with the Bernard-Soulier syndrome, in which platelets have a decreased concentration in glycoprotein I (Nurden and Caen, 1975; Caen et al., 1976; Jenkins et al., 1976), do not aggregate to ristocetin (Caen et al., 1973; Howard et al., 1973; Weiss et al., 1974) and show a reduced adhesion to the subendothelium (Weiss et al., 1974; Caen et al., 1976).

Treatment with proteolytic enzymes has been used in the studies reported in this thesis in an attempt to remove cell surface components which might be involved in platelet adherence to collagen or to the subendothelium. Although thrombin, plasmin or chymotrypsin hydrolyse specific peptide bonds in proteins; the identification of the protein

affected by the enzymatic treatment of intact platelets is complicated by the fact that the enzymes may have several substrates. Thrombin, for example, modifies fibrinogen (Ganguly, 1972), factor XIII (Kiesselbach and Wagner, 1966) and thrombosthenin (Cohen et al., 1969; Muszbek et al., 1976) in platelets.

A similar approach has been used with other cell systems. Enzymatic treatment of fibroblasts with various proteases has shown that a 250,000 dalton glycoprotein is involved in cell-cell adhesion (Zetter et al., 1976) and possibly in cell transformation (Hynes, 1974; Blumberg and Robbins, 1975).

(d) NEURAMINIDASE.

The treatment of platelets with purified neuraminidase devoid of proteolytic and phospholipase activity does not cause the platelet release reaction nor platelet aggregation. When platelets are treated with neuraminidase to remove up to 65% of total sialic acid, platelet aggregation by ADP, collagen, thrombin, ristocetin, polylysine and serotonin is either not affected or slightly enhanced (Greenberg et al., 1975). When up to 35% of total platelet sialic acid is removed, the adherence of platelets to collagen or to subendothelium is not modified. This indicates that removal of terminal sialic acid from exposed membrane glycoproteins does not alter platelet adherence to collagen or the subendothelium. A large part of the surface sialic acid of platelets is bound to glycoprotein I and glycocalicin (Pepper and Jamieson, 1970; Lombart et al., 1974; Okumura et al., 1976c). In

contrast to the effects of plasmin and chymotrypsin, which cleave glycopeptides from membrane glycoproteins and reduce adherence to collagen, removal of terminal sialic acid from the surface glycoproteins has no effect on platelet adherence to collagen.

(e) SODIUM PERIODATE.

Sodium periodate was used to treat platelets as another means of modifying platelet glycoproteins. Under the mild conditions (low concentrations of $NaIO_4$, short exposure time) used, $NaIO_4$ probably oxidizes mainly the terminal sialic acid of glycoproteins since some evidence has been obtained for such a site of action of periodate with lymphocytes (Novogrodsky and Katchalski, 1972; Zatz et al., 1972; Presant and Parker, 1976) and red blood cells (Blumenfeld et al., 1972; Liao et al., 1973).

The effects of $NaIO_4$ pretreatment on platelet response to aggregating and release-inducing agents are in sharp contrast to the effects of neuraminidase treatment. $NaIO_4$ pretreatment reduces the response of platelets to ADP, collagen, thrombin, arachidonic acid and ionophore A23,187. $NaBH_4$ partially reversed the effect of $NaIO_4$ treatment (Cazenave et al., 1976c).

$NaIO_4$ oxidation of platelets reduces their adherence to collagen and to subendothelium. The inhibitory effect of $NaIO_4$ pretreatment on adherence to collagen is lost when adherence is tested at a 40% hematocrit. This effect of hematocrit is similar to the loss of inhibitory effect of ASA at 40% hematocrit. However,

the interpretation of these results is not clear. One possibility is that oxidation of terminal sialic acids of glycoproteins favors their intramembraneous cross-linking through the formation of Schiff bases and thus modifies their interaction with collagen. Another possibility is that $NaIO_4$ oxidizes lipid components of the membrane (Blumenfeld et al., 1972) which are involved in adhesion to collagen.

In conclusion, we have used various treatments, which probably essentially affect glycoproteins, to modify the platelet surface. Removal of terminal sialic acid of membrane glycoproteins does not affect platelet adhesion to collagen. In contrast, oxidation of the sialic acid of membrane glycoproteins is associated with a decrease, although in adhesion, this decrease is only demonstrable at low hematocrits. Since removal of sialic acid with neuraminidase does not affect platelet adherence to collagen whereas treatment with proteolyetic enzymes decreases adhesion, it seems likely that removal of glycopeptides may be necessary to destroy the postulated collagen receptor on the platelet surface. Plasmin or chymotrypsin affects glycoprotein I and thrombin affects glycocalicin. The major lesion in Bernard-Soulier syndrome involves a marked reduction in glycoprotein I. This is associated with a reduced adherence of platelets to the subendothelium and a poor aggregation response to ristocetin. The experiments reported in the literature indicate that interaction of platelets with collagen and the subendothelium may be mediated through glycoprotein I. More recent evidence gives support

to the possibility that glycoprotein I may serve as a receptor or an acceptor for a component of the subendothelium. Recently (Tobelem et al., 1976) have isolated an IgG antibody from a Bernard-Soulier patient who had received multiple transfusions. This antibody agglutinated a panel of 25 control normal human platelets, but not the platelets from 2 other patients with the Bernard-Soulier syndrome. When this antibody was added to normal human platelets in vitro, a specific Bernard-Soulier-like defect was induced so that adhesion to the subendothelium was impaired and aggregation with ristocetin and bovine factor VIII was impaired while ADP and collagen aggregation were not influenced. The authors concluded that the antibody is directed against a specific platelet site, implicated in the interaction of platelets with the subendothelium. The platelet antigen recognized by the antibody could be the 155,000 molecular weight glycoprotein I.

2. <u>Platelet adherence to collagen and the collagen-glucosyltransferase hypothesis.</u>

The biochemical basis of the adhesion of platelets to collagen is not understood and the nature of the platelet receptor involved is not known, although accumulating evidence suggests that platelet membrane glycoprotein I might be involved.

Jamieson et al. (1971) and Bosmann (1971) have suggested that platelet adhesion may be mediated by the formation of an enzyme acceptor complex between collagen:glucosyltransferase present on the platelet surface and incomplete heterosaccharide chains (galactosyl residues)

present in collagen or basement membrane.

However, we found that UDP or UDPG (Cazenave et al., 1974a) did not inhibit platelet adhesion to collagen. These observations were interpreted as an indication that collagen:glucosyltransferase may not be involved in platelet-collagen adherence. More recently, Jamieson et al. (1975) have suggested that the interaction between collagen and the enzyme may be analogous to the interaction of a lectin with the cell surface, so that donation of glucose from UDPG need not to be involved in the process of adhesion. The hypothesis concerning the role of the platelet collagen:glucosyltransferase in adhesion to collagen has received further criticism by Menashi et al. (1976). These authors have pointed out that (1) the methods used (Jamieson et al., 1971; Bosmann, 1971) to demonstrate the collagen:glucosyl- and galactosyltransferase activities lack specificity; (2) the platelet collagen:glucosyltransferase requires the collagen substrate to be non-helical. When native triple helical collagen was used, no glucosylation reaction was detected, as measured by synthesis of ^{14}C-glucosylgalactosyl-hydroxylysine. These results are analogous to those reported for the collagen:glucosyltransferase involved in intracellular glucosylation of procollagen. Thus it seems that following vascular injury the adhesion of platelets to native collagen is unlikely to be mediated by the collagen:glucosyltransferase. (3) Finally, they questioned the observation of Barber and Jamieson (1971) that the enzyme is primarily located on the outer surface of the plasma membrane of human platelets. They found considerable enzyme activity

in the cytosol fraction of pig platelets, as well as in platelet membranes, and also high amounts of collagen:glucosyltransferase activity in plasma free of platelets. In a recent critical review, Keenan and Morré (1975) concluded that there appears, at the present time, to be no valid basis for the conclusion that glucosyltransferases are located on the surface of mammalian cells.

If the collagen:glucosyltransferase were shown to be located on the outer surface of the platelet plasma membrane the possibility would remain that collagen:glucosyltransferase functions as a "lectin" specific for collagen (Jamieson and Smith, 1976), that can be modified by acetylation with ASA. It is not known if glycoprotein I has collagen: glucosyltransferase activity, nor if Clq inhibits the activity of this enzyme.

3. Clq and the collagen receptor of human platelets.

Clq shares structural similarities with collagen (Müeller-Eberhard, 1968; Reid, 1974) and its activity is destroyed by heat denaturation and collagenase (Reid et al., 1972; Knobel et al., 1974). Collagen also has been found to interact with Cl (Takahashi et al., 1975) and to inhibit Clq fixation to immune complexes (Allan et al., 1975).

The results of our experiments (Cazenave et al., 1976a) using human platelet suspensions demonstrate that Clq inhibits platelet adherence to collagen and collagen-induced aggregation. The reaction was specific to collagen because Clq did not inhibit ADP- or thrombin-

induced platelet aggregation. Suba and Csákó (1976), have confirmed these results, demonstrating that Clq inhibited competitively and specifically collagen-induced platelet aggregation in platelet-rich plasma.

From these experiments, it can be concluded that Clq interacts with a platelet surface component or receptor which interacts also with collagen. The nature of the receptor is not known. Wautier et al. (1974, 1976) have demonstrated an association of Cl with human platelets. Pfueller and Lüscher (1972) have postulated the existence of a Clq-like receptor on the platelet surface for antigen-antibody complexes or aggregated IgG, because release and aggregation induced by aggregated IgG could be blocked by Clq. Wautier et al. (1976) have shown that Cls is bound to the human platelet membrane and that the presence of Clq is necessary for the aggregation of platelets by aggregated IgG. It was suggested that Clq is attached to Cls by its collagen-like moiety, which would leave its non-collagen portion free to react with aggregated IgG. The inhibition by Clq of platelet interaction with collagen is in agreement with Wautier's observation and indicates that collagen may interact with Cls bound to the platelet surface. Recently, it has been suggested (Chater, 1976) that collagen adhering to dog platelets may activate membrane bound complement that may be involved in the induction of the release reaction.

However, the nature of the Clq receptor remains to be elucidated. One possibility is that Cls bound to the platelet membrane acts as a receptor for Clq. Another possibility is that Clq binds to glycoprotein I.

These observations may mean that immune complexes and aggregated IgG, and collagen interact with common or closely related receptor sites located on the platelet membrane. It may be that Clq plays an important role in regulating platelet adherence to collagen of the subendothelium (Suba and Csákó, 1977).

In the preceding section we have discussed the presently available evidence that platelet adhesion to collagen is a highly specific biological interaction. Platelets have specific receptor(s) on their surface which interact with polymerized collagen. There is evidence that the platelet binding site(s) of collagen are located on the helical portion of the molecule (Michaeli and Orloff, 1976). The molecular nature of the platelet receptor for collagen and/or for the subendothelium is unknown at the present time. Glycoprotein I in the platelet membrane appears to be involved in adherence, because: (1) platelet adherence is decreased when platelets are modified with proteolytic enzymes which hydrolyse glycoprotein I; (2) platelet adherence to subendothelium is also decreased in Bernard-Soulier syndrome, a congenital platelet abnormality with reduced concentration of glycoprotein I; (3) and platelet adherence to the subendothelium is reduced when it is measured in the presence of an antibody directed against glycoprotein I. Using agents that inhibit several aspects of platelet function, we investigated some of the factors involved in platelet adherence to collagen-containing surfaces.

1. Role of divalent cations.

The results reported (Results, Sections I B and V) demonstrate that divalent cations are involved in the adherence of platelets to collagen-coated glass surfaces and to the subendothelium. Platelet adherence to collagen and the subendothelium is markedly decreased

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The results reported (Results, Sections I B and V) demonstrate that divalent cations are involved in the adherence of platelets to collagen-coated glass surfaces and to the subendothelium. Platelet adherence to collagen and the subendothelium is markedly decreased

if Ca^{2+} is omitted from the platelet suspending medium or if Ca^{2+} is chelated with EGTA. EDTA, which chelates both Ca^{2+} and Mg^{2+}, almost abolishes platelet adherence to collagen and to the subendothelium. A similar degree of inhibition of adherence to collagen is found when citrate is added to the suspending medium at concentrations used to anticoagulate blood. Earlier investigators (Hovig, 1963a,b; Spaet and Zucker, 1964; Hirsh et al., 1968b; Hovig et al., 1968; Spaet and Lejnieks, 1969; Lyman et al., 1971; Gordon and Dingle, 1974; Baumgartner and Muggli, 1976; Brass et al., 1976; Castellan and Steiner, 1976) used chelating agents and were not able to do control tests without chelating agents to study platelet adherence to collagen. They were therefore unable to draw valid conclusions concerning the possible involvement of divalent cations in platelet adherence to collagen.

Experiments have been done with glass surfaces in which the contribution of divalent cations in the suspending fluid has been assessed. When plasma or proteins were present in the medium, calcium was found to be involved in the adherence of platelets to glass surfaces (George, 1972; Mohammad et al., 1974, 1976) and to fibrinogen-coated glass surfaces (George, 1972; Jenkins et al., 1973). When platelets are resuspended in a medium devoid of proteins, they become more adhesive to glass (Packham et al., 1969) and the presence of either divalent cations or EDTA has no appreciable effect on platelet adherence under these conditions (Mohammad et al., 1976).

Anticoagulation with sodium citrate has also an effect on the interaction of platelets with the subendothelium in flowing blood (Baumgartner, 1976; Baumgartner et al., 1976; Baumgartner and Muggli, 1976). The extent of platelet adhesion to the subendothelium, after exposure to blood for 3 minutes, was similar when native blood (no anticoagulant) or anticoagulated blood (15 mM citrate in plasma) was used. However, generation of thrombin and fibrin deposition on the subendothelial surface occurred when native blood was used and thus it is not possible to make meaningful comparisons with anticoagulated blood. In addition, at concentrations of citrate higher than 28 mM in plasma, adhesion decreased and was virtually abolished at 91.5 mM citrate in plasma.

Our results indicate that platelet adherence to collagen and to the subendothelium is dependent on the presence of divalent cations. Thus, the results from studies using methods which measure platelet adherence using blood or plasma anticoagulated with citrate, EDTA or EGTA are open to question. For example, the presence of anticoagulants in plasma modifies the effect of ASA on platelet adherence to a damaged aorta (Cazenave et al., 1975). One of the main advantages of the method reported in this thesis for measuring platelet adherence is the use of a platelet suspending medium containing physiological amounts of calcium and magnesium. Aggregation is inhibited by inclusions of apyrase in the medium. The absence of plasma precludes the generation of thrombin and the formation of fibrin. Anticoagulation of blood

with hirudin, an inhibitor of thrombin, would make it possible to study platelet adherence in the presence of physiologic concentrations of divalent cations. The cost of doing so is prohibitive. Heparin, which may cause the platelets to release and to aggregate (Zucker, 1975), does not appear to be a suitable anticoagulant. In high concentrations, heparin inhibits collagen-induced aggregation (Rowsell et al., 1967).

2. Role of platelet shape change and contractile processes.

It may be that there are several ways in which adherence to collagen can be prevented. One is by inhibition of the mechanisms involved in platelet shape change and pseudopod formation. Platelets contain a marginal bundle of microtubules believed to be important in maintaining platelet disc shape (Hovig, 1968; Behnke, 1970; White, 1971b). Loss of microtubules leads to a loss of this discoid shape (White, 1971a). When platelets interact with collagen they change shape and form pseudopods (White, 1974). Pseudopod formation is a contractile process and there is evidence that platelet microfilaments, composed of contractile protein, are involved in this process (White, 1971a). Similarly, activation of the platelet contractile system and platelet shape change (De Clerck et al., 1975) are necessary for clot retraction to occur. Microtubules have been found in association with microfilaments and with cell membranes and they appear to be involved in the control, distribution and mobility of some cell surface receptors (for review see Nicolson, 1976). Microtubules and microfilaments are associated with cell membranes.

This association is: (1) transient; (2) dependent on cell energy; and (3) affected by drugs that disrupt cytoskeletal components.

We have investigated the effects on platelet adherence to collagen of agents that cause platelets to change shape and we have also examined a number of inhibitors of platelet shape change in response to aggregating stimuli. These inhibitors are also inhibitory of clot retraction and would be expected to affect the contractile processes that could be involved in shape change and pseudopod formation.

(a) ROLE OF ADP AND PROSTAGLANDIN ENDOPEROXIDES.

Collagen-induced aggregation seems to be caused by at least two mechanisms (Packham et al., 1977): (1) the release of ADP and; (2) the formation of endoperoxides and thromboxane A_2. This second reaction can be blocked by non-steroidal anti-inflammatory drugs. Electron micrographic studies of platelets in contact with the collagen fibers on the glass surface have indicated that platelet shape is altered and long pseudopods stretch out along the fibers. Therefore, it seemed possible that prevention of platelet shape change might inhibit platelet adherence to collagen. In earlier studies we found that adding high concentrations of apyrase to destroy the small amounts of ADP that might be released from the platelets did not significantly reduce the adherence of platelets to collagen. The addition of ADP which caused the platelets that were not adherent to collagen to change their shape, did not enhance the adhesion of platelets to collagen; this was studied in the presence of EGTA which prevented ADP-induced

aggregation (Cazenave et al., 1974c). We also demonstrated that high concentrations of apyrase did not affect the adherence of platelets to collagen in the presence of EGTA. It, therefore, does not appear that the shape change which occurs when platelets adhere to collagen is an ADP-dependent process, although many of the inhibitors of ADP-induced shape change and aggregation inhibit platelet adherence to collagen. The contribution of prostaglandin endoperoxides and thromboxane A_2 to the shape change which occurs when platelets adhere to collagen is more difficult to evaluate. The effect of collagen on thrombin-degranulated platelets has been examined (Kinlough-Rathbone et al., 1975; Reimers et al., 1976). It was found that collagen-induced aggregation of degranulated platelets was not inhibited by CP/CPK (in concentrations that inhibited aggregation induced by 10 μM ADP). However, acetylsalicylic acid or indomethacin blocked collagen-induced shape change and aggregation of these degranulated platelets as measured by changes in light transmission through stirred suspensions of platelets. This indicates that collagen can induce shape change and aggregation through the prostaglandin pathway independently of the release reaction. When platelet adherence to collagen-coated rods is studied, ASA or indomethacin inhibits adherence of normal platelets but not of thrombin-degranulated platelets. Since thrombin-degranulated platelets can interact with collagen and form thromboxane A_2 and since non-steroidal anti-inflammatory drugs can block this reaction, but ASA did not decrease the adherence of

thrombin-degranulated platelets it would appear that formation of prostaglandin endoperoxides and thromboxane A_2 is not involved in platelet adherence to the subendothelium.

A direct effect of anti-inflammatory drugs on cell membranes has been reported. ASA has been shown to modify lymphocyte function (Coeugniet and Dirksen, 1976) by a direct effect on the plasma membrane. Similarly, exposure of lymphocytes to indomethacin leads to a transient loss of the surface theta antigen (Bach, 1974). More recently, Kinlough-Rathbone (personal communication) has examined the adherence of platelets to collagen-coated glass slides by scanning electron microscopy. She found that platelets adhered to collagen, changed shape and formed pseudopods even in the presence of concentrations of CP/CPK and indomethacin that blocked changes in oscillations of light transmission through a stirred suspension in an aggregometer. Thus it appears that platelets can interact with collagen and undergo shape change through a process that is presumably independent of the release of ADP and the formation of prostaglandin endoperoxides and thromboxane A_2.

(b) ROLE OF CYCLIC AMP.

PGE_1 was found to be a strong inhibitor of the adherence of platelets to collagen-coated surfaces and to the subendothelium. This may be related to the increase in cyclic AMP produced by PGE_1 (Bergstrom, 1967; Wolfe and Shulman, 1969). Caffeine and adenosine are also known to increase cyclic AMP levels in platelets; the former

inhibits phosphodiesterase (Marquis et al., 1969), and the latter stimulates adenyl cyclase (Mills and Smith, 1971). Caffeine and adenosine were shown to inhibit platelet adherence to collagen-coated tubes (Cazenave et al., 1974c). Pyrimido-pyrimidine compounds (which have been reported to inhibit phosphodiesterase) (Mills and Smith, 1971, Vigdahl et al., 1971) also inhibit platelet adhesion to collagen. Most of the compounds that increase platelet cyclic AMP levels have been shown to inhibit clot retraction. It may be that any compound which increases the concentration of cyclic AMP in platelets affects the contractile process and thereby inhibits the ability of platelets to change their shape (Kinlough-Rathbone et al., 1970; Shio et al., 1970). Inhibition of platelet shape change appears to relate to a strong inhibition of adherence to surfaces in all test systems. PGE_1 also inhibits granulocyte adherence to glass surfaces (Bryant and Sutcliffe, 1974).

(c) ROLE OF MICROFILAMENTS AND MICROTUBULES.

Another agent that is known to interact with contractile protein and inhibit clot retraction is cytochalasin B (Shepro et al., 1970; White, 1971a,b; Haslam, 1972). It inhibits ADP-induced aggregation and maintains platelets in their disc shape (White and Estensen, 1972; Kay and Fudenberg, 1973; White, 1974). It was found to be an effective inhibitor of platelet adherence to the collagen-coated surface (Cazenave et al., 1974c).

Incubation of platelets with colchicine has been shown to inhibit ADP, thrombin, noradrenaline, or collagen-induced aggregation

(Jobin and Tremblay, 1969; Soppittand Mitchell, 1969; White, 1969; Sneddon, 1971). Shepro et al. (1969) showed that 10 mM colchicine inhibited clot retraction but White (1969) found that the loss of platelet capacity to retract clots was correlated with the extent of platelet damage caused by high concentrations. In recent studies, Chao et al. (1976) have demonstrated that colchicine at low concentrations (0.01 - 0.1 mM) inhibits clot retraction. The formation of pseudopods was impaired and no microtubules are found in the presence of colchicine. Colchicine reduces platelet adhesiveness to glass (Soppitt and Mitchell, 1969). Colchicine (1 mM) was found to inhibit platelet adherence to the collagen-coated surface, but there were indications of some platelet damage (Cazenave et al., 1974c). It is not known whether colchicine exerts its effect on adherence through its ability to disrupt platelet microtubules (White, 1969; Sneddon, 1971) which some investigators believe are involved in platelet contractile processes and maintenance of the disc shape (Shepro et al., 1969; White, 1969).

Lidocaine (20 and 80 mM) has been reported to inhibit ADP- or thrombin-induced aggregation and clot retraction (O'Brien, 1961; Aledort and Niemetz, 1968; Feinstein et al., 1976). Local anesthetics produce a wide variety of effects on biological membranes and may also induce direct breakdown of microfilaments and microtubules (Poste et al., 1975). Seeman (1972) has reviewed the evidence that local anesthetics such as lidocaine readily bind to biomembranes and

displace Ca^{2+} from phospholipid binding sites on the membranes; one membrane-bound Ca^{2+} competes with two amine (anesthetic) molecules. Significant inhibition of platelet adherence to collagen-coated tubes and of collagen-induced aggregation in the aggregometer was only apparent at concentrations of lidocaine of 2 mM or greater (Cazenave et al., 1974b). This concentration may be required before the lidocaine can compete successfully for the Ca^{2+} binding sites on the platelet membrane in the presence of 2 mM Ca^{2+} in the medium. This effect on membrane Ca^{2+} is in keeping with the observations that EDTA and EGTA reduce platelet adherence to collagen.

In addition to lidocaine, other agents with membrane stabilizing properties (Seeman, 1972) have been shown to inhibit platelet adherence. These agents are lipid-soluble drugs, they bind to and expand membranes and usually displace membrane bound calcium. Platelet adherence to collagen or to the subendothelium has been shown to be inhibited by sodium pentobarbital (Joist et al., 1973), promethazine (Cazenave et al., 1974b), imipramine and related tricyclic antidepressants (Cazenave et al., 1974 ; Mohammad and Mason, 1974), methylprednisolone (Cazenave et al., 1976b),reserpine (Cazenave et al., 1977c)and propranolol (Weksler et al., 1977).

(d) ROLE OF SULPHYDRYL GROUPS.

Although SH groups on the surface of platelets appear to be involved in ADP-induced aggregation (Robinson et al., 1963; Harrison et al., 1966), those involved in the release reaction and clot

retraction appear to be deeply placed (Aledort et al., 1968; Harbury and Schrier, 1974). On the basis of experiments with NEM, PHMB (p-hydroxy-mercuribenzoate), and PHMBS (p-hydroxy-mercurisulfonate) in EDTA platelet-rich plasma, Al-Mondhiry and Spaet (1970) suggested that an SH-containing substrate affecting the adhesion of platelets to collagen is probably also located beneath the plasma membrane of the platelets. Our findings with the penetrating sulfhydryl group reagent NEM support this conclusion (Cazenave et al., 1974c). With washed, prelabeled platelets in suspension, however, we found that a high concentration of NEM (1 mM) caused release or loss of ^{14}C-serotonin and loss of ^{51}Cr indicating that some release and/or lysis had occurred. Zucker and Jerushalmy (1967) have also reported that high concentrations of this penetrating sulfhydryl reagent cause the platelet release reaction and sphering of platelets. Behnke (1970) reported disappearance of microtubules and localized ballooning of the plasma membrane in response to NEM. Thus, in high concentrations, this agent appears to be unsuitable for investigaticn of platelet functions.

In agreement with Al-Mondhiry and Spaet (1970) we found (Cazenave et al., 1974c) that PCMBS (a nonpenetrating sulfhydryl reagent) did not inhibit platelet adherence to collagen after a 10 minute incubation period. At the concentrations of PCMB that could be used in suspensions of washed platelets without causing loss or release of platelet constituents, this penetrating sulfhydryl reagent did not inhibit platelet adherence to collagen. Al-Mondhiry and Spaet (1970)

did not determine whether release or loss of platelet contents occurred in their experiments, but the presence of plasma may have reduced the effective concentrations of the sulfhydryl reagents. Possibly the internal SH groups with which NEM reacts influence the platelet contractile protein.

(e) ROLE OF METABOLIC ENERGY.

Platelets require energy to carry out their functions of shape change, aggregation, release, phagocytosis and clot retraction (Mustard and Packham, 1970). The energy required to perform these functions is provided by a critical level of metabolic ATP. Platelet function can be inhibited if the level of ATP falls below a critical concentration although the adenylate energy charge may have more influence than the ATP concentration (Mills, 1973). Maintenance of the discoid shape of the platelets depends on metabolic energy which can be supplied by glucose (Kinlough-Rathbone et al., 1970). It has been demonstrated that during ADP-induced shape change, ATP is consumed (Holmsen et al., 1974), the adenylate energy charge is decreased (Mills, 1973) and the oxidation of [6-^{14}C]-glucose to $^{14}CO_2$ is increased (McElroy et al., 1971). Platelet adherence to collagen is inhibited if glycolysis is inhibited by iodoacetate. Inhibition of oxidative phosphorylation by antimycin does not inhibit adherence to collagen. A combination of iodoacetate and antimycin was slightly more effective than iodoacetate alone. These results are similar to those of Bettex-Galland and Lüscher (1960) who concluded that the

energy for clot retraction is largely derived from glycolysis. However, more recently Mürer (1969) has demonstrated that the effect of the inhibitors of glycolysis and respiration on clot retraction was due to a reduction in ATP concentration.

Interpretation of the mechanism of action of inhibitors is complicated by their many sites of action. For example, chelation of divalent cations by EDTA inhibits adherence to collagen, but also inhibits glycolysis in resting platelets and in platelets stimulated by collagen (Loder et al., 1968). NEM, a sulfhydryl inhibitor, inhibits adherence to collagen, but also inhibits glycolysis (Klebe, 1975) and hence inhibits energy production.

3. Coating of the platelet surface.

Penicillin G, cephalothin and related antibiotics inhibit platelet aggregation and the release reaction induced by a wide variety of agents (Cazenave et al., 1973a, 1977a). Among the agents which these antibiotics inhibit are some that do not act by inducing the platelet release reaction. The antibiotics also inhibit the adherence of platelets to collagen and to the subendothelium. It seems likely that their inhibitory effects result from their binding to the platelet surface and blockage of receptors on the platelet surface. However, since serotonin-induced aggregation and serotonin uptake are largely unaffected, there must be some receptors on the platelet surface that are not blocked by these antibiotics. It seems likely that the antibiotics become associated with the platelets since their inhibitory effects are not

completely removed by washing and resuspending the platelets and have been reported to persist in vivo (Cazenave et al., 1976d, 1977a). Coating of the platelet surface by penicillin G or cephalothin is not surprising, because it has been shown that these antibiotics can form stable conjugates with plasma proteins and with proteins of the red cell membrane (Vanarsdel, 1970).

In conclusion, at least three factors are involved in platelet adhesion to collagen: divalent cations, shape change and metabolic energy. Similar requirements have been demonstrated for the attachment and spreading of other mammalian cells on various substrates. Adhesion of fibroblasts to a culture dish coated with serum proteins or cellular products requires the presence of divalent cations (Takeichi and Okada, 1972; Culp and Buniel, 1976). The cells can attach to a clean glass or plastic surface in the absence of divalent cations (Ca^{2+} and Mg^{2+}), if serum-free medium is used (Okada et al., 1974). Fibroblasts attach and spread on a collagen substrate only in the presence of a collagen-dependant cell attachment factor (Klebe, 1974). The cell attachment factor purified from serum is a high molecular weight protein that binds to collagen in the absence of divalent cations, but divalent cations are required for cells to attach to the collagen-cell attachment factor complex (Klebe, 1974). Fibroblast attachment to collagen requires cellular metabolic energy and is decreased by inhibition of glycolysis (Klebe, 1975).

IV. PLATELET ADHERENCE TO COLLAGEN, HEMOSTASIS AND PLATELET SURVIVAL.

1. Platelet adherence to collagen and bleeding time.

The results obtained with the rotating probe device show that adherence measured with this method bears some relation to the hemostatic function of platelets in vivo. However, these results are preliminary and more work is necessary to evaluate completely the relationship.

The studies with platelets treated with thrombin, penicillin G, cephalothin or methylprednisolone show that when adhesion to collagen or to subendothelium is impaired, the platelets are not as effective in hemostasis. However, the studies with platelets treated with thrombin, penicillin G, cephalothin or methylprednisolone are complex to interpret. In the case of the thrombin-degranulated platelets, they have no releasable ADP (Reimers et al., 1976), their adherence to collagen is decreased and they are unresponsive to thrombin. In the case of the platelets treated with the antibiotics (Cazenave et al., 1977a) or with methylprednisolone (Cazenave et al., 1976b), they have a diminished adhesion to collagen and do not aggregate to ADP, thrombin or collagen. Thus when the effect of these treatments of platelets is examined in relation to hemostasis, the resulting impairment could be a summation of all these inhibitory effects.

(a) THROMBIN-TREATED PLATELETS.

Thrombin-treated platelets that have lost their releasable ADP can survive normally in the circulation of rabbits (Reimers et al., 1976). The hemostatic properties of thrombin-degranulated platelets were examined in rabbits that had been made very thrombocytopenic (platelet

count < 10,000 per mm^3). In these animals the bleeding time from a puncture wound in the exposed jugular vein was more than 900 sec. Upon infusion of untreated control platelets the bleeding time was shortened to 124 sec. whereas thrombin-degranulated platelets only shortened the bleeding time to 463 seconds. Thus, thrombin-degranulated platelets, although they showed some activity in hemostasis, were far less effective than untreated control platelets. There are at least three possible explanations for this: (1) since the in vitro experiments showed that adherence of these platelets to the exposed subendothelial tissue of aortas is diminished, it is likely that they do not adhere as readily to the puncture wound site as control platelets; (2) the thrombin-degranulated platelets are unresponsive to thrombin (Reimers et al., 1976); (3) these platelets lack releasable ADP and the release of ADP from platelets interacting initially with the wound site is thought to contribute to the accumulation of further platelets at this site, thus building up the primary hemostatic plug (Mustard and Packham, 1971). It seems likely that impairment of all these mechanisms contributes to the diminished hemostatic effectiveness of the thrombin-degranulated platelets.

(b) PENICILLIN G AND CEPHALOTHIN-TREATED PLATELETS.

The diminished ability of platelets preincubated with penicillin G or cephalothin to shorten the bleeding time of thrombocytopenic rabbits is in keeping with our observations from other studies of the effects of these antibiotics on platelet function (Cazenave et al., 1973a, 1977a).

We found that platelets incubated with penicillin G or cephalothin, washed and resuspended in fresh medium responded less strongly to aggregating and release-inducing agents (ADP, collagen, or thrombin) than control platelets. It seems likely that the antibiotics became bound to the platelets. Thus, it is not surprising that platelet function in vivo is inhibited by preincubation of the platelets with the antibiotics in vitro. The impairment of the ability of platelets to adhere to subendothelial structures and to react to aggregating agents would account for the observation that the antibiotic-treated platelets were much less effective than control platelets in shortening the bleeding times of thrombocytopenic rabbits.

Thus, the effects of penicillin G and related antibiotics on the bleeding times of patients (McClure et al., 1970; Lacombe et al., 1974; Roberts, 1974; Houbouyan et al., 1975) and volunteers (Brown et al., 1974, 1975, 1976) given high doses of these drugs is, at least in part, attributable to the inhibitory effects of the antibiotics on the platelets. Our results, however, do not exclude the possibilities that the antibiotics also affect the vessel wall and/or the plasma proteins.

(c) METHYLPREDNISOLONE-TREATED PLATELETS.

Methylprednisolone, in high concentrations, is a potent inhibitor of platelet shape change, aggregation and release induced by ADP, thrombin or collagen. The effect of methylprednisolone on platelets persists even when the platelets are washed and resuspended in a fresh medium (Cazenave et al., 1976b). Methylprednisolone is also a potent

inhibitor of platelet adherence to collagen and to the subendothelium. Thus, it is not surprising that platelets which had been pretreated with methylprednisolone washed, and resuspended in a medium not containing the drug were much less effective than control platelets in shortening the bleeding times of thrombocytopenic rabbits. However, methylprednisolone can affect the bleeding time through effects on the vessel wall as well as on platelets. Intravenous injection of methylprednisolone to severely thrombocytopenic rabbits shortens the bleeding time to normal. Similar results have been reported by Senyi et al. (1975) with hydrocortisone, and have been interpreted as evidence for a direct effect of the drug on the vessel wall.

2. Platelet adherence to collagen and platelet survival.

Platelet survival is one of the tests of platelet function that shows a relationship to the clinical manifestations of arterial disease. Shortened platelet survival and increased platelet turnover in subjects who have had clinical manifestations of atherosclerotic disease have been demonstrated by a number of investigators (Murphy and Mustard, 1962; Abrahamsen, 1968; Steele et al., 1973a; Harker and Slichter, 1974). There are drugs such as sulfinpyrazone, dipyridamole and clofibrate, which prolong shortened platelet survival, decrease platelet turnover and also appear to decrease the incidence of thromboembolic complications. These drugs have little effect on in vitro tests of platelet function at the blood levels achieved with therapeutic doses (Genton et al., 1975; Mustard and Packham, 1975).

There is also evidence that treatment of platelets with ADP (Mustard et al., 1966) or thrombin (Reimers et al., 1973b; 1976) does not influence platelet survival. Thus, it would appear to be important to study whether altering the ability of platelets to adhere to surfaces influences platelet survival.

(a) MODIFICATIONS OF PLATELET SURFACE.

The effect on rabbit platelet survival of agents that alter the platelet surface, some of which also cause the release of platelet granule contents, shows that there is no correlation with platelet adhesion to collagen or to the subendothelium.

(i) Thrombin-treated platelets.

When thrombin-treated platelets are infused into rabbits, about 45% are recovered in the circulation after 2 hours. This low recovery has been shown to be caused by the plasmin used in the preparation of thrombin degranulated platelets (Reimers and Greenberg, personal communication). These platelets that circulate show the same pattern of survival as normal platelets. Thus, thrombin does not appear to damage or alter the thrombin-treated platelets that circulate after reinfusion into rabbits in such a way that their survival is shortened (Reimers et al., 1973; 1976). It can be concluded that the release reaction as such and any modifications of the platelet surface caused by thrombin are apparently not factors that determine the lifetime of a platelet.

(ii) Neuraminidase- or periodate-treated platelets.

$NaIO_4$ pretreatment altered the platelets so that they were less

adherent to collagen and to subendothelial structures. This was in contrast to the effect of removal of surface N-acetylneuraminic acid which had no demonstrable effect on platelet adherence to these surfaces.

However, platelet survival was affected by either $NaIO_4$ pretreatment or removal of surface N-acetylneuraminic acid (Greenberg et al., 1975) and the platelets were rapidly removed from the circulation of rabbits into which they had been infused. These results indicate that removal of sialic acid (as suggested by Grøttum and Jeremic (1973)) or alteration of sialic acid resulted in the recognition of the platelets as "foreign" by the reticuloendothelial system. The results indicate that there is no correlation among alteration in platelet function, platelet adherence to vessel surfaces, and shortening of platelet survival. $NaBH_4$ apparently reduced the groups involved in this recognition so that the platelets were no longer subject to rapid removal from the circulation (Cazenave et al., 1976c). Removal of sialic acid from red blood cells (Gattegno et al., 1974; Jancik and Schauer, 1974; Durocher et al., 1975; Aminoff et al., 1976) and from some serum glycoproteins (Ashwell and Morell, 1974) has been shown to cause their rapid clearance from the circulation. Oxidation of sialic acid of membrane glycoproteins of mouse lymphocytes has also been shown to impair their recirculation in vivo and $NaBH_4$ reversed this effect of oxidation (Zatz et al., 1972). Durocher et al. (1976) have demonstrated that mild periodate oxidation of rat red blood cells shortens their survival and that sodium borohydride partially reverses the effects of

periodate oxidation on survival.

The mechanism of clearance by the reticulo-endothelial system of the platelets treated with neuraminidase or $NaIO_4$ is not clear at the present time. There are at least two possibilities: treatment by neuraminidase and $NaIO_4$ is such that the cells are recognized by the reticuloendothelial system as "foreign." Neuraminidase cleaves terminal sialic acid residues of glycoproteins, exposing a galactose residue. It has been shown that desialylated plasma glycoproteins are cleaved by the liver cells which have a receptor for the exposed galactose of sialoglycoproteins (Ashwell and Morrell, 1974). That such a mechanism operates for circulating cells is not known. The second possibility is that neuraminidase or $NaIO_4$ treatment exposes new antigenic determinants on the platelets which are recognized by preexisting immunoglobulins. Then these immunoglobulin-coated platelets could be recognized by the macrophages of the reticulo-endothelial system and removed from the circulation by phagocytosis. Although this second possibility is at the present time speculative, there are 3 pieces of evidence in its favor. (1) Treatment of cells with neuraminidase or $NaIO_4$ creates new antigenic determinants (Reisner, 1975; Schmidtke and Simmons, 1975). (2) The serum of most mammalians and birds contains natural antibodies specific for the treated cells (Moskowitz and Treffers, 1950; Kipnis and Sacks, 1955; Rogentine and Plocinik, 1974; Durocher et al., 1976). (3) $NaIO_4$ treated erythrocytes of chicken are rapidly cleared from the circulation of normal birds, but not of agamma globulinemic birds (Cazenave, unpublished observations).

It is possible that naturally circulating immunoglobulins which react with altered membrane glycoproteins may participate in the recognition process of senescent or damaged platelets by the reticulo-endothelial system and thus may be one of the factors governing platelet survival in the circulation.

(b) MODIFICATION OF PLATELETS BY PENICILLIN G.

Pretreatment of rabbit platelets with a high concentration of penicillin G in vitro did not affect their recovery or survival in vivo following reinjection. Although penicillin G inhibits adhesion to subendothelium and collagen, inhibits ADP, thrombin and collagen-induced aggregation and prolongs the bleeding time, it could not be demonstrated that it affects platelet survival. These studies were done by infusing ^{51}Cr-labeled penicillin-treated platelets into rabbits. It has been shown that the effect of carbenicillin, a penicillin derivative, remains throughout the lifespan of the platelets (Brown et al., 1974). This may mean that platelet interaction with surfaces is not an important determinant of platelet survival. (It has already been shown that neither exposure of platelets to ADP (Mustard et al., 1966), nor to thrombin (Reimers et al., 1973b,1976) influences platelet survival.)

It remains to be seen whether the penicillin antibiotics can prolong shortened platelet survival.

There is evidence from experiments with dogs that carbenicillin and ticarcillin (two penicillin derivatives) inhibit arterial thrombosis induced by damaging an isolated segment of artery with pronase (Lyman et al., 1976).

Another possible mechanism of shortened platelet survival is the alteration of platelets when they take part in the response of the blood to injury and then return to the circulation. Since it has not been possible to show that thrombin (Reimers et al., 1973b,1976) or ADP (Mustard et al., 1966) alter the platelets sufficiently to shorten their survival, it is important to establish whether platelet interaction with surfaces can lead to platelet alterations that shorten platelet survival. When platelets interact with a release-inducing stimulus they form a collagenase activity (Chesney et al., 1974). There is evidence that platelets that adhere to a damaged vessel surface may eventually leave the surface (Baumgartner, 1973). Harker et al. (1976) have injected homocysteine continuously into baboons. They demonstrated that such a treatment induced a loss of endothelial cells with subsequent platelet interaction with the exposed subendothelial tissue. Platelet interaction with the vessel wall resulted in a shortened platelet survival. Administration of dipyridamole produced a significant prolongation of platelet survival and prevention of the development of vascular lesions of arteriosclerosis.

Thus there are two lines of evidence about factors that may influence platelet survival. Those derived from the studies in this thesis that indicate that modifications of the platelet surface, particularly sialic acid, lead to shortened platelet survival. It has recently been found (Greenberg et al., 1976) that removal of platelet surface proteins and glycoproteins by chymotrypsin treatment

also causes a rapid removal of the platelets from the circulation. In all these studies we were unable to show a correlation between treatments which modify platelet adherence to surfaces and changes in platelet survival. The second line of evidence comes from the work of Harker et al. (1976) and Ross and Harker (1976), in which they found that damage to the endothelium by homocysteine, by hyperlipemia or by balloon injury causes shortened platelet survival. One would expect in these studies that the explanation for shortened platelet survival would be platelet adhesion to the damaged vessel wall. Further work would have to be carried out to determine the relative importance of the subendothelium in influencing platelet survival versus alterations in platelet membrane glycoproteins.

SUMMARY

SUMMARY

The aim of these studies was to develop a quantitative method for measuring platelet adherence to collagen-coated surfaces and to the subendothelium in the absence of platelet aggregation and to study the role of the components of the vessel wall, the role of platelet modifications and the role of the composition of the platelet suspending medium on platelet adherence to these surfaces.

The aspects of this thesis which represent new developments and have advanced our knowledge in relation to thrombosis and hemostasis are:

1. A quantitative method for measuring the adherence of individual platelets to collagen-coated surfaces or to the subendothelium has been developed. The features of this method which are distinct from those used by other investigators and which are improvements over earlier methods are:. (a) Platelets are labeled with ^{51}Cr. This label is not released or lost from adherent platelets and allows accurate quantitation of adherence over a large surface area without any possibility of the observer bias which is a drawback of the morphometric techniques. (b) The platelets are suspended in an artificial medium for the adherence measurements. Thus the constituents of the medium can be varied to determine their effects on platelet adherence and the platelet count can be adjusted in any way that is required. (c) Platelet adherence is studied in a medium containing approximately physiological concentrations of calcium and magnesium. (d) The adherence of single platelets is measured because apyrase is incubated in the suspending fluid

to degrade any ADP released or lost from the platelets. Platelet aggregates are not formed on the surfaces under the conditions used. (e) The absence of plasma permits platelet adherence to be studied without the complications that would be introduced by the thrombin generation and the formation of fibrin. (f) Adherence is measured at 37°C. (g) The surfaces to which platelets adhere (collagen-coated glass rods or everted rabbit aortas damaged by passage of a balloon catheter or by scraping) are mounted on rotating probes. The rate of rotation can be accurately controlled and is reproducible. Thus, the characteristics of flow around the surfaces on the rotating probes are constant.

2. It was found that platelet adherence to collagen-coated glass or to the subendothelium is greatly reduced in the absence of divalent cations. This finding affects the interpretation of all platelet adherence studies of other investigators which have been done in plasma anticoagulated with EDTA or citrate, or in artificial media without calcium.

3. The optimum pH for platelet adherence studies was found to be pH 7.35 under the conditions used. Adherence is less at lower pH values and aggregate formation occurs at higher pH values.

4. The albumin content of the medium was found to affect the number of adherent platelets. Fewer platelets adhered in a medium containing 4% albumin than adhered when 0.35% albumin was used. However, at the high albumin concentration there was less variation among

replicate samples.

5. The hematocrit also was shown to affect the number of adherent platelets. Progressively, more platelets adhered at hematocrits of 10, 20 and 40% than at zero hematocrit.

6. It was observed that platelets that became adherent to collagen or the subendothelium were not readily dislodged. Rinsing the surfaces to which platelets had adhered in solutions which cause immediate disaggregation of platelets (EDTA, EGTA or prostaglandin E_1) did not remove adherent platelets. This indicates that platelets adherent to the subendothelium at injury sites in vivo probably remain on the damaged wall, even if platelet aggregates which initially form on top of them are broken up and washed away.

7. It was found that when an endothelial surface which had not been damaged deliberately was exposed to thrombin and then rinsed before platelet adherence was studied, many more platelets adhered to the thrombin-treated surface than to an endothelial surface which had not been exposed to thrombin and this was blocked by heparin. This observation may indicate that local generation of thrombin in vivo could result in platelet adherence to the undamaged endothelial surface.

8. After these investigations were well underway it was found that some of the inhibitory effects of drugs or of modifications of the platelet surface which were readily demonstrable at 0.35% albumin and zero hematocrit were not apparent at 4% albumin with a 40% hematocrit. However, any drug or treatment which was inhibitory at 4% albumin and

40% hematocrit was also shown to be inhibitory at 0.35% albumin and zero hematocrit. For initial screening tests the simpler system (0.35% albumin, zero hematocrit) was found to be useful, but for final rigorous testing the more physiological system was used.

9. The effects on platelet adherence of modification of the platelet surface with several enzymes and with sodium periodate were examined. Removal of surface sialic acid with neuraminidase did not affect the number of adherent platelets whereas treatment with the proteolytic enzymes thrombin, plasmin or chymotrypsin decreased adherence by 30 to 50%. Sodium periodate also was shown to reduce platelet adherence at low hematocrits but not at 40% hematocrit.

10. UDP and UDPG were shown to have no effect on platelet adherence to collagen-coated glass surfaces. These observations are not in keeping with the collagen:glucosyltransferase theory of platelet-collagen interaction but may be compatible with the later concept that a lectin-like type of binding may occur.

11. A subcomponent of the first component of complement, Clq, was found to inhibit collagen-induced aggregation but not aggregation induced by ADP or thrombin. Clq also inhibited platelet adherence to collagen-coated surfaces. These inhibitory effects are most probably attributable to competition between Clq and collagen for the collagen receptor on the platelet membrane since there are structural similarities between Clq and collagen.

12. Platelet adherence to the collagen-coated surface and to the subendothelium was shown to be inhibited by a wide variety of drugs and agents which inhibit platelet aggregation and the release reaction. The components that were shown to be inhibitory by the simple screening system (0.35% albumin, zero hematocrit) were: (a) EDTA, EGTA and citrate. (b) non-steroidal anti inflammatory drugs - acetylsalicylic acid, sodium salicylate, sulfinpyrazone, and indomethacin; (c) the metabolic inhibitor iodoacetate; (d) agents that increase platelet cyclic AMP levels - prostaglandin E_1 and pyrimido-pyrimidine compounds; (e) reserpine; (f) penicillin G and related antibiotics; (g) methylprednisolone.

Of these inhibitors, only some were found to decrease platelet adherence under more rigorous testing at 4% albumin and 40% hematocrit. These were EDTA, EGTA, citrate, indomethacin, prostaglandin E_1, dipyridamole, RA 433, penicillin G, cephalothin and methylprednisolone.

13. Experiments were done in which acetylsalicylic acid was administered to rabbits. The ability of the platelets that had been exposed to aspirin in vivo to adhere to the subendothelium from aspirin-treated and normal rabbits was examined. It was shown that the acetylsalicylic acid affected the platelets and not the vessel wall.

Although the inhibitory effect of acetylsalicylic acid on platelet adherence was demonstrable in suspensions of washed platelets (0.35% albumin, zero hematocrit), it was not apparent when citrated platelet-rich plasma was examined. The lack of effect of acetylsalicylic acid in the presence of citrate is in agreement with the findings of other

investigators who used citrated platelet-rich plasma.

14. The possibility of a correlation between the ability of rabbit platelets to adhere to collagen-coated surface or the subendothelium and platelet survival was investigated. Platelets treated with thrombin survival for a normal length of time after infusion into recipient rabbits whereas platelets treated with sodium periodate or neuraminidase were rapidly cleared from the circulation. These observations indicate that if platelets take part in reversible thrombus formation in vivo they may survive for a normal length of time whereas if the sialic acid on their surface is removed or altered they are recognized as "foreign" and removed from the circulation. Platelets treated with penicillin G or methylprednisolone before infusion into animals survived normally. These observations indicate that treatments which inhibit the ability of platelets to adhere to collagen or the subendothelium do not necessarily affect their survival in vivo.

15. The possibility of a correlation between the ability of rabbit platelets to adhere to a collagen-coated surface or the subendothelium and their hematostatic effectiveness was investigated by examining the effect of various platelet treatments on their ability to shorten the bleeding time from the punctured jugular vein of thrombocytopenic rabbits. Thrombin-treated platelets did correct the bleeding time to some extent but they were less effective than normal platelets. Platelets that had been pretreated with penicillin G, cephalothin, or methylprednisolone did not correct the long bleeding times of

thrombocytopenic rabbits. Thus with respect to these treatments, there is a correlation between the ability of platelets to adhere to a collagen-coated surface or the subendothelium, and their hemostatic effectiveness. This is in accord with the earlier findings of other investigators that platelet adherence to collagen in the vessel wall is an early step in hemostatic plug formation.

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