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PROTHROMBIN *and* OTHER VITAMIN K PROTEINS

Volume I

Walter H. Seegers
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Prothrombin and Other Vitamin K Proteins

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THE EDITORS

Walter H. Seegers, Ph.D., is William D. Traitel Professor Emeritus, Wayne State University, and served as Chairperson of the Department of Physiology for 34 years. There he received the Alumni Faculty Service Award, School of Medicine Distinguished Service Award, The Distinguished Graduate Faculty Award, the Medical Alumni L.W. Weiner Award and membership in the Academy of Scholars. He is an honorary member of the Harvey Society of New York, the New York Academy of Sciences, the Chilean, Mexican, and Turkish Hematology Societies, the Turkish Medical Society, Alpha Omega Alpha, Phi Beta Kappa, Pi Beta Pi, and the Japanese Society on Thrombosis and Hemostasis. He is a 50-year member of the American Chemical Society, and a charter member of the International Society of Hematology. He is the recipient of honorary doctorate degrees, and had broadly based experience with the experimental enterprise during the golden age of U.S. biomedical research.

Walter Seegers contributed research on nutrition and reproductive biology, and to the isolation of enzymes from blood. He achieved the industrial production of thrombin as an hemostatic agent, and contributed to understanding biochemical abnormalities in blood coagulation, participated in the discovery of blood coagulation factors, and conducted research on blood platelets. He collaborated with University Professor John M. Dorsey, M.D. in authoring the book *Living Consciously: The Science of Self.*

Daniel A. Walz, Ph.D., is Professor of Physiology at Wayne State University. Dr. Walz received his undergraduate education at St. John Fisher College in Rochester, New York and the Ph.D. degree in Physiology at Wayne State University in Detroit. He is a member of the American Chemical Society, the American Heart Association, and The International Society on Thrombosis and Haemostasis. He has authored and co-authored over 60 articles in the field of thrombosis.

Dr. Walz's research interests include protein structure, particularly of those proteins involved in blood coagulation, platelet secreted proteins, and the chromatographic separation of proteins and peptides.

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Chapter 1

INTRODUCTION

Walter H. Seegers and Daniel A. Walz

The main objective in the production of this book is to achieve a summary of valuable information useful for anyone knowledgeable in the fields of Physiology, Biochemistry, Pharmacology, Molecular Biology, Medicine, or related backgrounds. A tendency to be didactic and brief is preferred to a show of being critical and analytical. Conciseness, with well-selected references, should make it attractive to individuals needing to invest reading time economically. It has been over half a century since vitamin K was discovered and found to be needed for the synthesis of prothrombin by the liver. At midcentury, prothrombin of high potency was isolated from bovine and human plasmas. Its molecular weight was approximately the same as that of serum albumin. This acheivement was in the vanguard of the purification of other coagulation factors done with modern equipment and techniques not available when prothrombin was first isolated.

Using purified prothrombin, at the midcentury period, it was discovered that about half of the molecule was not needed for the structure of thrombin. What was the function of the nonthrombin protein? It participated in the formation of thrombin. Part of this function depended upon the availability of γ-carboxyglutamic acid which was discovered in 1974, and located in the nonthrombin region of prothrombin. Thus, a precise function of vitamin K at the molecular level was found. That occurred 46 years after the first intimations that the vitamin existed. During that interval, inhibitors of the vitamin, and other vitamin K-dependent proteins were discovered, as well as other factors essential for the formation of thrombin. One of the vitamin K-dependent proteins has two functions; namely, the inhibition of thrombin formation and the induction of fibrinolysis. The platelets also have a role in the process. In 1962, the complex consisting of platelet factor 3 as lipoprotein, Factor V, Factor Xa, prothrombin, and calcium ions was discovered as the physiological condition for thrombin formation.

Each chapter is written by outstanding scholars qualifying with achievements in career contributions. Writing of their chapters was completed in July 1984. In the numbering of amino acid sequences, the trend is to use the human structure where completely available. However, in this book the numbers for the bovine structure are sometimes used.

Figure 1 is the product of periodic modifications made during the past four decades. It features three basic reactions of blood coagulation as the formation of fibrin, the formation of thrombin, and the formation of Factor Xa. Platelets are represented as having a major role. The contact activating system, as shown, does not bring attention to the fact that plasma deficiencies in that area are clinically asymptomatic. Participation of the blood vessel endothelium in the procoagulant, as well as anticoagulant function, is indicated. As inhibitor, antithrombin III alone or with heparin is the major substrate for thrombin and Factor Xa, as well as other enzymes. Some of the multiple functions of thrombin are indicated.

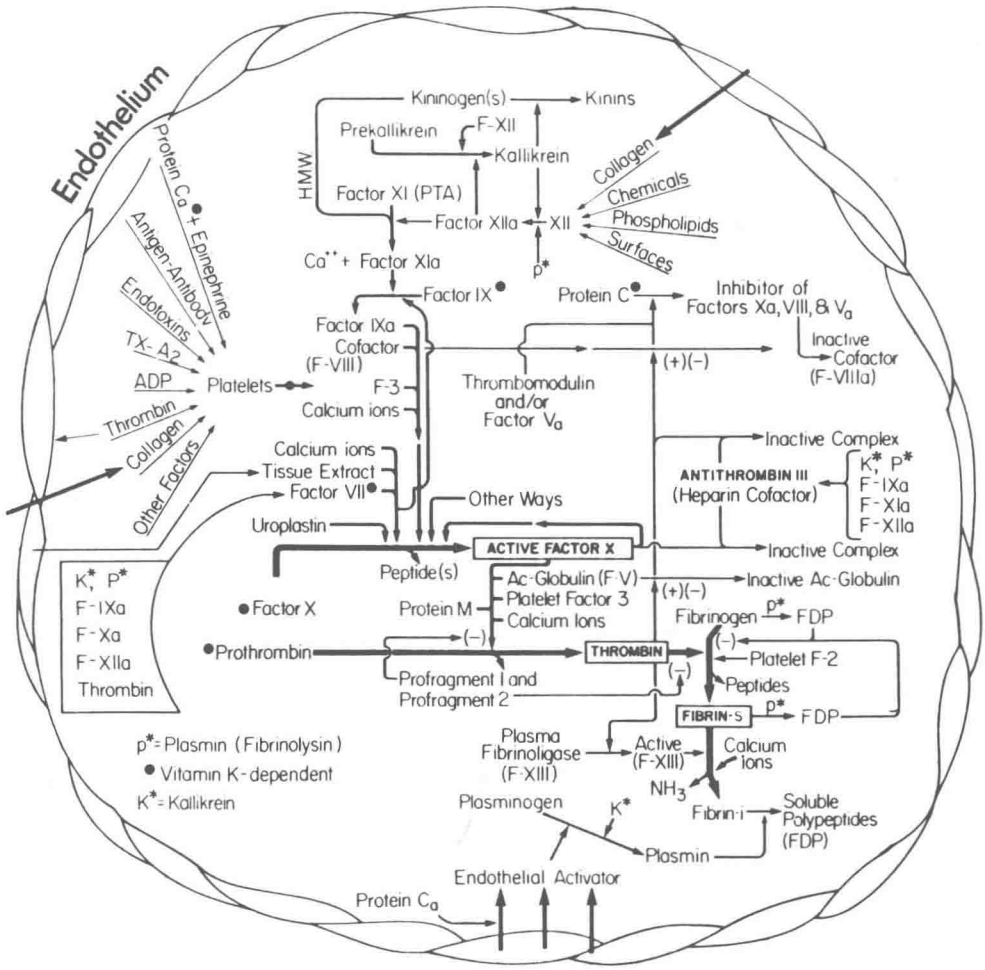

FIGURE 1. The global interrelationships between the endothelium, platelets, coagulation factors, and fibrinolysis are organized in such a manner as to depict the multiple interactions while emphasizing the central role of prothrombin and the other vitamin K-dependent proteins.

Chapter 2

BLOOD CLOTTING MECHANISMS AND FIBRINOLYSIS

Eberhard F. Mammen

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I. INTRODUCTION

Hemostasis is the physiological process by which the organism facilitates arrest of bleeding from an injured blood vessel. It is accomplished by the harmonious interplay between vessel wall, platelets, and the coagulation system. Hemostasis is triggered when the endothelial lining is injured, and when anatomical structures of the vessel wall other than the endothelium are exposed to the blood. Platelets will become attracted and adhere to the site of injury, they will release a number of substances ordinarily only kept within the platelets, they will aggregate, and through aggregation, they will bring about initial arrest of bleeding from the injured site. This process leading to the initial arrest of bleeding is called "primary hemostasis" and the platelet plug formed is referred to as the "first hemostatic plug".

While platelets adhere and aggregate, the coagulation system becomes activated through the absorption of some proteins, collectively called "contact factors", onto the same anatomical vessel wall structures which initiated platelet activation. Enzymes are generated which will activate not only the clotting cascade but also eventually the fibrinolytic system.

The activation of the clotting system will, through a series of complex formations in which proteins associate on surfaces to form active enzymes, result in the formation of fibrin from fibrinogen. Fibrin interweaves with the already existing platelet plug to give it strength and stability. This plug is now called the "second hemostatic plug", and the process leading to it "secondary hemostasis". In preparation for tissue repair, the fibrin slowly will be removed by the activation of the fibrinolytic system.

Each step in this complicated interplay between vessel wall, platelets, and coagulation is balanced by appropriate inhibitor systems, so that hemostasis is kept localized at the site of vessel wall damage.

Two major sets of disease entities are associated with the hemostasis system; bleeding disorders and thromboembolic diseases. Bleeding disorders can result from a defect in any one of the steps leading to the formation of fibrin, or by an excessive activation of the fibrinolytic system. Thromboembolisms arise when the system becomes activated at an inappropriate time and in an inappropriate blood vessel, and many conditions associated with vessel wall damage or blood flow alterations are recognized factors contributing to this form of pathology. Certain congenital or acquired changes in the hemostasis system may further contribute to it.

The following will present a conceptual description of the various events leading to primary and secondary hemostasis. It is not the objective of this article to go into extensive details. More detailed aspects will be discussed in this volume by other contributors. For this reason, the references listed are selective and not comprehensive.

II. VESSEL WALL

Blood circulates freely through the blood vessels as long as the endothelial lining of the vessel walls remains undamaged, and therefore the continuous endothelial lining represents the first line of defense against any loss of blood from the vasculature. Until recently, the vessel wall was generally considered as an inert layer of cells that would separate the blood from the extravascular spaces. It is now appreciated that the endothelium is a highly metabolically active layer of cells with numerous regulatory functions, as reviewed recently in a number of excellent articles.[1-6] Besides being a selective filter for blood constituents, the endothelial lining is now recognized as a site of synthesis of a number of substances which regulate several physiological processes, including hemostasis. A number of prostaglandin derivatives,[7] especially prostacyclin,[8] fibronectins,[9] basement membrane collagen,[10] a part of the Factor VIII macromolecular complex (von Willebrand factor),[11] and an activator of plasminogen[12] are synthesized in the endothelial cells. The endothelial cells bind heparin and heparan sulfate,[13] and were recently found to modify a number of vasoactive sub-

stances.[2,5] One of such modulators is thrombomodulin,[14,15] which was discovered on the endothelial cells. It binds thrombin, whereby thrombin loses its ability to convert fibrinogen to fibrin, activate Factors V and VIII, and activate platelets. Instead, thrombin now converts protein C, a powerful inhibitor of clotting, to its enzymatic form, protein Ca.[14,15] The endothelial cell lining is thus not an inert layer of cells, but rather a nonthrombogenic surface which is actively involved in a number of regulatory functions.

The hemostatic response is initiated when an injury is sustained to the endothelial lining. Upon exposure of subendothelial vessel wall structures, especially collagen fibers, the blood platelets will adhere promptly to this site. Although differences have been noted between certain forms of collagen, and their response to platelets differs,[2] collagen remains one of the primary structures to which platelets will adhere. This adhesion does not require calcium ions. The collagen mediated adhesion will then bring about platelet aggregation, which is dependent on calcium ions. Collagen is also a structure onto which the contact factors of clotting, especially Factor XII and high molecular weight (HMW) kininogen, will adhere, thereby initiating the clotting sequence.

Tissue damage in general will release clot-promoting material, commonly referred to as tissue thromboplastin. This release will activate the clotting system via the extrinsic route.

An additional input of the vessel wall to hemostasis is provided by the contractile properties of some vessels. Vasoconstriction may be mediated through the autonomic nervous system, and hormonally through prostaglandin derivatives, most notably thromboxane A_2, which is derived from platelet membranes.

There seems to exist a close relationship between this platelet-vessel wall interaction, subsequent clotting, and arteriosclerosis, as was recently reviewed in detail.[16]

III. PLATELETS

Platelets are derived from megakaryocytes in the bone marrow by a system of demarcation membranes. Their production and release from the megakaryocytes seem to be controlled by thrombopoeitin, which was recently partially purified from plasma.[17,18] There are between 200,000 and 350,000 platelets found in 1 $\mu\ell$ of human blood. They circulate as round discs with a diameter of about 3 μ and a thickness of about 1 μ. About one third of the entire platelet population is stored in the spleen, from which it can be released.[19] The life-span of platelets is about 7 to 10 days in blood. After that time, they are phagocytized by the RES. Also incorporation into the endothelial cytoplasm has been described.[20] The turnover and distribution of platelets in health and disease, as measured by radioactive tracers, was recently reviewed in detail.[21]

A. Platelet Function-Structure Relationship

Platelets assume several complex functions during hemostasis, and adhesion, aggregation, contraction, and release are just some examples. The relationship between these functions and anatomical structures of a platelet were reviewed in detail.[22,23]

Adhesion and aggregation involve the *peripheral zone* of the platelet, and receptor sites have been identified from which a signal can be transmitted to the interior of the cell. This zone is comprised of the actual cell membranes and the walls of the open canalicular system, as illustrated in Figure 1. This open canalicular system represents a communication device to the interior of the platelet, and serves as a channel system during the release reaction when substances from the platelet interior are expelled to the outside. The platelet unit membrane is like other cell membranes composed of phospholipids and proteins.[23] The phospholipids are arranged in a bilayer form, and serve later on as surfaces on which the clotting enzyme reactions are bound. This property of the cell membrane is encompassed in the term "platelet factor 3".

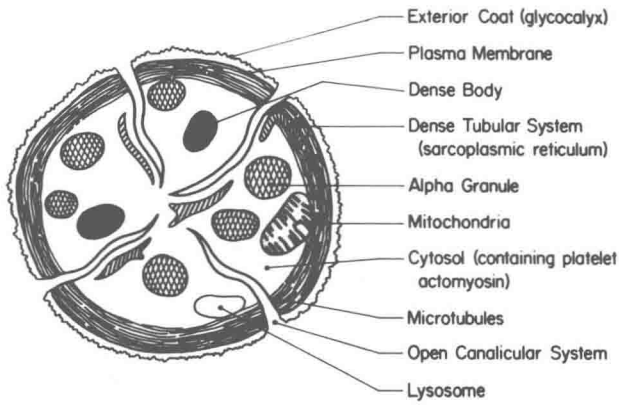

FIGURE 1. Schematic diagram of a platelet and its constituents. (From Henry, R. L., *Semin. Thromb. Hemost.*, 4, 96, 1977. With permission.)

The outer platelet unit membrane surface is covered by a glycocalyx in which mucopolysaccharides and a number of glycoproteins can be identified.[24-26] Some of the glycoproteins are involved with platelet adhesion and aggregation, and their congenital absence is related to certain thrombocytopathies, such as Bernard-Soulier syndrome and thrombasthenia.[27,28] Also, most of the clotting factors can be found in the glycocalyx.[21]

The *submembrane region* is located beneath the cell membrane, and is composed of a regular system of filaments.[23] These seem to maintain the actual cell shape, and are also involved when the platelet, upon stimulation, extrudes its pseudopods.

The *sol-gel zone* is comprised of microfilaments and microtubules, in which the organelles and glycogen are found. The microtubules — which are arranged in a circumferential way, as seen in Figure 1 — are important participants in platelet release and contraction.[26] The contractile protein (thrombosthenin), which facilitates platelet contraction and thereby release, is associated with the microfilaments.[23] The calcium needed for the contraction of the protein seems to derive from the dense tubular system.

The *organelle zone* is composed of dense bodies, mitochondria, different granules, peroxisomes, and lysosomes.[23] The dense bodies contain the nonmetabolic adenine nucleotide pool, serotonin, and also calcium. These are released during the release reaction, and their absence, on a congenital basis, is associated with the so-called storage pool disease and the Hermansky-Pudlak syndrome.[29]

Among the granules, the α-granules contain platelet factor 4, a protein with heparin neutralizing properties, β-thromboglobulin, a protein with yet unknown functions, platelet-derived growth factor, which is involved in endothelial repair, and fibrinogen. The absence of these proteins, due to a congenital absence of the α-granules, characterizes the so-called Gray platelet syndrome.[30]

B. Platelet Adhesion

Upon damage to the endothelial lining of the vessel wall, platelets will adhere to the underlying structures, especially collagen fibers. This process requires several plasma proteins, and fibrinogen, possibly fibronectin, and especially the von Willebrand factor, a part of the Factor VIII macromolecular complex, play a major role. Von Willebrand factor (F.VIII:vWF, F.VIII:RAg, or F.VIII:Rist.Cof.) is produced by the endothelial cells, and apparently binds to the subendothelial structures first. Then platelets will attach to it.[31] This adhesion is calcium-dependent. The actual site on the platelet where the von Willebrand factor attaches has been identified as glycoproteins of the subtype I, located in the glycocalyx,

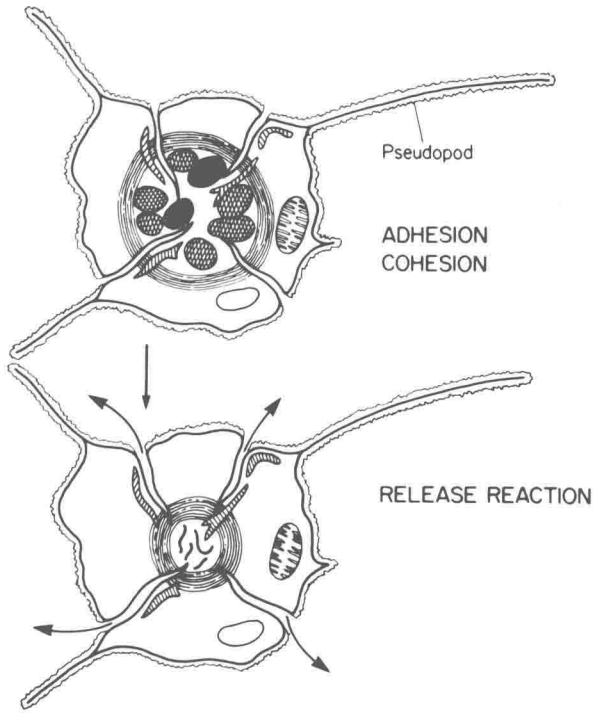

FIGURE 2. Diagrammatic rendering of the platelet release reaction. (From Henry, R. L., *Semin. Thromb. Hemost.*, 4, 99, 1977. With permission.)

as mentioned above. Two diseases characterized by absent platelet adhesion relate to this physiological process, von Willebrand's disease, and Bernard-Soulier syndrome. Patients with von Willebrand's disease lack the part of the Factor VIII macromolecular complex which is needed for platelet adhesion, while patients with Bernard-Soulier syndrome lack glycoprotein Ib, onto which the von Willebrand factor attaches.

As platelets adhere, they undergo a marked shape change in that pseudopods are extruded and platelet spreading is noted. This change in shape is associated with an internal contraction, as a result of which the organelles become centralized. This contraction is facilitated through the contractile protein, as illustrated diagramatically in Figure 2. Further contraction leads to the expulsion of the organelles to the outside of the platelet, either via the open canalicular system, or through a rupture of the cell membrane. This process is called the *release reaction*.

C. Platelet Aggregation

The release reaction facilitates the release of several substances, as described above. Adenosine diphosphate (ADP) is one of the prime release products which mediates platelet aggregation, which, by definition, is a process where platelets adhere to each other. However, serotonin, catecholamines, and certain prostaglandin derivatives can also aid in this process. Even substances carried to the site of injury, such as thrombin, vasopressin, and others, can facilitate platelet aggregation.[32] Many of these agents interact with specific receptor sites on the platelet surface, possibly glycoproteins, and thereby transmit the signal for further release to the platelet interior. Calcium ions may serve as a transmitter,[33] since calcium has to be available for the release reaction, and therefore for platelet aggregation.

Under pathological conditions, platelets can become activated by a number of alternate mechanisms related to the immune system. Antibody-antigen complexes, together with

complement, can activate platelets,[34] and leukocytes together with complement, or immune leukocytes in the presence of antigen but absence of complement, can produce a platelet release response.[35]

Platelet aggregation and subsequent release will thus produce more aggregation, so that eventually the injured vessel will be sealed off by a platelet plug, which is referred to as the "first hemostatic plug". This form of primary hemostasis will result in the occlusion of vessels smaller than 50 μ in diameter.

Platelet release, and therefore platelet aggregation, is regulated by the level of unbound calcium in the platelet. This in turn is determined by the level of cyclic adenosine monophosphate (AMP). cAMP can facilitate a complex formation in the platelet between a number of substances, one of which is unbound calcium. Higher cAMP levels thus lead to a greater binding of calcium,[36] and therefore a blockade of the release reaction.

D. Prostaglandins and Platelet Aggregation

The level of cAMP in the platelet can be regulated by components of the prostaglandin pathway.[8,37] The prostaglandin derivatives originate from arachidonic acid, which in turn is formed from membrane-bound phospholipids. The production of arachidonic acid from phospholipids by phospholipases can be fostered by thrombin and collagen.[37] Arachidonic acid is converted to the first prostaglandin derivative, prostaglandin G_2 (PGG_2), through the action of cyclooxygenase. PGG_2 is converted to prostaglandin H_2 (PGH_2). In the platelet membrane, PGH_2 is converted to thromboxane A_2 by a thromboxane synthetase. In the vessel wall, PGH_2 becomes prostaglandin I_2 (PGI_2) or prostacyclin. This is facilitated through a prostacyclin synthetase.[8] Both end products have a very short life-span (seconds)[8] and prostacyclin becomes prostaglandin $F_{1\alpha}$, while thromboxane A_2 becomes thromboxane B_2. These products today can be determined with radioimmunoassays (RIAs).[38]

These two prostaglandin derivatives, thromboxane A_2 and prostacyclin, influence adenylate cyclase, which is responsible for the formation of cAMP from ATP.[37] Prostacyclin stimulates adenylate cyclase, and thus leads to a formation of more cAMP in the cell, while thromboxane A_2 inhibits adenylate cyclase and permits only little cAMP from being formed. Under the influence of prostacyclin, therefore, the cAMP levels will rise, more calcium ions will be bound, and the release reaction is blocked. Indeed, prostacyclin is one of the most powerful anti-aggregating substances known at this time.[8] In contrast then, thromboxane A_2 will mediate only little cAMP formation, and thus little calcium binding; therefore, it will foster the release reaction, and thereby platelet aggregation.

This entire complex interrelationship between prostaglandins and cAMP is summarized in Figure 3.

Platelet aggregation can be therapeutically influenced by acetyl salicylic acid and a variety of other nonsteroidal anti-inflammatory drugs. These drugs, by and large, block the enzyme cyclooxygenase, and therefore the formation of the first prostaglandin derivative from arachidonic acid. Dipyridamole (Persantine) also blocks aggregation, but this compound inhibits phosphodiesterase, the enzyme responsible for the conversion of cAMP to AMP.

IV. BLOOD COAGULATION

The blood coagulation system is also activated as endothelial damage is encountered, in that collagen fibers or other nonendothelial structures of the vessel wall will adsorb certain clotting factors, which leads to an activation of both the clotting and the fibrinolytic systems. A number of authors have reviewed blood coagulation in considerable detail,[39-45] and additional information may be found in these articles.

A. Clotting System

The clotting system is a rather complex process, in which several clotting factors partic-

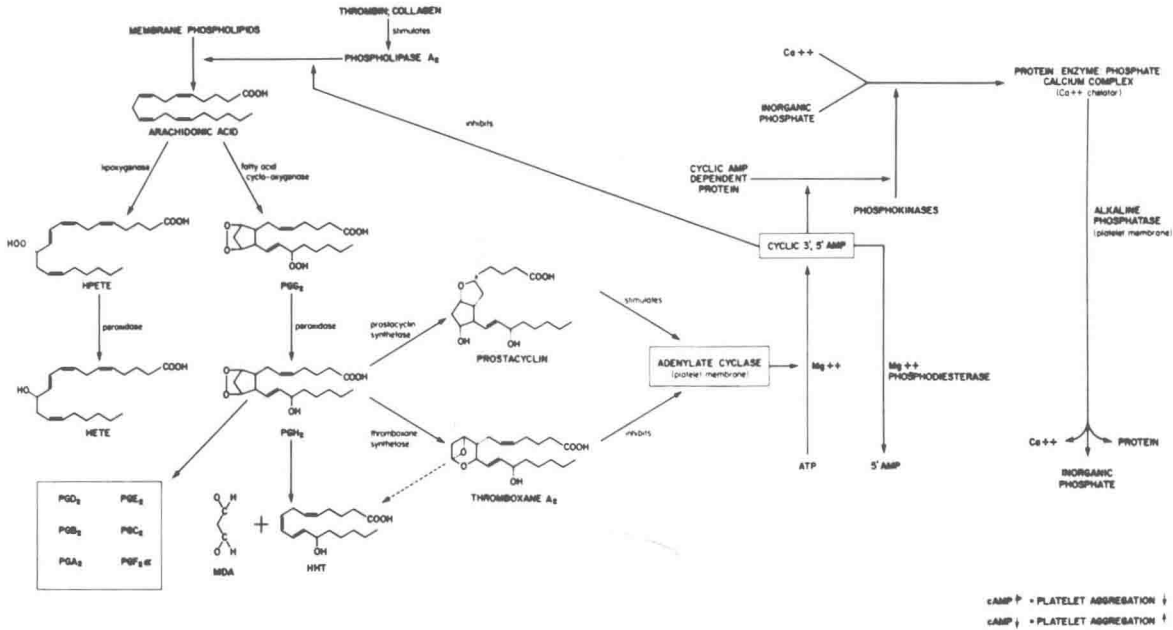

FIGURE 3. The prostaglandin pathways and their effects on cAMP formation in the platelets. Cyclic AMP regulates the amount of ionized calcium which is required for the release reaction. (From Nalbandian, R. M. and Henry, R. L., *Semin. Thromb. Hemost.*, 5, 104, 1978. With permission.)