PHYSIOLOGICAL AND PHARMACOLOGICAL ASPECTS OF THE PALTELET[1,2]

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Relatively few reports concerning the platelet appear in pharmacological literature, but it is certain that this cell will be given increasing attention in the future. This prediction is based on the following. (*a*) The platelet is a cell which can be readily isolated, and as will be seen is a useful model with which to study certain parameters of drug action. In particular, the introduction of a nephelometric method which can accurately record the events of platelet aggregation has enabled much knowledge to be accumulated on the specificity of the cell receptor sites and its interactions with a variety of compounds (1–4). (*b*) Thrombocytopenia is one of the more troublesome and unpredictable side effects of many drugs which play an important part in modern therapeutic measures (5), and is also frequently a cause of the suspension or inability to employ otherwise effective chemotherapeutic or immunosuppressive regimes. On rare occasions this complication has occurred so frequently with useful pharmacological agents, e.g., Sedormid and ristocetin, that the drug has had to be withdrawn from therapeutic use (6, 7). Despite the obvious importance of drug thrombocytopenia, our knowledge of the effects of the involved drugs on platelet function and metabolism is fragmentary, and in only a few instances have well-documented investigations of basic mechanisms been undertaken (8, 9). (*c*) Thrombosis is one of the major problems of modern medicine, and current concepts of hemostasis and thrombosis universally propose a central role for the platelet and, in particular, the phenomenon of platelet aggregation. At present, the pharmacological effects of drugs on platelet aggregation are being extensively examined with the realisation that in arterial thrombosis platelet aggregation is of major importance (10), and that a valuable therapeutic approach would be the use of compounds which inhibited platelet cohesion. This ap-

[1] The survey of the literature pertaining to this review was concluded in April 1968.

[2] The following abbreviations will be used: ADP (adenosine diphosphate); AMP (adenosine monophosphate); ATP (adenosine triphosphate); EDTA (ethylene diamine tetraacetate); EGTA (ethyleneglycol diaminoethyl tetraacetate); 5-HT (5-hydroxythyptamine, serotonin); PRP (platelet-rich plasma).

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proach is feasible in man since it has been demonstrated that platelets obtained from some patients with uraemia have a severe impairment of their ability to aggregate with only a mild bleeding diathesis (11). This abnormality may be rapidly reversed by dialysis (12).

It should be emphasised that hemostasis and thrombosis are interactions of events involving the blood vessels, the platelets and perhaps other formed elements of blood, blood viscosity, and coagulation. The major components may vary from situation to situation, as is demonstrated in the architectural differences between venous and arterial thrombi. These appearances can be readily reproduced *in vitro* by subjecting blood to varying degrees of shear during the progress of coagulation. At low rates of shear a few scattered platelets are seen on a background of fibrin, and histological examination reveals findings which are identical to those seen in a venous thrombosis and are similar to a test-tube clot. On the other hand, exposure of the blood to a high rate of shear with subsequent microscopic study reveals large platelet aggregates surrounded by leucocytes (13). This is the classical appearance of an arterial thrombus (10). The contribution of all of these factors in hemostasis is underlined by clinically anomalous situations wherein platelets may be reduced in number, or may have little ability to aggregate, without a resultant bleeding diathesis of any marked degree.

This review will be divided into two sections. The first section will consider presently held concepts of platelet ultrastructure and physiology, whilst the final section will deal with pharmacological aspects of the platelet.

Ultrastructure

The mammalian platelet is an anucleate cell approximately 2 to 3 μ in diameter and is produced by cytoplasmic fragmentation from the megakaryocyte in the bone marrow (14, 15) or in the lung capillaries (16), but like the red cell the platelet is believed to remain intravascularly throughout its life span. There is now ample evidence which supports the concept of "pooling" of platelets in certain organs, particularly the spleen, and these pooled cells are interchangeable with those circulating (17–19). The spleen may act as a safety monitor sequestering excessively adhesive platelets, or reversible platelet aggregates, or both, until they become less sticky or disaggregation occurs, or both, and in this way, may avert the initiation of thrombosis (20). The ultimate fate of the platelets and their possible metabolic and functional interrelationships with the vascular endothelium are two major questions which recent work has failed to answer satisfactorily. Some hold that platelets are being constantly consumed in a continuous process of subliminal intravascular coagulation (21) but the present, more widely held view, is that these cells, in man, live a finite life span of approximately ten days, during which time they may make metabolic contributions to the vasculature, whilst fulfilling the role of a maintenance work force ready to pinpoint and repair any breach which may occur in the vascular lining (22, 23).

There seems little doubt that in man the major and possibly sole function of this cell is that of hemostasis, although it possesses some remarkable properties, the functional significance of which are quite obscure. These latter properties include: (*a*) the cell's ability to phagocytose small particles (24–26); (*b*) its avid uptake and transport of serotonin (27–30); and (*c*) its high content of acid phosphatase (31) and taurine (32).

The way in which the platelet achieves hemostatic control may best be illustrated by outlining the sequence of events which occur following trauma to a blood vessel. Initially, platelets adhere singly to the exposed collagen of the damaged vessel wall, and this adhesion results in the release from the platelets of ADP with the consequent commencement of reversible platelet aggregation (33–36). The platelets carry with them a number of plasma coagulation factors which are adsorbed to the cell's surface, with varying degrees of affinity, in the form of a plasma atmosphere (37–39). If the vessel wall has been ruptured, extravasated blood enters the extravascular space, and surface contact results in the initiation of the intrinsic pathway of thromboplastin generation by the activation of factor XII (Hageman factor). Tissue thromboplastin may also be released and, at the same time, extravasated red cells may break down and cause a further increase in concentration of ADP (40). The intrinsic or extrinsic pathways of coagulation, or both, result in the formation of small amounts of thrombin which are initially unable to overcome the various antithrombin factors present in plasma, and convert fibrinogen to fibrin with resultant clot formation, but are able to bring about platelet aggregation. Prior to the formation of thrombin, the platelet aggregates could disperse and the contained platelets recirculate without any apparent effect with regard to their viability (41), hence the term "reversible aggregation." The enzyme thrombin acts on the platelet causing a release reaction with the resultant loss from the cell of ADP, serotonin, potassium, and amino acids (42). The cells also lose their contained granules (43) (*vide infra*) and become irreversibly aggregated. The released granules (44) or the altered platelet surface membrane are believed to contain platelet factor 3 or platelet thromboplastin (4). Platelet factor 3 combines with the numerous other plasma factors (some of which are present in the platelet atmosphere) concerned with the generation of thromboplastin (prothrombinase) which converts prothrombin to the active enzyme thrombin (43). Thrombin is thereby produced in sufficient quantities to overcome its antagonists and a clot is formed.

Figure 1 is a schematic representation of the ultrastructure of the human platelet. Its plasma membrane has the characteristic trilaminar appearance of other cell membranes, and its outer layer has frequently a nonspecific fuzzy outline which could represent some adsorbed protein. The surface is sometimes invaginated and pseudopods are often observed, although their frequency varies with the method of collection. Immediately beneath the membrane, nondescript fibrillar structures 70 Å in diameter may be noted and are usually arranged in a haphazard fashion except in pseudo-

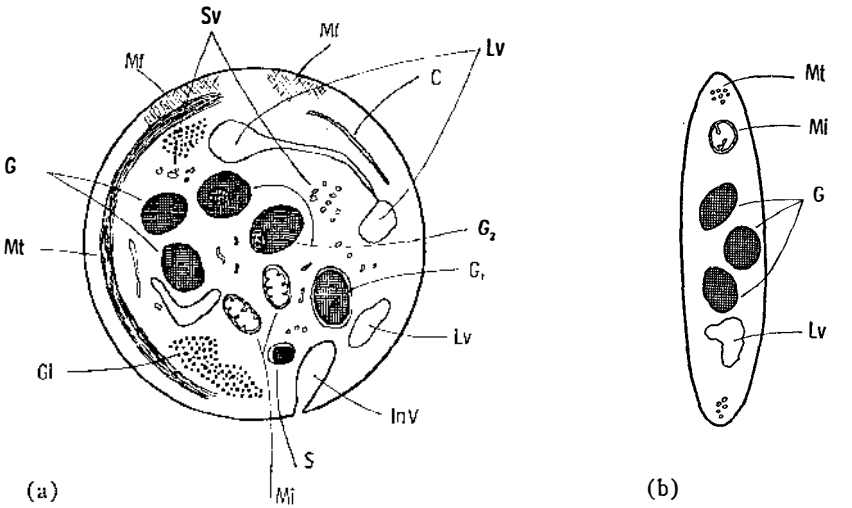

FIG. 1. Schematic representation of a human platelet sectioned in two planes. C = canal; G = α-granules; G_1 = α-granule contained in a large vesicle; G_2 = α-granules with dense osmophilic circular area; Gl = glycogen particles; InV = invagination into platelet's hyaloplasm—possibly due to pseudopodal formation and may later result in the formation of a large vesicle; Lv = large vesicle; Mf = microfibrils; Mi = mitochondria; Mt = microtubules; S = serotonin-containing organelle; Sv = small vesicles.

podia, where they are aligned parallel with the long axis. These have been termed microfibrils (45). Normally, the cell circulates in the form of a disc (46), and microtubules—which can be seen in Figure 1 (a) and 1 (b) cut in both the equatorial and transverse planes—are believed to have an important, possibly skeletal, role in maintaining the cell's shape (47–49). This is supported by the disappearance of microtubules when the cell's shape is changed from a disc to a sphere by cooling (50–52). The microtubules are 200 to 250 Å in diameter and are arranged in bundles of 5 to 7 (48, 49). They were only identified following the introduction of glutaraldehyde fixation and subsequent treatment with osmic acid, and are not seen in fresh platelet preparations fixed with osmic acid alone (49). Under certain conditions, e.g., incubation for 6 hr at 37° C in plasma prior to osmic acid fixation, microtubules are observed as well as an intricate canalicular system. It has been suggested that changes in lipid composition during the incubation renders the microtubules and the canals osmophilic and thus visible to electron microscope examination (53, 54). The canals seen in these prepara-

tions frequently contain granules, often link one large vesicle to another, and on occasions extend to the platelet's surface. This canalicular system may be represented in standard preparations by the large and small vesicles. The recent discovery of microtubules emphasises that further improvement in electron microscope fixation and staining techniques may result in new observations in ultrastructure.

The α-granules are the most striking feature of the platelet and are present in all mammalian species so far examined. In man they are usually 0.3 to 0.4 μ in length and 0.2 μ in diameter, having an ovoid shape. An eccentrically placed, rounded dense osmophilic area is often present in the otherwise granular matrix of this organelle which is enclosed in a membrane usually, but not always, closely applied (G_1 versus G_2, Fig. 1). After incubation of platelet-rich plasma at 37°C, some granules have been observed in the cannular system and it has been speculated that normally there may be interconnections between these structures, the large vesicles, and the cell's exterior (53, 54). Morphological changes in stored platelets support the concept that these granules are lysosomes (53) but the most direct evidence has been the demonstration of acid phosphatase, β-glucuronidase, and cathepsin in isolated platelet granules (55). Recently, the latter findings have been challenged by Siegel & Lüscher who used similar techniques but could demonstrate only acid phosphatase activity in the α-granules and found cathepsin and β-glucuronidase in isolated microvesicles (56). The role of the α-granules in platelet physiology is still uncertain. They may have some lysosomal function, important in the platelet release reactions and aggregation (57, 58), in clot resolution, or in the platelet's phagocytic capacity. On the other hand, Fonio's original suggestion (44) that the granules are the source of platelet factor 3 has been supported by recent morphological studies which show the transition of the platelet granule to a micellar structure similar in appearance to lipid particles with thromboplastic activity (59).

Another granule which has been overlooked by earlier investigators has now been identified (60, 61). This organelle ranges from 0.05 to 0.13 μ in diameter and is contained within a unit membrane approximately 50 Å wide. It is extremely osmophilic and is the densest structure seen in the platelet. Occasionally it is contained within a small vacuolar space. Evidence has been cited which strongly suggests that this organelle represents the site of 5-HT storage in the platelet. The evidence for this is as follows: (*a*) 5-HT is extremely osmophilic; (*b*) the number of these granules present varies in platelets obtained from species to species in strict accord with the 5-HT content of that species' platelets (for example, the rabbit has a high platelet 5–HT level and has a large number of these organelles whereas man and the guinea pig have relatively low platelet 5-HT levels and have few granules); (*c*) treatment of platelets with reserpine, which lowers their 5-HT content, also results in a disappearance of these granules; (*d*) platelets obtained from 5-HT-depleted animals and incubated with

5-HT showed a reappearance of the granules; and (*e*) isolation of the granules by gradient centrifugation following platelet rupture has demonstrated that they contain both a high 5-HT and ATP content (61). This confirms previous suggestions that 5-HT is stored in the platelet bound to ATP (62). Pletscher and his co-workers have been able to demonstrate that the membranes of these granules differ physiologically from that of the platelet membrane in transport properties (61). There is also a similarity between these descriptions and the catecholamine granules which have been reported in the adrenal medulla and mast cells (63, 64). In addition the platelet contains a small number of mitochondria which are somewhat smaller and contain fewer cristae than mitochondria observed in other cells. With suitable preparation, clumps of dark conglomerates 100 to 200 Å in diameter are found in aggregates in the platelet hyaloplasm. These are believed to be glycogen particles and vary considerably in amount from cell to cell. Scattered through the platelet's cytoplasm are numerous small vesicles probably representing transverse sections of an extensive canalicular system which ramifies throughout the platelet. This system may connect with the large vesicles which occasionally contain some nondescript material loosely adherent to the membrane, or more rarely hold an α-granule. Some of these vesicles appear to be arising from pseudopodal formations in the platelet (Fig. 1). Pinocytosis has not been observed by the author (B.F.) in human platelets.

Attempts have been made to correlate important aspects of platelet biochemical and physiological function with the cell's ultrastructure. Many puzzling features remain and much of the evidence is unsatisfactory or speculative since the techniques employed are still relatively crude and often nonspecific and indirect. This may be illustrated by the two proteins, fibrinogen and thrombosthenin, which are important in platelet aggregation and the property of clot retraction. A significant proportion of fibrinogen cannot be removed from the platelet by repeated washing (65) and is believed to be an integral part of the platelet (66). Its intracellular identification relies on the use of ferritin-labelled antibody (67), which can only enter the cell to react with fibrinogen after the plasma membrane has been sufficiently damaged (as is the case with frequent washing). In this instance, a widespread distribution in the platelet's hyaloplasm is seen, but whether such is the case in the intact circulating cell is debatable. Following viscous metamorphosis, fibrillar structures are identified by this technique to be antigenically related to fibrinogen. These extend from the α-granules in the platelet masses to fibrin and interconnect with α-granules in neighbouring clumps of platelets (68). The contractile protein, thrombosthenin, which has similar properties to actomyosin of muscle cells has been isolated from human platelets (68a). Attempts have been made to localise the protein intracellularly by identifying ATPase activity, since this is an integral component of thrombosthenin as well as of muscle actomyosin. The ATPase activity connected with cation transport may be decreased by the use of ouabain and is mainly located in the plasma membrane. The remaining

activity may be due to nonspecific ATPase as well as thrombosthenin (69, 70). Several laboratories have recently reported the incorporation of amino acids into human platelet proteins and in particular into thrombosthenin and fibrinogen (71–73). Transport of various amino acids into the human platelet has been found to be independent of the anticoagulant employed but there is considerable variation for individual amino acids. Glutamic acid is the most poorly transported. The following amino acids are listed in ascending order of their incorporation into the platelet: glutamic acid <glycine <proline <leucine <serine <lysine (74). The paucity of ribosomes in the human platelet (75) raises the question of the site of this amino acid incorporation (? mitochondria) and whether this represents true *de novo* synthesis of platelet protein.

One of the most important platelet properties is its ability to release ADP and perhaps its other components, such as 5-HT and amino acids, following appropriate stimuli. This is not a generalised release, since ^{14}C-labelled amino acids taken up by the platelet are not exchanged at any faster rate, following the addition of thrombin, than occurs in platelets incubated in plasma without thrombin. Holmsen has also reported that there is compartmentalisation of platelet ADP, and that ADP released from the cell is derived from a metabolically inactive pool (76); it is possible this may also be true for the amino acids (77).

Comparative studies of platelets from different species may answer some of the above questions by their individual and sometimes unique properties, allowing better opportunities for unravelling physiological functions, e.g., the rabbit's platelets in the understanding of 5-HT storage sites (61) (*vide supra*). A comparative approach could be useful in the investigation of subcellular fractionation of platelets; for example, acetylcholinesterase (78) might be an extremely useful membrane marker in some mammalian platelets. From our survey of platelet ultrastructure in the mammalian species it would appear that the sheep's platelets offer the best opportunity for a study of ultrastructural alteration or transformation in the α-granule during viscous metamorphosis. In this animal, the α-granule is 1μ in diameter, is larger than those of other species, and has a matrix with striking periodicity (75).

Relatively little attention has been paid to the thrombocytes in the submammalian classes. These cells, at least in many instances, share the mammalian platelet function in hemostasis from the viewpoint of aggregation and coagulation. The term "thrombocyte" is usually reserved for these nucleated cells although plastid forms are occasionally seen in submammalian classes, but their functional significance is unknown. Ultrastructural studies have been undertaken on thrombocytes obtained from elasmobranchs, amphibia, and aves (47, 75, 79, 80). In the elasmobranch, *Heterodontus portusjacksoni*, two types of thrombocytes are seen; one is spherical in shape and the other spindle to oval, and these correspond to Jordan's description of certain amphibian thrombocytes (81). He attributed the property of aggregation to the rounded cells and that of coagulation to the spindle cells. In the shark, the spherical cells are also the most frequently observed in aggre-

gates. The spindle cell has an inclusion myelin-like figure and it could be speculated that this organelle may be of importance in contributing platelet lipid to coagulation (82). Similarly in the duck and the chicken, single or occasionally double inclusion granules are present, and frequently one or both of these resemble a myelin figure (79). Attempts to detect glass contact or Hageman's activity in these animals has been unsuccessful (82–84), and intrinsic generation of thromboplastin appears to be grossly defective in comparison with mammals (82, 83). This could imply that if intrinsic thromboplastin generation occurs in these classes it may involve a completely different mechanism to that of mammalian blood (82). The extrinsic clotting system, however, is well developed and in elasmobranchs and aves is very specific for tissue thromboplastin obtained from the particular species being examined (82, 83). Clot retraction does not occur, or is extremely poor, but coagulation is grossly prolonged in blood of ducks or chickens from which the thrombocytes have been separated (82, 83). Obviously, therefore, the thrombocytes contribute to coagulation in these animals and possibly the inclusion bodies play some role in this. Thrombocytes from elasmobranchs, amphibia, and aves exhibit phagocytic properties similar to those of the mammalian platelet (75, 79, 80). Thrombocyte aggregation, however, is not induced by the addition of ADP (80, 85). In one species studied (*Heterodontus portusjacksoni*) adenosine does not affect aggregation of the shark's thrombocytes but aggregation may be inhibited by sulphydryl-containing compounds in the same concentrations which inhibit aggregation of human platelets. Thrombocytes do not accumulate ^{14}C-serotonin, nor have 5-HT storage granules been seen in elasmobranch, chicken, or duck thrombocytes (79, 80). Microtubules, however, are most prominent and, indeed, Fawcett suggested this structure's role in the maintenance of cellular shape following observations on amphibian thrombocytes (44).

In summary, thrombocytes share a number of properties with mammalian platelets but are strikingly different in others. Whether the mammalian platelet evolved from the thrombocyte is questionable, but it is interesting to note that cells which do not have the property of clot retraction and active accumulation of 5-HT, lack the characteristic mammalian acid-phosphatase-containing granules and 5-HT storage organelles. They do contain a nucleus, a well-developed microtubular system and, in some instances, a myelin figure inclusion body. This would be consistent with the speculation that α-granules may be important for participation of platelet factor 3 in intrinsic thromboplastin formation and also in clot retraction. Microtubules which are present in both thrombocytes and platelets may be of major importance in the phenomenon of cell aggregation (86).

Platelet Pharmacology

The most outstanding effects of drugs on the platelet's normal physiological function are: (*a*) the promotion or antagonism of the cell's tendency to

adhere to surfaces (adhesion) or to other platelets (aggregation); (*b*) enhancement or inhibition of the platelet's ability to take up, transport and release substances—particularly the biogenic amines—by active or passive processes; and (*c*) to cause or resist certain morphological changes which allow the cell to perform its function. The limited space allows only a cursory glance at the cell's most interesting responses to drugs and restricts discussion to only some of the major platelet-drug interactions.

Platelet aggregation.—Platelet aggregation is defined as the ability of blood platelets to cohere in the absence of an immunological mechanism. Hellem's observation in 1960 that platelet adhesiveness *in vitro* could be enhanced by a dialysable factor derived from red cells (87), and later identification of this factor as ADP by Gaarder et al. (88) resulted in a great interest being directed to the study of ADP in relation to platelet function, hemostasis, and thrombosis. A number of chemical agents have been shown in recent years to produce platelet aggregation. These include certain adenine nucleotides, 5-HT, the catecholamines, various lipids, thrombin, collagen, and a number of miscellaneous substances.

Adenosine diphosphate produces immediate and, in low concentrations, reversible aggregation of platelets in PRP (1, 88–90). The most potent aggregating agent described so far is the recently synthesized 2-chloro substituted analogue 2-Cl-ADP which is nearly ten times more effective than ADP (91, 92). Other nucleotides with significant aggregating ability, but less active than ADP, are adenosine tetraphosphate (93, 94), 3-deoxyadenosine diphosphate, and adenosine-diphosphate-1-N-oxide (95). There is not complete agreement on the effect of ATP on platelet aggregation. Adenosine triphosphate is used by some to aggregate platelets (96) and to show a potentiating effect of catecholamines and some fatty acids on platelet clumping (97, 98). The aggregating effect of ATP is believed by others to be the result of contamination by ADP (88, 99) and is found together with AMP to be inhibitory (2, 89). Non-adenine nucleotides such as uridine triphosphate (UTP), uridine diphosphate (UDP), and uridine monophosphate were reported inactive (88) but recently UDP was found to aggregate, and UTP and AMP to inhibit this aggregation (100). Epinephrine and norepinephrine (96, 99) also produce aggregation of most mammalian platelets, as does 5-HT when added to PRP (96, 99, 101, 102). Among other substances causing aggregation of platelets are enzymes such as thrombin and trypsin (42, 103, 104), certain fatty acids (104, 105), collagen (36, 106), triethyl tin (99), oxime esters (107), nicotine (108), high molecular weight dextrans (109), latex and polystyrene particles (26), ristocetin (7), bacterial endotoxins (110), staphylococcal toxins (111), antigen-antibody complexes (112), snake venoms (113), kaolin (114), sodium fluoride (115), and neuraminidase (116). Divalent cations, calcium or less effectively magnesium, are necessary for platelet aggregation and can themselves induce clumping (90, 117–120) possibly by releasing nucleotides and 5-HT from platelets (121).

Measurement of platelet aggregation.—Platelet aggregation is possibly the only platelet function which can be measured with a degree of reliability and validity *in vitro*. The nephelometric technique described by Born in 1962 (1, 89), soon after used by O'Brien (2), and further modified by Mustard and co-workers (122), has proven to be one of the most valuable tools in platelet research. The method takes advantage of the reduction in optical density of PRP, or platelets suspended in an appropriate "plasma-free" medium, when a substance causing them to clump is added. Changes in optical density, which on the whole are directly proportional to the log concentration of the aggregator, can be recorded by a suitable photometric recording device. When the temperature of the system and the collision forces between platelets (constantly stirred) are accurately controlled, it is possible to measure platelet aggregation and its inhibition in a highly quantitative manner (92, 123–125). The system has a certain appeal to the experimental pharmacologist by virtue of simplifications possible in the analysis of drug-receptor interactions.

It might be assumed that the diffusion barrier through which the agents have to pass before having access to the platelet receptor is negligible and it might further be assumed that the response is proportional to the number of receptors occupied, since the concentrations of agonists or antagonists at the respective receptor sites can be achieved almost instantaneously. The affinity constants can then be obtained by reference to the log dose-response curves when at 50 per cent of the maximal response—with half the receptors presumed to be occupied—the affinity constants would be the reciprocal of the concentration of the agonist (92).

Platelet clumping induced by ADP, 5-HT, and epinephrine is immediate and reversible. Aggregation in response to thrombin occurs after a delay of about 10 sec and triethyl tin after several minutes (99); it has been suggested that aggregation by agents other than ADP is due to ADP released from the platelets (99, 104, 126). After an initial decrease in optical density in response to ADP and epinephrine, the rate of aggregation often increases again (127, 128) in a second wave of clumping—the biphasic response—accompanied by the release of platelet contents including nucleotides and 5-HT (50, 129). This release reaction is similar to the liberation of platelet material by thrombin (42, 130) and collagen (36, 131). The platelet concentration in the second-phase aggregates is greater than in the initial stage (132). Furthermore, the second phase of *in vitro* aggregation is similar to the release occurring during viscous metamorphosis *in vivo* in which the liberated material, particularly 5-HT, could play a part in vasoconstriction. At any rate, there appears to be a synergism between different clumping agents (99). Thus, the release could lead to an acceleration of the "chain reaction" which was proposed by Born to account for the rapid accretion of platelets from a few adherent cells to a coherent hemostatic plug (133).

Platelets may be studied in whole blood media in the presence of other

cells, in PRP, or in "plasma-free" systems. The addition of any constituent, such as anticoagulants, stabilizing agents etc., to these systems must be carefully considered. Platelets aggregate in the presence of low concentrations of heparin and trisodium citrate but suspensions of platelets containing EDTA or EGTA are not readily responsive since Ca^{++} ions are needed for aggregation. In "plasma-free" systems, while thrombin aggregation is preserved, ADP frequently fails to clump platelets resuspended in buffered media. Platelet damage accompanied by a rapid release of ADP is the common outcome of platelet centrifugation and washing (3, 117, 130). This may be the reason for failure to observe the second phase of aggregation in washed, resuspended platelets (129). Nevertheless, "plasma-free" systems are often successfully used in the study of plasma co-factors, surface proteins, and platelet ionic requirements (104, 134, 135).

Mechanism of ADP-induced platelet aggregation.—Although platelet aggregation by ADP is probably the most rigorously tested single function of the platelet, there is not general agreement as to the mode by which ADP induces platelets to adhere to one another. The understanding of the mechanism of ADP aggregation is of paramount urgency if effective pharmacological agents are to be "tailor-made" for the antagonism of aggregation *in vitro* and the inhibition of thrombotic states *in vivo*. Following is a list, in more or less chronological order, of some of the more significant hypotheses proposed to explain the way by which ADP brings about platelet aggregation.

It has been suggested that platelets which carry a net negative charge adhere to other negatively charged surfaces (glass or other platelets) by attracting positively charged cations such as Ca^{++} to form cationic bridges between platelets (136). This idea was expanded later to include ADP and calcium in the so-called "calcium bridge" theory (95) which proposes ADP-Ca-ADP links between platelets and could explain why nucleotides lacking free valency for calcium binding (AMP, ATP) do not clump platelets, while ADP and adenosine tetraphosphate (93, 94) are effective aggregators. The above hypothesis also suggests the involvement of a protein co-factor thought to be the von Willebrand factor. The attachment of ADP molecules to the platelet membrane was seen to be the binding of the adenine moiety of the nucleotide by hydrogen bonds between the -CO and -NH groups in membrane protein with calcium then bridging the negatively charged terminal phosphates of adjacent platelet-ADP combinations (95). Another suggestion that the reduction in net surface charge or charge density by the presence of ADP molecules on or near the platelet membrane is instrumental in reduced platelet repulsion (96, 137), had further support in the observation that ADP reduced the mobility of platelets in an electric field (138).

Since washed platelets require the addition of fibrinogen for ADP aggregation it was suggested that it was fibrinogen which was the co-factor required for aggregation (139). Furthermore, a hypothesis proposed by

Born suggests that fibrinogen plays a vital part in aggregation; ADP molecules are said to "bombard" a specific receptor on the platelet membrane, causing changes in platelet membrane which enable the exposure of sites for links with fibrinogen (123, 140). The source of fibrinogen is not identified and one might presume that plasma fibrinogen is implicated; on the other hand, carefully washed platelets still aggregate (134) and therefore platelet fibrinogen cannot be excluded in the ADP-platelet reaction (141).

While the last hypothesis does not stress the involvement of calcium, the possibility remains that the polar ends of the phospholipid micelles uncovered in platelets by some triggering mechanism are bridged by calcium (142). Following the observation that ADP is hydrolysed in plasma prior to aggregation, it has been suggested that the process of aggregation requires energy and that this energy is supplied by the conversion of ADP to AMP; the binding reaction was not clearly specified (143). Salzman and associates postulated that platelets require a constant source of energy to remain in a contracted nonadhesive state analogous to the contracted state of skeletal muscle and that this energy is supplied by the activity of an ATPase situated on the platelet membrane. Platelet inhibition of the Ca^{++}-Mg^{++} dependent "ecto-ATPase" by ADP may arrest the regulatory process and expose a greater number of receptor sites for secondary fibrinogen and Ca^{++} links (144). Further support for equating the contracted with the nonadhesive state of the platelet has been proposed by the suggestion that the membrane actomyosin-like protein, thrombosthenin, contains both negatively charged carboxyl groups and positively charged side chains. In the contracted state, the positive charges are apparently neutralized by adjacent negative charges but, due to protein folding, there is an increase in the negative surface charge density. Relaxation then leads to a decrease in charge density and results in a decrease in repulsive force (145). The effect of ADP on platelets could then be the product inhibition of the energy-supplying system suggested earlier.

An interesting variation of the Pauling-Miller theory of anaesthesia has also been called upon to explain the stimulus leading to aggregation, due to the increase in the "ice-likeness" of water around platelets by the hydrophobic properties of some aggregating agents (146). However, it remains to be demonstrated that the local concentrations of long chain alcohols used in the study were sufficient to induce clumping by altering the ordered structure of water and aggregation was not a secondary effect. The effect of ADP on the solubility of plasma protein was examined by yet another hypothesis, claiming that the change caused by specific adsorption of an acidic ADP-Ca complex caused fibrinogen precipitation leading to interplatelet bridging (147). More recently, a suggestion has been put forward that platelets in the normal discoid form are in a "relaxed" state and that ADP changes the cell wall contour thus triggering a reversible, contractile wave within the platelet hyaloplasm associated with the movement of granules and marginal microtubules towards the centre of the cell—a process which is reversible when the ADP influence is removed and the platelet returns to its relaxed state (86).

From the outline above, it can be seen that the emphasis in explaining platelet aggregation has slowly shifted from a simple surface charge or cationic bridge effect, through the involvement of protein co-factors and specific ADP receptors, to the modification by ADP of the metabolic processes involving membrane biochemistry and perhaps also the maintenance of the basic nonadhesive morphological state. No single hypothesis so far completely explains all the phenomena observed during the aggregation of platelets and much work still remains to be done to elucidate the exact mode of action of ADP. It is not unlikely that the final picture will come close to a reconciliation and partial integration of the existing and perhaps as yet unspoken views.

Mechanism of aggregation by substances other than ADP.—It has been repeatedly proposed that the final common pathway in all platelet clumping is the interaction of ADP with platelet surface, with perhaps fibrinogen involvement, irrespective of the aggregator used. This view rests largely on the indirect evidence that the removal of ADP from the platelet system results in no aggregation taking place. In the presence of pyruvate kinase and phosphoenolpyruvate (PEP) ADP is removed, probably by a direct transfer of the phosphoryl group from the donor to the acceptor substrate in the formation of pyruvate and ATP. Under these conditions substances such as thrombin and sodium salts of fatty acids do not aggregate platelets (104). Similarly, in the presence of leucocytes which cause a rapid hydrolysis of ADP (148), aggregation does not occur (for norepinephrine *vide infra*). Furthermore, it has been shown that competitive inhibitors of ADP such as adenosine and 2-chloroadenosine (92, 123) inhibit aggregation by such agents as 5-HT, epinephrine, collagen, and thrombin (99, 149). The release of ADP from platelets by collagen (36, 76, 131) and a similar liberation of nucleotides and fibrinogen by thrombin (42, 103, 131, 150) implicates the ADP-fibrinogen pathway. However, some recent evidence suggests that aggregation by thrombin may come about by a different mechanism than ADP since there is at least one report of thrombin clumping being unaffected by the ADP antagonist, adenosine (151), and nonmammalian thrombocytes are aggregated by thrombin but not by ADP (85).

Some kind of a specific 5-HT receptor site has been forecast for a number of years since antiserotonin substances (96, 99) inhibit 5-HT clumping, but only recently a detailed analysis of 5-HT-induced aggregation has been undertaken. This study suggests that 5-HT aggregates platelets by its effect at receptors identical to those involved with the active uptake of the amine by the cells and that the number of these receptors, at least on human platelets, is 10^4 per platelet (101, 152). Furthermore, the receptors appear to resemble the classical D-type receptors on the smooth muscle of the intestine (153, 154). Since adenosine, a competitive inhibitor of ADP, also inhibits 5-HT clumping in a concentration-dependent manner, the possibility is not excluded that ADP might be released during the active uptake of 5-HT through specific receptors and that the nucleotide might contribute to the clumping (99, 102). Unlike ADP, the 5-HT effect reaches an optimum at a

certain concentration after which there is a reversal and platelets become refractory not only to further additions of 5-HT but also to ADP and epinephrine. It has been suggested that saturation of the 5-HT receptor may lead to self-regulatory inhibition (101, 155).

Catecholamines, particularly epinephrine and norepinephrine (isoproterenol is inactive), cause platelet aggregation (96, 99) and also potentiate ADP clumping (97, 156). The aggregation may be mediated by the ADP-fibrinogen pathway or, by stimulating the production of cyclic AMP from ATP (activation of adenylic cyclase), thus depleting the platelet's energy store (97). On the other hand, epinephrine-induced aggregation is antagonized by sympatholytics such as phentolamine and dihydroergotamine and may be thus mediated via a specific α-receptor on the platelet membrane (128). Furthermore, the presence of leucocytes which inhibit ADP, 5-HT, and thrombin-induced clumping (*vide supra*), does not interfere with norepinephrine-induced clumping (148). In addition, platelets exposed to epinephrine do not change shape, in contrast to the rounding and swelling produced by ADP or 5-HT (50, 157).

Consequently, it seems that at least in the case of ADP, 5-HT, and epinephrine, specific receptors are present on the platelet membrane, and the possibility exists that each of these substances could produce aggregation independently.

Inhibitors of ADP aggregation in vitro.—Platelet cohesion is inhibited by a number of substances but the potential use of these as antithrombotic agents has been restricted by either toxicity *in vivo* or the wide spectrum of their biological activities. Inhibitors include chelating agents, blockers of specific receptor sites, and compounds causing membrane changes. Specific inhibitors of ADP are substances closely related to ADP, such as ATP, AMP (2, 89), adenosine (3, 93), and a series of adenosine analogues (93, 149, 158–160). Nucleotides substituted in the 2-position, 2-chloro-AMP and 2-methylthio-AMP, were recently found to be potent inhibitors of aggregation and potentially useful antithrombotic agents; 2-methylthio-AMP in particular, had little effect on the cardiovascular system and respiration (F. Michal & Fylia Penglis, results to be published). It has been proposed that adenosine inhibition is competitive and that its effect is mediated at the specific ADP receptor (3, 92, 123, 125); the affinity constants of ADP, of 2-Cl-ADP, and of the antagonists adenosine and 2-chloroadenosine for this receptor have been calculated. Chlorine substitution in the 2-position increases the affinity of these compounds for the postulated receptor site (92). According to another suggestion, adenosine is not a competitive antagonist of ADP but has to be taken up by the platelet; similarly, hydrolysis of AMP to adenosine may be required before inhibition by AMP (144). The inhibition could be caused by a competition for the energy required for platelet aggregation, adenosine transport, or the nucleoside phosphorylation process (161, 162). Other workers have failed to detect any incorporation of ^{14}C-labelled nucleoside into the platelet or conversion of AMP to adeno-

sine during the inhibitory period (135, 163). These observations, together with the apparent structural specificity of adenosine analogues, would favour the ADP receptor concept.

Salzman & Chambers have shown that clumping induced by ADP and thrombin can be inhibited by esters of certain amino acids (arginine methyl ester, benzoyl arginine methyl ester, and tosyl arginine methyl ester) and proposed that an enzymatic reaction is responsible for the aggregation of platelets by ADP (164). However, it has been shown that platelet esterase activity does not directly correlate with the property of platelet aggregation (165). The sulphydryl inhibitors (*N*-ethyl maleimide, methyl mercuric nitrate, *p*-hydroxymercuribenzoate) but not the glycolytic enzyme inhibitors (potassium cyanide, sodium fluoride, and monofluoroacetate) were reported to inhibit aggregation, suggesting the involvement of platelet thiol groups (166, 167). Inhibition of ADP can also be achieved by compounds which break disulphide bonds (e.g. dimercaptol, dithiothreitol) (140). Compounds which bind divalent cations (citrate, EDTA, and EGTA) prevent clumping by chelating calcium and perhaps magnesium (3) which is then not available for the active complex with ADP whereas, arcaine and 1,4-diguanidino diphenyl sulfone compete with calcium for ADP forming a complex which does not aggregate (125). Among inhibitors are certain antihistaminics (2, 160), antimalarials and local anaesthetics (2), methyl xanthines (168), histamine (169), toluidine blue and promethazine (96), lecithin and linolenate (105), fibrinogen degradation products (170), prostaglandin E_1 (171), and papain and trypsin (116). Dipyridamole may inhibit aggregation by a direct effect on the platelet or perhaps by preventing deamination of adenosine (172), as adenosine deaminase antagonists such as ouabain can enhance the inhibitory effect of adenosine (173). Finally, although experiments using different animals are not in complete agreement on the effect of aggregators and inhibitors, variations—at least in mammals—appear to be more a function of technique rather than being attributable to actual differences existing between the species.

Platelet adhesion.—Platelets adhere to the damaged vessel wall, collagen, and *in vitro* to glass and other surfaces. In the laboratory, platelet adhesion is usually assessed by a method utilizing the tendency to adhere to glass. Three techniques are presently in general use: (*a*) the rotating glass flask method (174); (*b*) columns of glass wool (175); or (*c*) glass beads (87, 136). The platelet count in native, anticoagulated blood or PRP before processing with the glass surface is compared with the platelet count after contact with glass. An adhesive index obtained indicates the ratio of adhesive to nonadhesive platelets (for an excellent review of techniques in the study of platelet functions including adhesion see ref. 176). Adhesion is promoted by ADP, 2-chloro-ADP, and 5-HT and is inhibited by 2-iodoadenosine, 2-methylthio-AMP, 2-Cl-AMP, and some antiserotonin substances (177). Inhibition is also achieved by adenosine, AMP, ATP (117), cocaine (2), monoamine oxidase inhibitors (178), monoiodoacetate,

iodine, parachloromercuribenzoate, and cysteine (179). Although adhesion usually precedes aggregation *in vivo*, its mechanism is not clearly understood. There appear to be many similarities between adhesion and aggregation; both phenomena require calcium, ADP, and sulphydryl groups (179) but in contrast to aggregation, adhesion is relatively unaffected by temperature reduction below that of the body (2, 117).

Electrophoretic mobility.—It has been suggested that the net negative charge on platelets keeps them separated and some modification of this charge is necessary for the cells to adhere to each other. A technique based on the horizontal capillary electrophoresis apparatus has been developed for the measurement of the platelet movement in an electric field (138, 180, 181). Norepinephrine, ADP, ATP, thrombin, and collagen cause a dual change in the electrophoretic mobility when added to PRP; in subthreshold concentrations they increase and in aggregating concentrations they reduce platelet mobility (180). Changes in platelet electrophoretic mobility most probably reflect alterations in the surface charge. Negatively charged phosphate groups of ADP molecules contribute to the net negative charge of the platelet; hence the initial increase in electrophoretic mobility. Calcium binding to the phosphate reduces the charge and produces the subsequent decrease in the electrophoretic mobility (180). In addition to calcium these changes, like aggregation, require fibrinogen. Norepinephrine, thrombin, and collagen depend on some additional co-factor (138). The positive charge borne by norepinephrine, thrombin, and collagen suggests that a release of ADP is the most likely cause of the reduction in electrophoretic mobility produced by these substances. Inhibitors of aggregation such as adenosine and sulphydryl group inhibitors also abolish the electrophoretic mobility response (181, 182). On the basis of measurement of the surface charge, Hampton & Mitchell estimated the number of ADP-binding sites on human platelets as 0.85×10^5 per platelet (183). This value is in close agreement with that obtained by Born who estimated the number of these sites by using ^{14}C-labelled ADP and adenosine (123).

Drugs and platelet shape.—It is believed that the normal discoid shape of platelets is maintained by the activity of the contractile protein thrombosthenin and can be modified by a number of stimuli. Contact with surfaces such as collagen, damaged vessel wall, or glass, and exposure to cold, chelating agents, and aggregators, produce alterations in platelet morphology leading to sphering and volume changes. Although some limited information may be obtained by light microscopy and photometric methods (157), the measurement of shape changes depends largely on electron microscope techniques (86, 184) and electronic particle-counting procedures (50). Platelets sphere and increase in volume on cooling (50–52, 184, 185) and in the presence of ADP (50, 157, 186, 187), 5-HT, triethyl tin, collagen (157), and thrombin (50, 157). In contrast, catecholamine concentrations sufficient to cause aggregation have no effect on platelet shape (157, 186). The most dramatic morphological alterations occur as platelets change from

discs to "spiny spheres" (96) and the marginal microtubules disappear. On rewarming or disaggregation, the microtubules return to their peripheral location (50, 51, 86, 184). The factors which induce platelet shape changes are believed to act on the microtubules (51) and the disappearance of this reinforcing structure from the periphery causes the cells to sphere and become more sticky. It is perhaps significant that specific inhibitors of ADP-induced platelet aggregation—adenosine and AMP—prevent sphering and volume changes by the diphosphate (50, 157, 187), whereas EDTA, cocaine, and sulphydryl inhibitors block aggregation without preventing the morphological changes (50, 157, 186, 187). At least some of the alteration in platelet morphology could be attributed to a gain in water and sodium and to a loss of potassium as volume changes were found to be independent of Na-K ATPase activity (not blocked by ouabain) (188). It has been proposed that calcium, which is essential for platelet cohesion, is not required for the morphological alterations preceding aggregation since EDTA does not prevent these changes (157). However, platelets sphere in the presence of EDTA (50, 157, 185) and calcium added to PRP causes an increase in optical density consistent with increased platelet volume (3, 189).

Platelet uptake and release reactions.—Blood platelets transport around the body 5-HT which they take up from plasma against high concentration gradients by an active uptake process (30, 152, 190). The intraplatelet concentration of serotonin is many times the plasma 5-HT level (191) and is usually associated with ATP (62). The amine enters the cell via specific uptake sites (61, 192) and two separate mechanisms have been proposed for its uptake which may involve two different membranes; one at the intracellular storage organelle surrounded by its own membrane and one at the platelet plasma membrane (192). The plasma membrane site is possibly involved in the clumping effect of 5-HT (101). The antagonism of 5-HT uptake and its release may be divided into three groups: (*a*) inhibitors of the plasma membrane transport which is linked to the cell's metabolism include ouabain, KCN, NaF, atropine, imipramine, cinchocaine, and local anaesthetics (29, 61, 101, 192, 193); (*b*) specific antiserotonin substances such as LSD-25, BOL-148, UML-491, and others, occupy the plasma membrane receptor, not necessarily involving the cell's metabolism (29, 96, 99, 102, 153); and (*c*) inhibitors of the intracellular storage mechanism such as reserpine, chlorpromazine, and the amphetamines (61, 62). Inhibitors of serotonin uptake at the plasma membrane are generally also effective in antagonizing 5-HT-induced platelet clumping (96, 102, 153). Among the more potent inhibitors are the thymoleptics; monoamine oxidase inhibitors and the amphetamines are less effective (194, 195). Both uptake and release are inhibited by quinidine and related substances (196).

In most species histamine probably enters the cell by passive diffusion which cannot be inhibited by local anaesthetics. Only in the rabbit is there a high intraplatelet concentration of histamine (197) which is localized in the same or similar organelles as 5-HT (193, 198, 199) and its accumulation

could involve an active transport system. Epinephrine and norepinephrine levels are much higher in human PRP than in platelet-poor plasma, and, in contrast to the epinephrine of erythrocytes, platelet epinephrine is much more susceptible to environmental conditions and to drugs (200). The uptake of epinephrine and norepinephrine is not by the same pathway as 5-HT although an active transport is involved (201) and calcium is required by both processes (202). The uptake is blocked by phentolamine, dihydroergotamine, reserpine (201), and antidepressants of the imipramine type (126).

The liberation of amines is a process dependent on temperature and the presence of calcium. Release may be caused by mechanical trauma, factors normally present in blood and factors in pathological conditions, and drugs (203). The washing of platelets, contact with unsiliconized glass, sonication, extravasation of blood in extracorporeal circulation, and clotting, all cause a liberation of 5-HT and other platelet constituents (130). Thrombin (42, 103, 204), trypsin (193, 204, 205), and antigen-antibody (206) release 5-HT and histamine. In addition, it has been shown that the collagen release reaction includes a tissue permeability factor (207, 208). Papain, pronase (193, 204), reserpine (201, 209) and fatty acids in the presence of calcium (210), papaverine (198), tetrabenazine, chlorpromazine (which causes increased permeability of the membrane), and other phenothiazines (194, 211, 212) liberate amines. Guancthidine lowers 5-HT and epinephrine content (203). Nicotine releases 5-HT, catecholamines, and histamine (213).

Platelets also contain high concentrations of potassium, which is mainly in the free ionized state, and amino acids (205). The amino acids are accumulated against a concentration gradient by an energy-dependent mechanism (214) and may be incorporated into the platelet contractile protein, thrombosthenin (72). The uptake of amino acids is inhibited by cyanide, 2,4-dinitrophenol, and by thrombin which probably alters the character of the cell membrane (215). Potassium and 5-HT may be released differentially. Ouabain liberates K without affecting 5-HT release; reserpine, on the other hand, releases the amine without potassium while fluoride and iodoacetate cause a loss of both (205). Potassium is also released by chlorpromazine together with histamine and 5-HT (209, 216).

Taurine is present in platelets in large amounts, probably as a result of the metabolism of -SH containing amino acids or is a cysteine derivative (4, 217). A number of possible biological functions has been suggested for taurine, such as osmoregulation and control of rate of cholesterol elimination (217) but the true significance of its high concentration in platelets is still uncertain.

Effect of drugs in vivo.—While the effects of drugs on *in vitro* systems are a valuable indication of their possible therapeutic usefulness, the final criterion is their action and effectiveness in the body. After the administration of drugs to the experimental animal or man, platelet behaviour may be modified and this in turn may reflect on the measurement of platelet func-

tion *in vitro*. Local applications of active aggregators, 5-HT, ADP, and epinephrine have been found to enhance the formation of white thrombi at the site of injury in the whole animal (218, 219), as well as intravascular aggregation and a reduction in the circulating platelet count (41, 219–221). Experimental thrombosis usually depends on damage to the vessel wall by pinching, cooling, electrical, or chemical stimuli (for extensively documented review see ref. 222). The more potent inhibitors of platelet aggregation, adenosine and 2-chloroadenosine, have been reported to inhibit thrombus formation induced experimentally by pinching cortical blood vessels (219, 223). An effective inhibition by 2-chloroadenosine, 2-chloro-AMP, and 2-methylthio-AMP of the electrically induced thrombosis in the rat cortical arterioles has been observed with doses well tolerated by the animal, while ADP and 2-chloro-ADP promoted thrombus induction (Michal, F. & Wiesner, Diane: results to be published).

Drugs in common therapeutic use can have an effect on platelet function, as recently demonstrated by the observation that after the ingestion of acetylsalicylic acid the secondary aggregation effect of ADP and epinephrine in human PRP was prevented (224, 225). It has been proposed that aspirin may hinder the release of nucleotides and 5-HT from the aggregating platelets (225) possibly by permanent damage to some enzyme pathway in the platelet or the platelet membrane (226). Infusions of sulfinpyrazone and phenylbutazone in rabbits were also found to inhibit the release of nucleotides and 5-HT (227). In addition, sulfinpyrazone inhibited thrombus formation and apparently prolonged platelet survival *in vivo* (228). Infusions of amines, 5-HT, and epinephrine have been reported to increase platelet adhesiveness and to promote thrombus formation (218, 219, 229) but in high doses this effect was abolished. An interesting speculation has been put forward by Baumgartner & Born as to the "biological purpose" of 5-HT in platelets. Depending on the availability and rate of uptake, 5-HT may play a self-regulatory role in hemostasis at first enhancing, and later, by partial saturation of the serotonin receptors, leaving them refractory to ADP and 5-HT, thus limiting the spread of intravascular coagulation (101). A similar self-regulatory mechanism could exist for the catecholamines. The apparent disagreement on the effect of depletion of 5-HT by reserpine and the failure to induce hemostatic disorders (230) could also be partially explained on this basis. These observations may well be a further impetus to the study of the physiological function of 5-HT and catecholamines in hemostasis and in thrombosis.

Within the space limitations of this review we have illustrated how platelets have been employed to investigate particular aspects of ultrastructure, biochemistry, and pharmacology. As well as possessing special properties which may give opportunities to study a variety of receptor sites under controlled conditions, the platelet is readily accessible and can be isolated by simple procedures. Like the red cell it may therefore be usefully employed in investigations of more general biochemical and pharmacological effects.

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