review

The role of lipids in platelet function : with particular reference to the arachidonic acid pathway

Aaron J. Marcus

Hematology Section, New York Veterans Administration Hospital, New York, NY 10010, and Department of Medicine, Division of Hematology–Oncology, New York Hospital–Cornell Medical Center, New York, NY 10021

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INTRODUCTION

From coagulation to metabolism to prostaglandins

Prior to 1950 it was acknowledged that lipids and lipoproteins were involved in the blood coagulation process (1). Early studies of McLean (2) and Chargaff (3) indicated that lipids, and in particular phospholipids, were capable of accelerating the conversion of prothrombin to thrombin. These and other investigators were exploring what is now recognized as the extrinsic or tissue factor system. The contribution of individual phospholipids to this system was later defined by Nemerson (4). In 1953 Biggs, Douglas, and Macfarlane (5) discovered that platelets and certain plasma protein components were capable of generating their own procoagulant activity in the absence of tissue factor. This was designated the intrinsic system of coagulation. These classical studies focused attention on the contribution of platelets to the coagulation process. During the next two decades a great deal of research was carried out in an effort to establish a direct or indirect relationship between platelet (6), dietary (7–9) and plasma lipids and coagulation, thrombosis, and atherosclerosis. Although the relationships of these studies are extremely interesting (10, 11), they remain inconclusive.

The aforementioned observations came at a time when lipid chemistry was in a state of rapid transition. For example, individual lipid classes were separable by column chromatography on silicic acid (1). Gas–liquid chromatography (GLC) was developed, making it possible to study fatty acids and aldehydes in single species of lipids (12, 13). Thin-layer chromatography (TLC) permitted accurate qualitative and quantitative analysis of complex lipid mixtures (14). Thus, in the mid-1960's, studies of platelet lipid metabolism were initiated in several laboratories. Since platelets could incorporate radioactive isotopic precursors, such as phosphate, acetate, glycerol, and fatty acids, changes in platelet function could be correlated with alterations in platelet biochemistry. Platelets could be prelabeled with a compound of interest and its metabolic fate, after stimulation with agents such as thrombin and epinephrine, could be followed by radiochromatographic techniques (15).

Silicic acid column chromatography, TLC, and GLC, combined with mass spectrometry, were in large part responsible for progress in prostaglandin research—currently one of the most rapidly advancing fields of biochemical investigation. In the early 1970's such groups as the one at the Karolinska Institute in Sweden and that at Unilever Laboratories in Holland began to characterize the oxygenation and transformation of platelet arachidonic acid into biologically active intermediates which have important

implications for platelet function, and which provide new information on the role of essential fatty acids in platelet structure and biochemistry. In 1976 the same basic approach was used to characterize a new prostaglandin, prostacyclin (16, 17), which only a few months before had been recognized because of its potent biological properties as a vasodilator and inhibitor of platelet aggregation (18–21). Recently endothelial cells have been shown to be a source of PGI_2 (22), although its presence in other cell types has been reported.[1]

In this review an attempt will be made to summarize recent developments in our knowledge of platelet lipid metabolism and to relate them to a possible role for platelets in hemostasis, coagulation, thrombosis, and atherosclerosis. It is not intended to be encyclopedic, and in some instances the author's comments will be admittedly speculative.

[1] See Sun, Chapman, and McGuire. 1977. *Prostaglandins* **14:** 1055–1074.

GLOSSARY OF ABBREVIATIONS

ADP	adenosine diphosphate
ASA	acetylsalicylic acid
ATP	adenosine triphosphate
cAMP	cyclic adenosine 3′,5′-monophosphate
cGMP	cyclic guanosine monophosphate
dBcAMP	dibutyryl cyclic AMP
EFA	essential fatty acids
ETYA	5,8,11,14-eicosatetraynoic acid
GLC	gas–liquid chromatography
HETE	12L-hydroxy-5,8,10,14-eicosatetraenoic acid
HHT	12L-5,8,10-heptadecatrienoic acid
15-HPAA	15-hydroperoxy arachidonic acid
HPETE	12L-hydroperoxy-5,8,10,14-eicosatetraenoic acid
5-HT	serotonin
MDA	malonaldehyde
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	prostaglandin, e.g., PGE_1
PGG_2	prostaglandin endoperoxide G_2
PGH_2	prostaglandin endoperoxide H_2
PGI_2	prostacyclin
PHD	thromboxane B_2 (TXB_2)
PI	phosphatidylinositol
PLA_2	phospholipase A_2
PRP	platelet-rich plasma
PS	phosphatidylserine
RCS	rabbit aorta contracting substance
SRS	slow-reacting substance
TBA	thiobarbituric acid
TLC	thin-layer chromatography
TX	thromboxane, e.g. TXA_2
20:4	arachidonic acid

CURRENT CONCEPTS OF PLATELET FUNCTION

The platelet is the anucleate derivative of the largest cell in the bone marrow, the megakaryocyte. It contains no DNA and, although there are reports of protein synthesis in platelets, we have been unable to detect RNA in large quantities of platelets analyzed for its presence. Furthermore, most electron microscopic studies of platelets have not revealed the presence of rough endoplasmic reticulum or ribosomes, although there are some reports to the contrary (23). Normally about 300,000 platelets/mm^3 remain in the circulation for a life span of 7–10 days (24).

In the course of development from megakaryocytes, the platelet acquires a plasma membrane that has several specific biochemical and physiological properties (25, 26). Many important platelet interactions are surface membrane-associated phenomena, and studies of isolated platelet membranes have increased our understanding of some of these processes (27–31). Unfortunately, many of the significant biochemical properties of whole platelets are lost in the preparation of platelet homogenates or subcellular fractions. Thus, subcellular platelet organelles no longer aggregate, take up and store serotonin (14), or interact with other stimuli in a manner comparable to that of the intact platelet. This phenomenon has been an obstacle to the isolation and characterization of platelet receptors and/or acceptors as carried out with many other cell types.

The first important platelet function is one that will not be covered in this or any other review, since there is little to no information on its mechanisms. This is the ability of platelets to maintain the integrity of the vasculature. In general, blood vessel fragility and permeability can be correlated with the quantity of platelets in the circulation, and only viable platelets can circulate. An abrupt fall in the platelet count can be accompanied by hemorrhage into the skin and mucous membranes. This defect is rapidly corrected by infusion of platelet concentrates.

A second important platelet function, which will be considered separately, is the promotion of coagulation via the intrinsic system. This is a highly efficient mechanism which in fact requires relatively few stimulated platelets. The clot-promoting function is one that does survive fractionation, and this has permitted studies of the role of platelet lipoprotein surfaces in coagulation (27). Platelets also mediate the phenomenon of clot retraction, as is easily observed in vitro; but this will not occur in the setting of thrombocytopenia. The in vivo significance of clot retraction is unknown, although it is a well-defined metabolic process as studied in the test tube (24). Moreover,

patients with the platelet disorder thrombasthenia have absent or defective clot retraction, indicating that it may have an as yet undefined role in hemostasis.

Finally, the ability of platelets to respond to blood vessel damage and to release biologically active substances during the process will be discussed in the ensuing description of primary and secondary hemostasis.

Primary hemostasis

The initial response of platelets to interruption of continuity of vascular surfaces is known as primary hemostasis. The process can be arbitrarily subdivided into phases that represent biochemical and morphological transitions of the platelet response to blood vessel severance or injury (32, 33). The initial phase is that of platelet adhesion to vascular tissues located below the endothelial layer, such as collagen (which is the most important), basement membrane, and microfibrils. Such adhesion is a property unique to platelets and is defective in disorders such as von Willebrand's disease and the Bernard–Soulier syndrome (23, 34, 35). I suggest that a major consequence of the adhesion reaction is a sequence of steps during which the platelet membrane develops new biochemical properties. For example, the stimulated plasma membrane of the platelet develops the capacity to catalyze interactions between proteins of the coagulation system (also known as platelet factor 3). These reactions take place alternately on the lipoprotein surface and in the fluid phase in the immediate environment of the platelet, which thus becomes a locus for thrombin formation (27, 36). In addition, the activated platelet surface develops other interesting properties, such as the capacity to reduce cytochrome *c* and nitroblue tetrazolium in far greater quantities than that reduced by resting platelets (37).

The stimulated platelet membrane then transmits an intracellular signal, resulting in a release reaction (38) characterized by secretion of several components previously stored in at least three types of platelet organelles (39). The release reaction has been likened to secretory processes in other cell types (endocrine, neuronal, leukocytes); but this must be regarded as conjectural because true exocytosis (see, for example, reference 40) has never been conclusively demonstrated in platelets and, after exposure to thrombin, platelet storage organelles disappear en masse, with no evidence of fusion with the plasma membrane. Moreover, the platelet cannot be regarded as a phagocytic cell in the classical sense because it cannot form bona fide phagocytic vacuoles; nor is the platelet oxygen burst associated with the formation of superoxide radicals (37). Furthermore, in many secretory cells, release is associated with increased levels of cyclic AMP, but in platelets aggregation and secretion are frequently accompanied by a fall in cyclic AMP. On the basis of present evidence I propose that the release reaction in platelets bears only a superficial resemblance to secretory processes in other cells.

One of the most important substances released from platelets is adenosine diphosphate (ADP), which aggregates platelets by a direct effect on the plasma membrane. ADP probably also acts in a synergistic manner with most other aggregating agents, including those derived from arachidonic acid oxygenation. In some instances fibrinogen can act synergistically with or in place of ADP.

Other substances released by stimulated platelets are also of great biologic interest, such as serotonin (5-HT), platelet factor 4 (a heparin neutralizing protein), beta-thromboglobulin (a platelet-specific protein of unknown function), certain lysosomal enzymes, and factors involved in the promotion of cell growth, which are currently the subject of intensive research (41, 42). A recent report suggests that experimentally induced smooth muscle proliferation in blood vessels is markedly reduced in thrombocytopenic animals (43). Activated platelets also release material that is chemotactic for leukocytes (44, 45) and that increases vascular permeability (46, 47).

Released ADP aggregates other platelets in the immediate environment, and these in turn undergo their own release reaction. If the release reaction is impaired, as in certain rare platelet disorders (23) or after ingestion of aspirin, the primary wave of aggregation in response to ADP will occur but is reversible: i.e., the second wave of aggregation is absent. In the platelet disease thrombasthenia the platelet does not aggregate in response to ADP, although intracellular granules do indeed release it.

Secondary hemostasis

Primary hemostasis merges into secondary hemostasis when the platelet membrane-catalyzed coagulation sequence culminates in the evolution of the serine protease, thrombin. Thrombin itself is a powerful aggregating agent, operating via three mechanisms: ADP release, arachidonic acid oxygenation, and another mechanism, the nature of which has yet to be determined (48, 49). Thrombin also acts on its natural substrate, fibrinogen, by sequentially splitting off two polypeptides, fibrinopeptides A and B, permitting the remaining fibrin monomer to polymerize into insoluble fibrin strands held together by hydrogen bonds. The fibrin strands reinforce the primary hemostatic platelet plug, rendering it an impermeable, consolidated mass of stimulated platelets. Permanence of

the fibrin strands is further enhanced by concomitant activation of factor XIII by thrombin in the presence of calcium. Activated factor XIII is a transamidase that catalyzes the formation of covalent amide bonds between fibrin polymers which were previously held together by hydrogen bonds.

If we consider that aggregated platelets can obstruct normal blood vessels that have been severed or traumatized, it follows that thrombosis may in fact be an aberrant form of normal hemostasis. Thus atherosclerotic blood vessel surfaces may locally stimulate platelets, which would then develop clot-promoting properties, inflict further vascular injury, release mitogenic materials, and oxygenate arachidonic acid. Pharmacologic approaches that might interfere with platelet stimulation without deleterious effect on hemostasis are currently under study (50).

Fig. 1 is a photomicrograph, kindly provided by Dr. Dorothea Zucker-Franklin, in which the salient features of platelet ultrastructure are highlighted.

TRANSFORMATION OF ARACHIDONIC ACID IN HUMAN PLATELETS

Background

One could have anticipated a role for prostaglandins and intermediates in their synthesis in platelet physiology, since they can induce vasoconstriction, vasodilatation, and smooth muscle contraction (51–55). Vasoconstrictors such as epinephrine, prostaglandin endoperoxides G_2 (PGG_2) and H_2 (PGH_2), and thromboxane A_2 cause platelet aggregation and lower platelet adenosine 3′,5′-cyclic monophosphate (cyclic AMP) content. Vasodilators such as prostaglandin E_1 (PGE_1), prostacyclin (PGI_2), and dipyridamole block platelet aggregation. The latter agents elevate levels of platelet cyclic AMP (56–65). Although prostaglandins E_2 and $F_{2\alpha}$ are formed and released by stimulated platelets (66), it is the unstable intermediates in prostaglandin synthesis—PGG_2, PGH_2, and thromboxane A_2 (TXA_2) (67)—that are the important mediators of biological activity derived from essential fatty acids in platelets.

Essential fatty acids

Three presumably unrelated areas of biological investigation began to merge in the mid-'60's. At the time of this writing it is obvious that they are part of the same discipline. Considered in chronologic order, the first involves the role of fatty acids in nutrition, with particular emphasis on those known as essential fatty acids (EFA) (68). The pioneering studies of Burr and Burr (69, 70), and later of Thomasson (71), indicated that the clinical and biochemical consequences of feeding fat-free diets to rats could be reversed by the introduction of linoleic (18:2) and arachidonic (20:4) acids to the diet. Thomasson (71) suggested that EFA be called vitamin F, with one unit of vitamin F activity representing the equivalent of 10 mg of linoleic acid. It was also proposed, and later borne out, that arachidonic acid could be formed from linoleic acid (72). In the light of present knowledge it is of interest to re-examine the results of these classical investigations. For example, during the period of EFA deficiency, animals were observed to develop increased capillary fragility, decreased capillary resistance, impaired reproduction and lactation, and degeneration of seminal vesicles. Adrenal and thyroid atrophy were also reported, as well as cholesterol deposition in various tissues. Burr and Burr (69) observed gross hematuria as an early symptom of EFA deficiency, and the most common cause of death was hemorrhagic renal necrosis. It is plausible to consider that the gross and microscopic pathology observed in these animals was at least in part attributable to the absence of arachidonic acid in cell membranes of affected tissues, as well as to its unavailability for the synthesis of prostaglandins and their intermediates. A recent monograph on polyunsaturated fatty acids is recommended (73).

Arachidonic acid is one of the most abundant fatty acids in platelet membranes, granules, and soluble fractions (12, 13, 74). The presence of arachidonic and other long-chain unsaturated fatty acids in extra- and intracellular membranes may allow for more efficient interaction between the cell and its external environment (75, 76). The C_{20}-polyenoic fatty acids are thought to be necessary for metabolic activities that depend on respiration and oxidative phosphorylation. It is therefore probable that arachidonic acid is essential for organisms that utilize oxygen (77).

Prostaglandins

The second evolving area of research was that of prostaglandins. The identification and chemical characterization of the material responsible for the biological activity of prostaglandins were initially undertaken by Bergström and associates (78, 79) and by van Dorp and colleagues (80). The link between EFA and prostaglandins was suggested by van Dorp et al. (80) after they incubated tritiated arachidonic acid with a homogenate from sheep seminal vesicular glands. Conversion of the ^3H-labeled 20:4 to PGE_2 was demonstrated by TLC. Furthermore, after elution from the plate, PGE_2 had biologic activity when added to guinea pig ileum. In a companion paper Bergström, Danielsson, and Samuelsson (81), using a

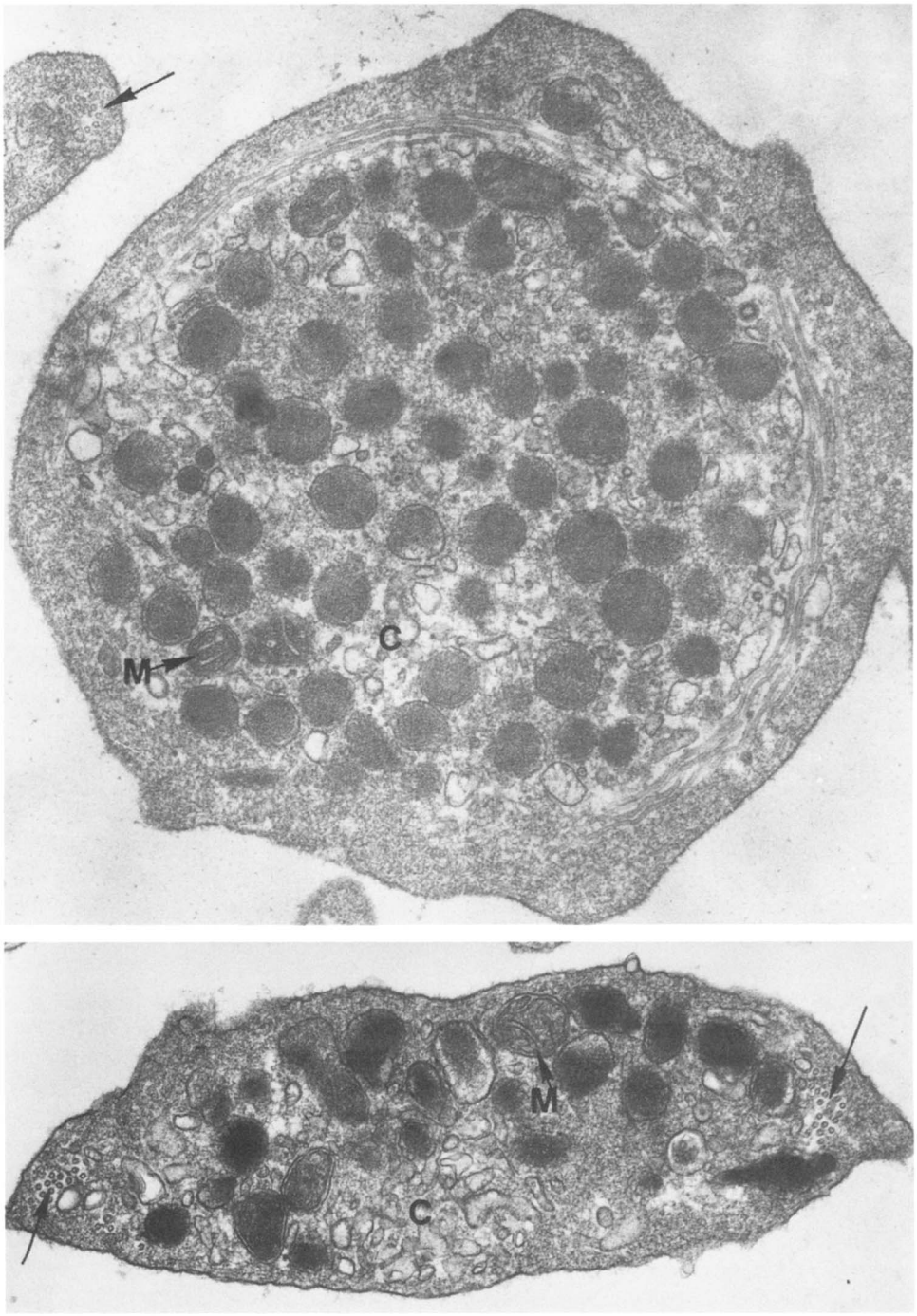


Fig. 1. Electron micrograph of human platelets in cross-section (top) and in longitudinal section (bottom). Platelet cytoplasm consists mainly of intracellular granules and mitochondria (*M*). The open canalicular system (*C*) can be seen in both sections. The peripheral band of microtubules is well depicted. Arrows indicate the tubules in cross section. Magnifications: top × 35,000; bottom × 36,000 (kindly provided by Dr. Dorothea Zucker-Franklin, New York University School of Medicine).

sample of the same tritiated 20:4, showed formation of prostaglandin E_2 from arachidonic acid. Both groups concluded that EFA were obligatory precursors for biosynthesis of prostaglandins. In addition, it was suggested for the first time that some symptoms of EFA deficiency might actually be due to inadequate biosynthesis of prostaglandins. Bergström and colleagues (81) also postulated a route of synthesis involving direct cyclization of dihomo-γ-linolenic acid (8,11,14-eicosatrienoic acid, 20:3), arachidonic acid,

or 5,8,11,14,17-eicosapentaenoic acid (20:5). It is now known that 20:3 is the precursor of prostaglandins E_1 and $F_{1\alpha}$, while 20:5 is the precursor of prostaglandins E_3 and $F_{3\alpha}$. Both 20:3 and 20:5 are absent from platelet phospholipids and neutral lipids (74, 13). These studies indicated that, if platelet prostaglandin synthesis was to be studied later, the most favored starting material would be arachidonic acid which, as already mentioned, is quite prominent in the phospholipids of platelets and subcellular platelet organelles.

Rabbit aorta contracting substance (RCS)

The third line of investigation, which initially did not appear to be related to EFA or prostaglandins, concerned an unidentified, unstable substance released from guinea pig lung during anaphylaxis (82). This transient material caused contraction of rabbit aorta and, most pertinently, its action was blocked by anti-inflammatory agents such as aspirin and indomethacin. The locution 'rabbit aorta contracting substance' was coined by Piper and Vane (82). Evidence was presented that RCS could not be bradykinin, prostaglandin E_2, an adenine nucleotide, lysolecithin, or kallikrein. Vargaftig and Zirinis (83) subsequently showed that when platelets were aggregated with arachidonic acid, RCS and prostaglandins were generated and the effects of both could be blocked by anti-inflammatory drugs. Gryglewski and Vane (84) later suggested that RCS was an endoperoxide intermediate in prostaglandin synthesis, but Hamberg et al. (85) determined that the half-life of prostaglandin endoperoxides was longer than that of RCS. In a subsequent study Svensson, Hamberg, and Samuelsson (86) found that RCS had two components, one with a $t½$ of 5 min and a more potent constituent with a $t½$ of 30 sec. The latter was soon identified as thromboxane A_2, and the more stable but less potent activity was attributed to PGG_2 and PGH_2 (67). Another important consequence of research on RCS was the concept, suggested by Vane (87), that the mechanism of action of aspirin-like drugs was due to their capacity to inhibit prostaglandin synthesis. Smith and Willis (88) demonstrated that aspirin inhibited prostaglandin production in platelets treated with thrombin (5 units). In this instance, release of serotonin was normal, thereby suggesting a dissociation between prostaglandin synthesis and the platelet release reaction. This dissociation has been postulated by others (37, 64, 89, 90) but is currently a subject of controversy. Also, it is now known that thrombin induces aggregation and release by more than one mechanism (48, 49). Indomethacin reduced prostaglandin production in stimulated platelets by 50–83%. When *low concentrations* of collagen or thrombin were added to aspirinized platelets, the release reaction, as well as prostaglandin production, was blocked. These results served to explain previous observations of Zucker and Peterson (91, 92), who showed that high concentrations of collagen could induce aggregation in aspirinized platelets. Inhibition of prostaglandin production in platelets by anti-inflammatory drugs was also shown by Glenn, Wilks, and Bowman (93).

Endoperoxides and thromboxanes produced by human platelets

From 1973 through 1975 a series of papers appeared from the Karolinska Institute and the Unilever Laboratories describing the isolation of intermediates in prostaglandin synthesis. These publications revolutionized our concepts of the role of prostaglandins in biology and medicine. The investigative approach toward elucidation of the pathways of arachidonic acid transformation in platelets can be appreciated by a chronological review of the research.

Isolation of endoperoxide PGH_2

Samuelsson (94–96) and Nugteren, Beerthuis, and van Dorp (97) had previously proposed that an endoperoxide derivative was formed during synthesis of prostaglandin E_1 from 8,11,14-eicosatrienoic acid in sheep vesicular glands.[2] When the biosynthetic reaction was carried out in the presence of $^{16}O_2$/$^{18}O_2$, the two oxygens of the 5-membered ring were found to contain either two atoms of ^{16}O or two atoms of ^{18}O. In no case did the ring contain one atom of ^{16}O or one of ^{18}O. Thus, the oxygen of the keto group at C-9 and the oxygen of the hydroxyl group at C-11 of PGE_1 originated from the same O_2 molecule. Therefore a cyclic peroxide derivative had formed which could isomerize into a 1,3-hydroxy ketone (the PGE group) or which could undergo reduction to a 1,3-diol (the PGF_α group) (**Fig. 2**). Techniques used in these early experiments for isolating prostaglandin intermediates set the general pattern for future work (98, 99). Microsomal preparations from sheep vesicular glands were incubated with radioactive arachidonic acid for short periods of time. In some studies the incubation chamber was fitted with an oxygen electrode. Reactions were stopped by addition of ethanol, followed by dilution with water and acidification. After rapid extraction with diethyl ether, the evaporated residue was esterified with diazomethane and

[2] A theoretical postulation of endoperoxide structure was made by Beal, Fonken and Pike in 1964 (Upjohn Company, Belgium patent 659,884).

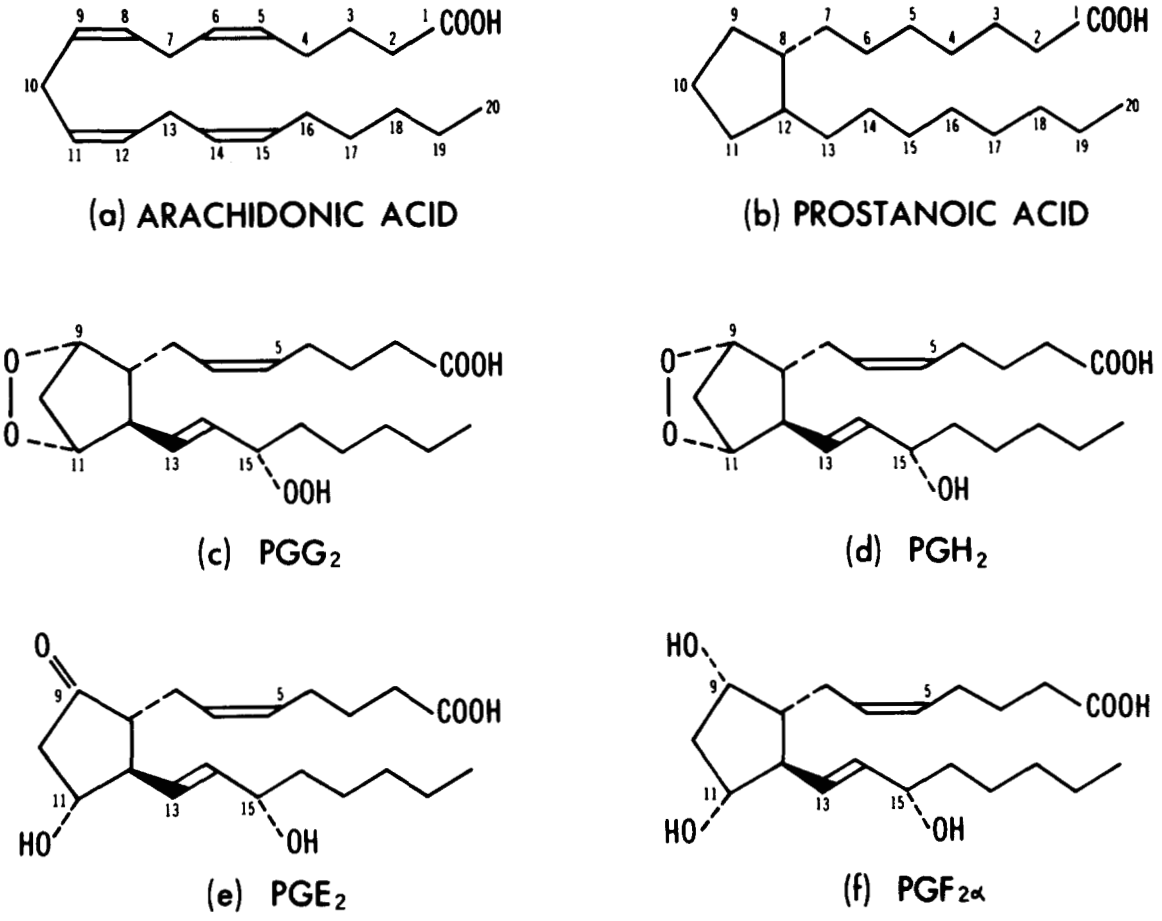


Fig. 2. (*a*) All-*cis*-5,8,11,14-eicosatetraenoic acid (20:4). A major component of phospholipids in all subcellular platelet fractions (74). After hydrolysis by the putative platelet phospholipase A_2, cyclization to prostaglandins of the "2" series ensues. Although mammals can synthesize saturated and mono-unsaturated fatty acids, they cannot form linoleic and γ-linolenic acids and must obtain these from plants. Linoleic acid is the precursor of 20:4. Arachidonic, linoleic, and linolenic are therefore essential fatty acids for mammals and are specifically required for prostaglandin synthesis (76, 225). In platelets arachidonic acid probably serves as an important structural component and as a precursor for endoperoxide and thromboxane formation. Classical prostaglandin synthesis is a small component of the platelet pathway (105). (*b*) Prostanoic acid is the theoretical precursor or parent compound of prostaglandins. It is a fully saturated C_{20} acid with a cyclopentane ring formed by closure from C_8 to C_{12} (221). (*c*) 15-Hydroperoxy-9α,11α-peroxidoprosta-5,13-dienoic acid (prostaglandin G_2). This is the first oxygenated compound formed from 20:4, and the reaction is catalyzed by cyclo-oxygenase (85). Oxygenation occurs at C_{11} and C_{15}. The Δ^{12} double bond isomerizes and a new bond forms between C_8 and C_{12}. An oxygen radical is present on C_{11}, which attacks C_9 to form a peroxide bridge. The oxygen atoms at C_9 and C_{11} were shown to originate from the same oxygen molecule (94, 98). Since cyclo-oxygenase preparations have been found to contain peroxidase activity (139), it has been speculated that PGG_2 may have a short life span in vivo, and that it is converted to PGH_2. When placed in hydroxylic media, PGG_2 has a half-life of approximately 5 min, whereupon it isomerizes to 15-hydroperoxy-PGE_2 (85). (*d*) 15-Hydroxy-9α,11α-peroxidoaprosta-5,13-dienoic acid (prostaglandin H_2). This endoperoxide was discovered before PGG_2 (98). PGH_2 is more polar than PGG_2 and in aqueous media will yield principally PGE_2 (85). The metabolism and mechanism of action of endoperoxides on washed platelet suspensions and in platelet-rich plasma are discussed in the text. (*e*) Prostaglandin E_2. Although platelets form this classical prostaglandin during aggregation (66), PGE_2 does not play an important role in the process (67, 104). On the other hand, PGE_2 is a major prostaglandin produced by tissues such as cultured endothelial cells (22). (*f*) $PGF_{2\alpha}$. This prostaglandin is formed and released during platelet aggregation (66); however, as in the case of PGE_2, it does not appear to play a critical role in platelet function (67, 104).

subjected to radio-TLC. When larger yields were desired, extracted incubation mixtures were first separated on silicic acid columns, followed by methyl esterification and purification by TLC. Methyl esters were then analyzed by TLC in combination with GLC–mass spectrometry. In the first study (98) an oxygenated derivative of arachidonic acid was detectable within 15 sec, but the end product, PGE_2, was not completely formed until 2 min had elapsed. If the incubation mixture was treated with stannous chloride, the oxygenated derivative was reduced to $PGF_{2\alpha}$. When the oxygenated intermediate was allowed to remain on the TLC plate for 15–60 min, it underwent spontaneous rearrangement to a mixture of 11-dehydro-$PGF_{2\alpha}$ and PGE_2. The known structure of the latter two compounds indicated that a

hydroxyl group was present at C-15 in the intermediate as well. The first intermediate was thus designated 15-hydroxy-9α,11α-peroxidoprosta-5,13-dienoic acid, i.e., prostaglandin H_2 (PGH_2) (Fig. 2). It was also shown that PGH_2 elicited a contractile response when added to a rabbit aorta strip.

PGG_2, PGH_2, and platelet aggregation

Following the description of PGH_2, experiments were carried out in which an additional prostaglandin endoperoxide with a hydroperoxy group at C-15 was identified (85). Microsomal pellets from sheep vesicular glands were incubated with [^{14}C]arachidonic acid for 30 sec. Ether extracts were subjected to silicic acid column chromatography, with the elution of two major radioactive peaks. When these were subsequently examined by TLC, it was found that the two endoperoxides had been separated in pure form. PGH_2 was the more polar compound, and its structure was again established by treatment with stannous chloride or triphenylphosphine, resulting in its conversion to $PGF_{2\alpha}$. Dehydration of PGG_2 with lead tetraacetate, followed by reduction with triphenylphosphine, resulted in the formation of 15-keto-$PGF_{2\alpha}$, indicating that a hydroperoxy group was present at C-15. Additional biochemical reactions, in conjunction with TLC, GLC, and mass spectrometry, confirmed the previous identity of PGH_2 and established the structure of PGG_2 as 15-hydroperoxy-9α,11α-peroxidoprosta-5,13-dienoic acid (Fig. 2).

When these 15-hydroperoxy and 15-hydroxy endoperoxides were added to washed platelet suspensions, an aggregation response was elicited. Furthermore, both caused contraction of a rabbit aorta strip. After exposure of platelets to thrombin and treatment of the supernatants with stannous chloride, $PGF_{2\alpha}$ was identified by mass spectrometry, indicating release of endoperoxides from aggregating platelets (85). Since Smith and Willis (88) had already shown that aspirin inhibited prostaglandin synthesis in human platelets, Hamberg et al. (85) suggested that the inhibition was due to interference with the formation of PGG_2 and PGH_2 from platelet arachidonic acid. Subsequent studies by Roth and Majerus (100) and Roth, Stanford, and Majerus (101) documented the authenticity of these suggestions.

It appeared likely that PGG_2 was the first derivative formed from arachidonic acid by a prostaglandin synthetase system, with the introduction of oxygen at C-15 occurring via a dioxygenase reaction. The term fatty acid cyclo-oxygenase was introduced for the enzyme system catalyzing conversion of arachidonic acid into PGG_2 via oxygenation at C-11 and C-15. Nugteren and Hazelhof (99), at approximately the same time, also isolated these 15-hydroperoxy and 15-hydroxy endoperoxides from a similar incubation system. They used the term prostaglandin R_2 for PGH_2. The corresponding endoperoxide derived from dihomo-γ-linolenate during synthesis of PGE_1 and $PGF_{1\alpha}$ was designated PGR_1. As already mentioned, there is no 20:3 in platelet phospholipids (74) and therefore no platelet PGR_1 or PGE_1. It was also obvious that the intermediate reported by Willis et al. (102) and termed LASS was actually PGG_2 and/or PGH_2. The material observed in the 1-min supernatant of aggregated PRP by Smith and associates (103), which was reducible to $PGF_{2\alpha}$ by stannous chloride, was probably also endoperoxide in nature.

Platelet lipoxygenase and cyclo-oxygenase

Having shown that PGG_2 and PGH_2 from sheep seminal vesicles could aggregate human platelets, Hamberg and Samuelsson (104) investigated the transformation of 20:4 by platelet suspensions. [^{14}C]Arachidonic acid was incubated with platelets for 2 min. When the methyl esters derived from this reaction mixture were subjected to TLC, four peaks of radioactivity appeared. The least polar, running near the solvent front, was unchanged methyl arachidonate. Methyl esters of the other three compounds were designated I, II, and III, compound I being the least polar. The structure of compound I was established as 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE). This derivative had not been recognized previously. Compound II was identified as 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT). After an intricate series of procedures, compound III was identified as the hemiacetal derivative of 8-(1-hydroxy - 3 - oxopropyl) - 9,12L - dihydroxy - 5,10 - heptadecadienoic acid (PHD) (later designated thromboxane B_2). These compounds are depicted in **Fig. 3.**

HETE was formed by reduction of 12L-hydroperoxy-5,8,10,14-eicosatetraenoic acid (HPETE). HPETE itself could be isolated if a platelet homogenate was added to the system as an enzyme source. Since there was a pair of conjugated double bonds alpha to the hydroperoxy group, it was concluded that HPETE and HETE were formed by the action of a platelet lipoxygenase. HHT was thought to be a by-product of the chemical decomposition of PGG_2 and PGH_2. In both platelets and sheep vesicular glands, the mechanism of formation of HHT was proposed as fragmentation of the endoperoxide intermediate and release of malonaldehyde. It also appeared that PGG_2 (or PGH_2) was a common precursor of HHT and PHD. When platelets were incubated with radioactive 20:4 in the presence of

aspirin or indomethacin, formation of HHT and PHD was inhibited, but with 100 μg/ml aspirin HETE increased. When 5,8,11,14-eicosatetraynoic acid (ETYA) was included in the incubate, formation of all three compounds was blocked.

It was now obvious that human platelets contained a fatty acid cyclooxygenase that transformed arachidonic acid into endoperoxides PGG_2 and PGH_2, which then formed HHT with release of malonaldehyde as well as the hemiacetal derivative PHD. In addition, platelets contained a lipoxygenase that formed HPETE followed by reduction to HETE. Thus, in human platelets, transformation of arachidonic acid into nonprostaglandin metabolites derived from endoperoxides occurred to a greater extent than synthesis of prostaglandins themselves (105).

Quantification of HETE, HHT, and PHD

With the use of deuterated standards and mass spectrometry, Hamberg, Svensson, and Samuelsson (105) were able to quantify the amounts of HETE, HHT, and PHD released from thrombin-treated platelet suspensions. The effects of aspirin and indomethacin on this process were also evaluated. In addition, measurements were made for thiobarbituric acid (TBA)-positive material produced in response to thrombin. Since the TBA-positive product appeared with HHT, it was assumed to be malonaldehyde. Prior aspirin ingestion by the platelet donor reduced HHT and PHD production by 95%; and there was a precipitous drop in their precursor, PGG_2. Also, direct addition of aspirin or indomethacin to the platelets inhibited HHT and PHD synthesis. There was an average decrease in absorbancy at 532 nm of 82% in the TBA reaction following aspirin ingestion. It was of interest that formation of HETE actually increased threefold after aspirin—an effect also seen with indomethacin, although less pronouncedly.

Time course experiments indicated that HHT and PHD synthesis with thrombin was identical and proceeded faster than release of HETE. It was also noted that the quantities of PGE_2 and $PGF_{2\alpha}$ recovered were very low, as were the amounts of PGG_2 and PGH_2.

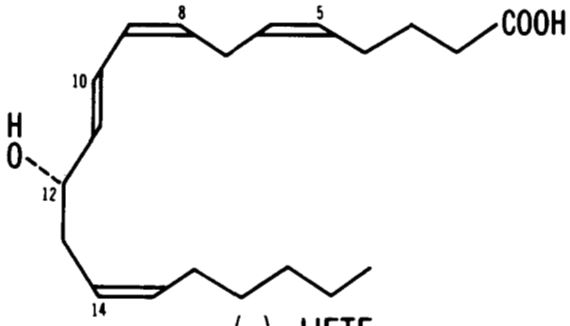
This work served to corroborate previous observations and led to the concept, which is now generally accepted, concerning the action of thrombin and other aggregating agents. These stimuli result in the liberation of free arachidonic acid from platelet phospholipids (research on the putative platelet phospholipase A_2 will be described in a later section). The free 20:4 is available to platelet cyclo-oxygenase for conversion to PGG_2 and PGH_2, followed by fragmentation and production of HHT plus malonaldehyde, as well as PHD (thromboxane B_2). Simultaneously, platelet lipoxygenase acts on arachidonic acid to produce HPETE, which is then reduced to HETE. Aspirin and indomethacin block the cyclo-oxygenase, thereby making more 20:4 available to the lipoxygenase for the formation of HETE. Most observers agree that responses to low concentrations of thrombin are cyclo-oxygenase-dependent. High concentrations of thrombin can elicit aggregation responses in the presence of inhibitors of cyclo-oxygenase.

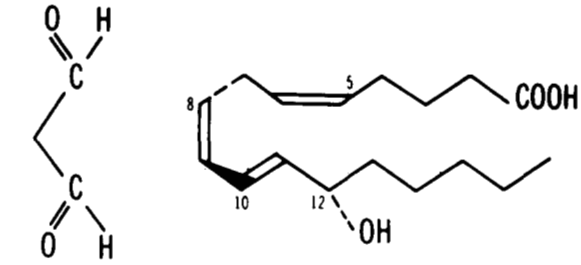
After the observation that aspirin blocked platelet prostaglandin synthesis (88), it was not clear how platelet aggregation would be affected since the prostaglandins produced by platelets, PGE_2 and $PGF_{2\alpha}$, were not particularly effective as aggregating agents. However, when aspirin inhibits platelet cyclo-oxygenase it blocks synthesis of endoperoxides. There was now more qualitative and quantitative evidence that the biological effect of the prostaglandin system centered around endoperoxide formation. At this time reports of the identification of endoperoxides and their effects in other tissues began to appear (86).

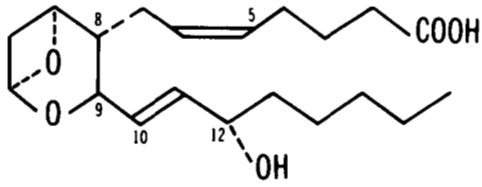
Endoperoxides and the platelet release reaction

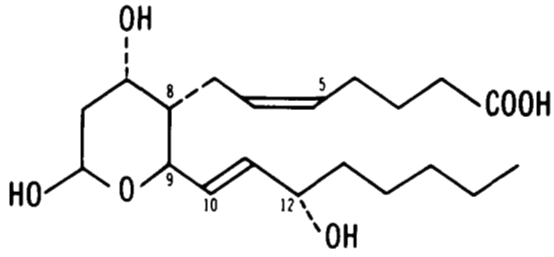
The aforementioned conclusions prompted a more detailed study of the effects of PGG_2 on platelet aggregation (106). PGG_2 at a concentration of 0.6 μM induced a single wave of irreversible platelet aggregation in platelet-rich plasma. Aggregation responses were accompanied by release of ADP and [^{14}C]serotonin, the latter occurring within 5 sec. Small amounts of PHD and HETE were detected after addition of PGG_2, but the synthesis of these substances was *not* blocked by indomethacin. Collagen and ADP also caused serotonin release and PHD and HETE formation; but, in contrast to PGG_2 stimulation, these effects *were* blocked by indomethacin, indicating that the effect of indomethacin was on cyclo-oxygenase. Furosemide, a competitive inhibitor of ADP-induced aggregation, also inhibited the aggregation response to PGG_2 (106).

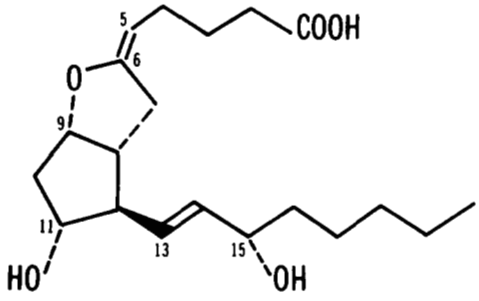
In the course of this work, the first patient with a potential deficiency of platelet cyclo-oxygenase was reported. The propositus was a 30-year-old male with a history of easy bruisability but uneventful surgery in the past. The bleeding time on two occasions was 8.5 and 9 min, respectively (normal, 3–7 min). Platelet counts and all coagulation tests were normal. The patient's platelets did not respond to collagen or arachidonate but aggregated and released normally when exposed to PGG_2. ADP and epinephrine-induced aggregation occurred in a single wave, but there was no serotonin release with ADP or collagen.











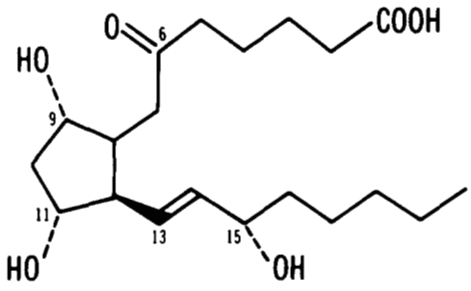


Fig. 3. (*a*) 12L-Hydroxy-5,8,10,14-eicosatetraenoic acid (HETE) is the end product of the lipoxygenase pathway in human platelets and has been reported to mediate neutrophilic leukocyte chemotaxis (45). HETE is formed by reduction of its precursor, 12L-hydroperoxy-5,8,10,14-eicosatetraenoic acid (HPETE). The positional specificity of platelet lipoxygenase on arachidonic acid differs from the action of previously reported lipoxygenases (104). Production of HETE is not inhibited by aspirin or indomethacin (105). In fact, Hamberg et al. showed that, in the presence of aspirin, HETE production actually increased (105). The stimulation of chemotaxis and random migration in polymorphonuclear leukocytes by HETE is the subject of a recent report (Goetzl et al. 1977. *J. Clin. Invest.* **59:** 179). (*b*) 12L-Hydroxy-5,8,10-heptadecatrienoic acid (HHT) forms as a by-product in the reduction of PGG_2/PGH_2. The endoperoxides are thought to be fragmented to a 17-carbon compound, accompanied by release of malonaldehyde (104). As expected, aspirin and indomethacin inhibited synthesis of HHT from arachidonic acid in platelets (105). As yet no biological function has been reported for HHT, although its involvement in chemotaxis has recently been described by Goetzl and Gorman. 1978. *J. Immunol.* **120:** 526. Regarding the malonaldehyde (MDA), it was noted that thiobarbituric acid-positive material was produced during thrombin-induced platelet aggregation. Since it appeared with HHT, it was assumed that the material was malonaldehyde. Thus, the TBA reaction has been used to quantify arachidonic acid transformation in platelets (105, 226). Aspirin or indomethacin will prevent MDA formation in platelets. It should be mentioned that malonaldehyde is a highly reactive molecule that is capable of causing cross-linking of proteins and disturbances in enzyme function, both in vivo and in vitro. Thus it is of interest that a by-product of a presumably beneficial platelet metabolic pathway is a potentially harmful substance (227). (*c*) Thromboxane A_2 (TXA_2) is an unstable intermediate ($t½$ = 32 sec) in the pathway between PGG_2/PGH_2 and thromboxane B_2 (formerly PHD) (67, 228). When 20:4 or PGG_2 was incubated with washed platelets (30 sec), TXA_2 was trapped in methanol, ethanol, or sodium azide. The oxane ring of thromboxane B_2 was present, but

When release of PHD (TXB_2) was monitored, little was detected after addition of collagen, thrombin, ADP, or epinephrine to the patient's platelet-rich plasma (106).

The patient's washed platelets were incubated with [^{14}C]arachidonic acid and the methyl esters were studied by radio-TLC. Only traces of PHD and HHT were detectable on the plates. On the other hand, HETE derived from the lipoxygenase reaction was formed to the same extent as in normal platelets. It was of interest to note that the radiochromatogram of the patient's platelets showed much less unconverted arachidonate near the solvent front than the normal control (106). Is it possible that the patient's platelets did not take up as much 20:4 as did those of the normal control?

Although the history of and laboratory findings on this patient are very convincing for a deficient or nonfunctional platelet cyclo-oxygenase, inadvertent aspirin ingestion must be ruled out unequivocally when comparable patients are studied. This can be done only by GLC analysis of the patient's plasma and urine for salicylates simultaneously with the platelet evaluation. There are so many over the counter drugs that contain acetylsalicylic acid that blood donors are frequently unaware of having ingested this medication. Of course GLC analysis of plasma and urine will only detect salicylates recently ingested. Newer approaches for evaluating aspirin ingestion will be described in the section on platelet cyclo-oxygenase (101).

Malmsten et al. (106) have concluded that PGG_2 caused platelet aggregation via release of ADP. Recent studies in our own laboratory of the oxygen burst associated with platelet stimulation have suggested that ADP plays an important role in aggregation induced by sodium arachidonate. When 20:4 was added to washed platelets or PRP there was an immediate burst of oxygen consumption and simultaneous aggregation. When the platelets were preincubated with apyrase, an enzyme that blocks the action of ADP, arachidonic acid induced the identical burst of oxygen consumption but no aggregation took place. Thus, in this system, oxygenated derivatives of arachidonic acid did not induce aggregation when ADP was unavailable (107).

Kinlough-Rathbone et al. (108, 109) have shown that arachidonate produced shape change and aggregation in washed platelets that had been previously degranulated by repeated thrombin treatment and therefore did not contain releasable ADP. However, their washed platelet suspensions contained fibrinogen which, according to Smith, Ingerman, and Silver (110), is sufficient to support aggregation by endoperoxides in a washed system. Charo and associates (89, 90) found that endoperoxides can cause platelet aggregation in the absence of nucleotide release. However, these experiments were carried out in platelet-rich plasma, which also may have been sufficient to support the aggregation process via its fibrinogen content (110). Finally, Weiss et al. (111) found that the platelets from patients with storage pool disease aggregated normally in response to added endoperoxides. In these studies the platelets were in plasma, or were processed by gel filtration with a small amount of plasma being added subse-

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the hemiacetal hydroxyl group of the latter was absent. Additional experiments demonstrated the presence of an oxane-oxetane ring system in TXA_2. Thromboxane A_2 is a powerful stimulus to platelet aggregation and release and is the major component of rabbit aorta contracting substance (RCS) (67, 82, 86, 228). One interesting exception has recently been reported (229). Dog platelets exposed to arachidonic acid or PGH_2 can generate TXA_2 but will not aggregate in response to its appearance; yet dog platelet TXA_2 will aggregate rabbit and human platelets and will contract rabbit aorta strips. (*d*) Thromboxane B_2 (TXB_2). This compound is the hemiacetal derivative of 8-(1 - hydroxy - 3 - oxopropyl) - 9,12L - dihydroxy - 5,10 - heptadecadienoic acid and was originally called PHD (104). In platelets endoperoxides PGG_2/PGH_2 are transformed mainly to TXA_2 and thence to TXB_2 rather than to classical prostaglandins. During the transition from PGG_2/PGH_2 to TXB_2 there is a rearrangement of the endoperoxide structure with the incorporation of one molecule of water, which is the origin of the hemiacetal hydroxyl group. The intermediate in TXB_2 formation, TXA_2 (*c*), does not contain the hemiacetal hydroxyl group (67). Hamberg et al. also showed that the two oxygens of the peroxide bridge in PGG_2 were retained in the oxane ring of TXB_2. Furthermore, hydrogens at carbons 5, 6, 8, 9, 11, 12, 14, and 15 of 20:4 and PGG_2 could all be accounted for in the structure of TXB_2 (67). (*e*) (5*Z*)-9-deoxy-6,9α-epoxy-Δ^5-$PGF_{1\alpha}$ (PGI_2) (17). Prostacyclin (formerly known as PGX) is an unstable intermediate in the 6(9)-oxy-cyclase pathway of 20:4 transformation (195). In an aqueous medium PGI_2 is highly unstable unless the pH is 8.5 or above. At pH 7.6 (37°C) PGI_2 spontaneously hydrolyzes to 6-keto-$PGF_{1\alpha}$ (196). Prostacyclin can be formed in human and bovine endothelial cells (22), rat stomach (16), arterial walls (203), and other tissues (195). It has been shown that arterial walls can utilize PGG_2/PGH_2 for the production of PGI_2 and subsequent inhibition of platelet aggregation (192, 203). Thus, Moncada et al. have proposed that endoperoxides released by stimulated platelets can be utilized by the 6(9)-oxy-cyclase pathway in blood vessels (prostacyclin synthetase) for the generation of PGI_2, thereby preventing accumulation of platelets on intact vascular endothelial surfaces (203). (*f*) 6-Keto-$PGF_{1\alpha}$ is the final product of the 6(9)-oxy-cyclase pathway in which PGI_2 is the most important intermediate (195). Because of the instability of prostacyclin, validation of its presence in a biological system is established by measuring 6-keto-$PGF_{1\alpha}$ in a TLC system. It has recently been found that 6-keto-$PGF_{1\alpha}$ can be synthesized by ram seminal vesicles when treated with low concentrations of 20:4 and in the absence of reduced glutathione (230). 15-Hydroperoxy arachidonic acid inhibits the 6(9)-oxy-cyclase pathway and blocks formation of PGI_2 and 6-keto-$PGF_{1\alpha}$ (187, 203, 195). This has led to the speculation that lipid peroxides may be involved in certain vascular diseases (19, 195).

quently. Thus fibrinogen was available as a cofactor in the system.

At the present time it would appear that prostaglandin intermediates induce platelet aggregation mainly in synergy with fibrinogen or ADP (110). However, whether endoperoxides (and thromboxane A_2) induce aggregation by direct stimulation of the platelet surface or whether they do so only via the release reaction is a question that will require additional investigation (110, 112). Gorman et al. (113) have proposed that PGH_2 does not induce platelet aggregation in PRP unless it is converted to thromboxane A_2. Platelet responses to aggregating agents that induce endogenous transformation of 20:4 may be quite different from responses to exogenously added arachidonic acid or PGG_2/PGH_2.

Thromboxane A_2: the unstable intermediate between PGG_2/PGH_2 and PHD (thromboxane B_2)

When the hemiacetal derivative PHD was first isolated (104), its formation was thought to occur via a rearrangement of PGG_2/PGH_2, followed by incorporation of one molecule of water. In 1975 (67) a new intermediate between endoperoxides and PHD was isolated from platelet suspensions when the incubation time with [^{14}C]arachidonic acid was shortened to 30 sec, and the intermediate was biochemically trapped. The compound was unstable ($t½$ = 32 ± 2 sec) and, in low concentrations, induced platelet aggregation and release of [^{14}C]serotonin. The name thromboxane A_2 was suggested for this intermediate, and thromboxane B_2 (TXB_2) was the term to be used for its inactive end-product, formerly known as the hemiacetal derivative PHD.

[^{14}C]Arachidonate was incubated with washed platelets in the presence of $^{18}O_2$ for 10 min. HETE, HHT, and PHD formed as expected. HETE and HHT were labeled with ^{18}O in the hydroxyl group, and PHD (hereafter called TXB_2) was labeled with three atoms of ^{18}O. The labels were present in the nonhemiacetal hydroxyl group of the oxane ring, the hydroxyl group at C-12, and the ether oxygen of the oxane ring. No label was present in the hemiacetal hydroxyl group, indicating that the two oxygens of the peroxide bridge of PGG_2/PGH_2 were retained in the oxane ring of thromboxane B_2 (see Fig. 3).

Trapping experiments with the nucleophiles methanol, ethanol, and sodium azide were carried out to determine the origin of the unlabeled hemiacetal hydroxyl group of thromboxane B_2. This was done by incubating platelets with ^{14}C-labeled 20:4 or ^{14}C-labeled PGG_2 for 30 sec, followed by addition of nucleophile. Formation of HHT and TXB_2 was noted; but, in addition, derivatives of TXB_2 were detected in which the hemiacetal hydroxyl group was replaced by methoxy, ethoxy, or azido groups. These derivatives were not detected when the incubation time was extended to 5 min. The experiments indicated that the hemiacetal hydroxyl group originated from water. Thus, thromboxane A_2 was an unstable intermediate in the conversion of PGG_2/PGH_2 to TXB_2. The acetal carbon between the two oxygens in TXA_2 was susceptible to attack by H_2O, thereby forming TXB_2. This structure confirmed previous observations wherein the hydrogens at carbons 5, 6, 8, 9, 11, 12, 14, and 15 in 20:4 and PGG_2 were all accounted for in TXB_2 (Figs. 2 and 3).

A series of experiments was then carried out in order to determine whether this unstable derivative of 20:4 or PGG_2 could induce platelet aggregation. The indicator system was indomethacin-treated platelets in an aggregometer, which therefore could not respond to 20:4 itself. Arachidonic acid was added to a separate platelet suspension and, after 30 sec, an aliquot therefrom induced aggregation in the indicator system. Measurement of the endoperoxide content of the transferred aliquot indicated that this quantity was insufficient to account for the degree of aggregation observed, i.e., another substance was involved. A time study of filtrates from arachidonic acid-treated platelets indicated that a short-lived substance ($t½$ = 41 ± 7 sec), which was *not* endoperoxide, was responsible for the aggregation observed.

Experiments with PGG_2 were designed to show that endoperoxide was the precursor of this unstable aggregating activity. Indicator platelets in the aggregometer, as well as platelets to be incubated with PGG_2, were pretreated with indomethacin. After a 30-sec exposure to PGG_2, aliquots from the test platelets induced aggregation which could not be attributed to the measured endoperoxide itself but to another substance that had formed in the incubation mixture.

Several lines of evidence indicated that incubation of platelets with PGG_2 or arachidonic acid resulted in formation of the same aggregating material: *1*) both substances were unstable and had similar half-lives; *2*) indomethacin inhibited PGG_2 formation from 20:4 in platelets and also blocked development of aggregating activity derived from 20:4. It was therefore obvious that PGG_2 was a precursor of this aggregating activity, and that the time course of its development and disappearance was identical to that of thromboxane A_2.

It also became clear that thromboxane A_2 was the major component of RCS. When the filtrate from a platelet suspension treated with arachidonic acid (30 sec) was added to isolated rabbit aorta strip there was a strong contraction which could not be elicited

if the filtrate was incubated for more than 3 min. Incubation of PGG_2 with an indomethacin-treated platelet suspension also produced a substance that contracted the rabbit aorta strip. The half-life of this material was 34 ± 6 sec (67).

Source of arachidonic acid for platelet prostaglandin synthesis

After it was established that direct cyclization of essential fatty acids gave rise to prostaglandins (114), additional aspects of the synthetic mechanism were reported. *1*) Synthesis was most rapid if hydrolysis of the phospholipid precursor preceded cyclization (115). *2*) The free carboxyl group of the fatty acid was necessary for conversion in the seminal vesicle incubation system. Thus, esters of fatty acids were not converted to prostaglandins unless they were first split by an esterase or by hydrolysis (116). Platelets contain only traces of free 20:4 and, as already indicated, this fatty acid is esterified to specific phospholipids in all subcellular platelet fractions (74). It was therefore of interest to determine the phospholipid source of 20:4 for prostaglandin synthesis in platelets. The pioneering work of Bills, Smith, and Silver (117, 118) has provided answers to this and other questions concerning arachidonic acid metabolism in platelets. When [^{14}C]arachidonic acid was incubated in human platelet-rich plasma, the label (90%) was incorporated into phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI). The labeled platelets were washed and treated with thrombin (10 U/ml, 5 min). Radio-TLC analysis of the phospholipids extracted from the thrombin-treated platelets indicated that the 20:4 was released mainly from PC and PI. The PS and PE fractions showed little or no decrease in radioactivity. A comparable study was carried out with rabbit platelets by Blackwell et al. (119) in which GLC analyses were performed in addition to radioisotope studies. Collagen and thrombin were the aggregating agents and induced release of up to 80% of the total platelet 20:4. PE, PC, and PI were the sources of released arachidonate. About one-half of the platelet arachidonate is present in the PE fraction, one-third is in PI, and the remainder is distributed between PC and PS. PC is the most prominent phospholipid in platelets and in subcellular platelet fractions (74, 119, 120). Although there was an apparent species difference, the results reported by Bills et al. (117, 118) and by Blackwell and associates (119) were in general agreement. It remains to be explained why arachidonate is not liberated from platelet PS after an aggregation stimulus (119), since 23% of the fatty acids in this phosphatide is 20:4 (12, 13, 120). Perhaps platelet PS is unavailable to phospholipase A_2, or the enzyme is not specific for this phospholipid.

Lapetina and associates (121) carried out a comparable study in which [^{14}C]arachidonic acid was incubated with horse platelet-rich plasma (PRP) for 2 hr. After incubation the platelets were washed, resuspended in buffer, and treated with thrombin (50 units/ml) for 5 min. Ninety-five percent of the incorporated radioactivity was in the phospholipid fraction. PE, PI, and PC were almost equally labeled. PS, lyso-PC, and sphingomyelin were not labeled. Phosphatidic acid and the neutral lipid fraction (free fatty acids, hydroxy acids, cholesterol, and triglycerides) contained less than 5% of the total radioactivity. After treatment with thrombin for 5 min, radioactivity in the three labeled phospholipids decreased, and this was accompanied by a corresponding increase in the neutral lipid fraction. The results with PS were in contrast to those of Blackwell et al. (119).

Russell and Deykin (122) incubated washed human platelets *in plasma* containing [^{3}H]arachidonic acid and found that more than half of the total radioactivity was incorporated into lecithin. Addition of thrombin to these platelets resulted in the release of radioactivity from PC, PI, and PS, but there was an enhancement of radioactivity in a lipid fraction heretofore unidentified. Subsequently Rittenhouse-Simmons, Russell, and Deykin (123), using a two-dimensional silica gel paper chromatographic technique, showed that this increase in specific activity in response to thrombin occurred in that portion of the PE fraction that is in the plasmalogen form. This unusual phosphatide has been identified in platelets and other tissues (12, 124), but its function has never been defined. The authors suggested that plasmalogen PE was a receptor for the arachidonic acid transferred from platelet PC and PI after thrombin stimulation. It had not been identified previously because of its acid lability in TLC systems. Schoene and Iacono (125) have reported in abstract form that stimulation of intact or lysed platelets with aggregating agents resulted in release of labeled 20:4 from platelet PI, which is not in agreement with the results reviewed above.

Working with isolated platelet membranes prepared by the nitrogen decompression technique of Broekman, Westmoreland, and Cohen (30), Jesse and Cohen (126) reported that 69% of released 20:4 originated from diacyl PE, but not from plasmalogen PE. Membranes were incubated with 10 mM Ca^{2+} at alkaline pH and, after extraction and addition of internal standards, TLC and GLC were carried out for

quantification of free fatty acids. These studies are not directly comparable to those using whole platelets and radioactive arachidonate at physiological pH, but they do measure 20:4 which is naturally present—though not newly incorporated radiolabeled 20:4, which may subsequently be diluted by unlabeled 20:4. These investigators also noted no preferential release of arachidonic acid from PE as compared to PC. Each of these phospholipids lost 25% of its endogenous 20:4. However, 25% of platelet membrane arachidonic acid is present in the PE component. Thus, studies of the source of 20:4 for prostaglandin synthesis in whole platelets gave different results from those carried out with subcellular platelet fractions. Contrasting findings may also be expected from investigations employing radioactive arachidonic acid rather than measurement of endogenous 20:4 release.

A role for cyclic nucleotides in the regulation of hydrolysis of 20:4 from its phospholipid source should be mentioned here. Agents that elevate intraplatelet cyclic AMP, such as dibutyryl cyclic AMP and PGE_1, probably inhibit platelet aggregation by several mechanisms. Minkes et al. (64) and Lapetina and colleagues (121) have recently shown that elevations of platelet cyclic AMP were accompanied by inhibition of thrombin-induced arachidonic acid hydrolysis from platelet phospholipids, and they suggested that release of arachidonic acid from phospholipids by phospholipase A_2 might be regulated by cyclic AMP levels. Gerrard and associates (127) have reported the same observations, although they elicited aggregation in response to thrombin. Hydrolysis of phospholipids to release 20:4 in the platelet is an important limiting step in prostaglandin synthesis. If arachidonic acid or PGH_2 is added to platelets that have been pretreated with either dibutyryl cyclic AMP or PGE_1, aggregation is inhibited although thromboxane A_2, B_2 and malonaldehyde are formed—indicating yet another mechanism(s) for the inhibitory effect of elevated levels of cyclic AMP (64, 121, 127).

Platelet phospholipase A_2 activity

Blackwell et al. (119) detected phospholipase A_2 activity in frozen and thawed platelets, as well as platelets in PRP. The substrate was PC that was labeled with [1-^{14}C]oleic acid in the 2 position, which they had prepared. Activity was preceded by a lag phase of 10–20 min, followed by a slow linear reaction rate which was terminated after 1 hr. Addition of aggregating agents to the lysate had no effect on phospholipase A_2 activity. Hydrolysis of the substrate was also demonstrable in PRP, and the addition of thrombin resulted in a marked increase in enzyme activity (119).

Derksen and Cohen (128) studied release of fatty acids from endogenous substrates in human platelet homogenates and membranes. Odd-chained fatty acids were used as internal reference compounds and were added at the time of lipid extraction. In membrane preparations there were two peaks of fatty acid release, both of which were accompanied by phospholipid degradation. Release of arachidonic acid was Ca^{2+}-dependent and occurred maximally at pH 9.5. In the subsequent study, mentioned above, Jesse and Cohen (126) reported that diacyl PE accounted for two-thirds of the liberated 20:4 under the conditions of this assay. Since Schick, Kurica, and Chacko (129) had found that choline phosphatides were located primarily on the outer surface of platelet membranes, and that anionic phospholipids such as PE were present on the inner surface, Jesse and Cohen (126) and Derksen and Cohen (128) suggested that platelet phospholipase A_2 might be located on the inner layer of the platelet membrane, where it would have better access to the 2-position fatty acids of PE or PI.

Pickett and Cohen (130) later correlated release of endogenous arachidonic acid with the thrombin-mediated burst in oxygen consumption by platelet suspensions. The same techniques of measuring endogenously released fatty acids were utilized in conjunction with polarigraphic tracings of oxygen consumption following stimulation with thrombin or arachidonic acid. When the incubation chamber was depleted of oxygen and thrombin was added to the platelet suspension there was an accumulation of 20:4, indicating that phospholipase A_2 had been activated but that cyclo-oxygenase was inoperative. Trypsin was later shown to be even more effective than thrombin in mediating 20:4 release and oxygen consumption in platelets (131). Both thrombin and trypsin stimulated more release of arachidonic acid in intact platelets than in membrane preparations. This might indicate participation of an intracellular constituent such as Ca^{2+} in the activation of platelet phospholipase A_2.

Since platelet membrane phospholipase A_2 had an absolute requirement for Ca^{2+} (128), it was of interest to examine the effect of ionophore A23187 on the activity of this enzyme in platelet suspensions (132). Ionophore mobilized five times more 20:4 than did thrombin, and release was accompanied by a marked increase in oxygen consumption. Furthermore, the ionophore effect took place in the absence of external calcium, indicating that the Ca^{2+} requirement

for phospholipase A_2 activity was satisfied by intracellular Ca^{2+} mobilization. Although the stimulatory effect of thrombin on phospholipase A_2 activity was inhibited by dibutyryl cyclic AMP, no such inhibition was seen with ionophore. The authors suggested that activation and release of phospholipase A_2 in platelets were mediated via mobilization of intracellular calcium.

The above concept has been further extended by Rittenhouse-Simmons and colleagues (133, 134), who compared the effects of ionophore and thrombin on 20:4 mobilization, as well as serotonin release. Their assay system for 20:4 mobilization measured the transfer of ^{3}H-labeled 20:4 from platelet PC and PI to plasmalogen PE (123). Thrombin required metabolic ATP for 20:4 liberation and serotonin release, whereas ionophore induced release and 20:4 mobilization in a Ca^{2+}-free medium. ATP-depleted platelets exposed to ionophore did not release serotonin, but arachidonic acid was mobilized in a normal fashion. Thus, platelet phospholipase A_2 can be activated solely by an increase in cytoplasmic Ca^{2+}. Thrombin activation of the enzyme, even in the presence of calcium, required intact platelet energy metabolism; but ionophore activation required no cofactors and was independent of the release reaction. It had previously been speculated that an intracellular proteinase might be liberated as part of the release reaction and that it subsequently activates a membrane-bound prophospholipase (131); but the experiments reported here revealed activation of phospholipase A_2 in the absence of a release reaction but in the presence of intracellular calcium mobilization.[3]

Bills et al. (118) have presented evidence that the putative endogenous phospholipase A_2 activities in platelets are specific in their preference for the 1-acyl-2-arachidonoyl forms of PC, PS, or PI. This is in contrast to most other phospholipase activities, which are generally positional in specificity. When platelets were prelabeled with [^{14}C]arachidonic acid and then treated with thrombin, there was a significant decrease in [^{14}C]20:4-labeled PC, as well as labeled PS and PI. In the case of PS and PI, trace amounts of other fatty acids were also released. In contrast to results with platelet membranes under different experimental conditions (128), no changes were detected in PE. The [^{14}C]20:4-labeled platelets were also preincubated with 5,8,11,14-eicosatetraynoic acid, which blocked oxidation of free arachidonate and allowed for its accumulation. Only 20:4 was seen to increase, and release of other fatty acids was insignificant. The incorporation data also suggested that free 20:4 in plasma might be the source of arachidonate in platelet PC and PI. Incubation of platelets with ^{14}C-labeled fatty acids 20:4, 20:3, 18:2, and 18:1 resulted in more fatty acids being incorporated into PC than into any other platelet lipid. However, only 20:4 was released from PC in response to thrombin. The results therefore indicate that the net effect of thrombin stimulation in human platelets is the specific release of arachidonic acid. It will be of interest to discern whether this unique group of enzymatic activities is specific to platelets.

[3] Participation of a stimulus-activated protease that can activate platelet phospholipase A_2 has been suggested by Feinstein et al. (1977. *Prostaglandins* **14:** 1075).

Platelet cyclo-oxygenase (prostaglandin endoperoxide synthetase): inhibition by aspirin and indomethacin

Histochemical studies have indicated that platelet cyclo-oxygenase activity is located in the dense tubular system, a component present in the microsomal fractions of ultracentrifuged (100,000 *g*) platelet homogenates (135, 136).

Miyamoto et al. (137) purified prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes by combining Tween 20 solubilization with DEAE cellulose column chromatography. The column eluates were electrofocused and the peak of activity catalyzing synthesis of PGH_1 from 8,11,14-eicosatrienoic acid was at pH 7.2. In the presence of hematin the enzyme converted 20:3 to PGG_1, and with hematin plus tryptophan PGH_1 was synthesized. Production of PGG_1 was inhibited by aspirin and indomethacin. The purified enzyme possessed both oxygenase (20:3 $\rightarrow$ PGG_1) and peroxidase activity, since it catalyzed degradation of the hydroperoxy group of PGG_1 to the hydroxyl group of PGH_1.

Working with sheep vesicular glands, Hemler, Lands, and Smith (138) purified fatty acid cyclo-oxygenase by a comparable sequence of procedures, i.e., detergent solubilization, ammonium sulfate fractionation, chromatography on DEAE-cellulose, and flurbiprofen–Sepharose, isoelectric focussing, and gel filtration. The final product had a molecular weight of 70,000, and treatment of the tissue with tritiated acetylsalicyclic acid yielded a radioactive product which co-electrophoresed with the protein of 70,000 molecular weight. Van der Ouderaa and colleagues (139) carried out a similar isolation of cyclo-oxygenase from sheep vesicular glands and found it to be a membrane-bound glycoprotein with a molecular weight of 126,000. The enzyme was isolated as a complex with Tween 20, wherein it behaved as a dimer.

On 5% SDS gels the molecular weight averaged 77,000. In agreement with Miyamoto et al. (137), the purified enzyme possessed both oxygenase and peroxidase activity, since arachidonic acid was converted to PGH_2. With the isolated enzyme, hemin was added as a prosthetic group and hydroquinone served as a hydrogen donor for reduction of the hydroperoxy group of PGG_2. Acetylation of the enzyme following incubation with tritiated acetylsalicylic acid was also shown (139). Peroxidase activity in sheep seminal vesicle microsomes has been reported (140).

More recently Hammarström and Falardeau (141) demonstrated the presence of both cyclo-oxygenase and thromboxane synthetase in human platelet microsomes. After treatment of microsomes with Triton X-100 and passage of the ultracentrifuged supernatant over a DEAE-cellulose column, cyclo-oxygenase and thromboxane synthetase were recovered separately. An interesting new finding was that thromboxane synthetase was inhibited by HPETE. The latter, a product of the lipoxygenase pathway and formed during platelet aggregation, may represent a mechanism for feedback regulation of thromboxane synthesis. Since HETE was not inhibitory, the hydroperoxy but not the hydroxy group must be a prerequisite for inhibition.

The first report of a biochemical interaction between platelets and acetylsalicylic acid was published by Al-Mondhiry, Marcus, and Spaet in 1970 (142). It was already known that therapeutic doses of aspirin produced a mild hemostatic defect in normal individuals, manifested by slight prolongation of the bleeding time. However, ingestion of aspirin by patients with coagulation disorders magnifies the severity of the defect. The aspirin effect is permanent in a given population of platelets, since complete reversal roughly coincides with the 7–9 day platelet life span (23, 33). Al-Mondhiry et al. (142) found that incubation of platelets with aspirin or acetic anhydride abolished the second wave of aggregation in response to ADP or epinephrine, i.e., aggregation was reversible. Aggregation induced by collagen was also markedly impaired. When aspirin labeled with ^{14}C in the acetyl position was incubated with PRP, radioactivity was irreversibly bound to the platelets. ^{14}C-Labeled acetic anhydride was also bound but no interaction occurred with carboxyl-labeled aspirin. This suggested that the mechanism of action of aspirin on platelets involved acetylation, since acetic anhydride produced a comparable functional defect. When platelets were labeled with acetyl-[^{14}C]aspirin and subcellular fractions were prepared, radioactivity was detected in membranes, granules, and the soluble fraction. It thus appeared that the acetyl group of aspirin was reacting with multiple sites on or in the platelet. However, the specificity and saturability of the acetylation reaction were not determined (142).

Roth and Majerus (100) synthesized acetyl-[^3H]-aspirin of high specific activity (197 μCi/μmol) and incubated it with washed platelets in concentrations of 50–500 μM. After sonication of the incubate, SDS–polyacrylamide gel electrophoresis revealed three peaks of radioactivity corresponding to molecular weights of 225,000, 85,000, and 55,000, respectively. The peaks of mol wt 225,000 and 55,000 took up radioactivity at 0 time. The second peak (85,000) incorporated radioactivity maximally between 20 and 60 min, but no incorporation was observed at 0 time. When the incubation mixture was ultracentrifuged to obtain a soluble and a particulate fraction, only the 85,000 mol wt material was particulate. The supernatant fractions were not saturable with increasing concentrations of ^3H-labeled aspirin, but the particulate fraction was saturable at 30 μM aspirin in 15 min, which correlates with the time required to inhibit platelet function in vitro with acetylsalicylic acid (ASA) (92). Preincubation of platelets with nonradioactive ASA blocked uptake by acetyl-[^3H]aspirin. When ASA was ingested by volunteers and their washed platelets were incubated with ^3H-labeled aspirin, the 85,000 mol wt protein was not labeled on day zero or the day following the last dose. More label was incorporated on the 3rd–6th day, and full uptake occurred by day 13. This roughly correlates with normal platelet turnover time and indicates that the in vivo acetylation reaction is permanent. Roth, Stanford, and Majerus (101) extended these observations by incubating [^3H]aspirin with microsomal preparations from sheep and bovine seminal vesicles and again confirmed acetylation of the 85,000 mol wt peak. The extent of acetylation correlated with the degree of inhibition of cyclo-oxygenase activity as measured by malonaldehyde formation. Arachidonic acid also inhibited acetylation, as did linolenic (18:3) and eicosatrienoic (20:3) acids (143). Indomethacin (100 μM) was an effective inhibitor of acetylation, and this inhibition could be blocked by arachidonic acid, a natural substrate for cyclo-oxygenase. These and other studies strongly indicate that aspirin acetylates and inhibits the active site of prostaglandin cyclo-oxygenase in all tissues thus far examined (144).

As will be pointed out in the section on prostacyclin, it is important to know the degree of sensitivity of platelet cyclo-oxygenase to acetylation (and, by definition, inactivation), as compared to cyclo-oxygenase in other tissues, especially the enzyme in blood vessels known to be the site of occlusion by platelet thrombi.

Burch, Stanford, and Majerus (145) measured the ability of ingested aspirin to block subsequent acetylation of platelet cyclo-oxygenase in vitro by ^{3}H-labeled ASA. A daily dose of 20 mg of aspirin inactivated platelet cyclo-oxygenase by more than 50%. The analgesic and anti-inflammatory dose of aspirin ranges from 325 mg to 4 g per day. A single aspirin tablet (325 mg) reduced cyclo-oxygenase activity in platelets by 89%, and the effect persisted for 2 days (megakaryocyte acetylation). When compared to sheep seminal vesicles, platelet cyclo-oxygenase was 31-fold more sensitive to ASA. Platelet cyclo-oxygenase is also more sensitive to aspirin than the enzyme in fibroblasts and smooth muscle cells in culture (146).

Platelet lipoxygenase

Lipoxygenase in bovine platelets has been studied by Nugteren (147, 148). In contrast to enzymes of the prostaglandin pathway, which are particulate, lipoxygenase was present in the supernatant of disrupted platelets. Arachidonic acid was the best substrate and, in the presence of semipurified enzyme preparations, HPETE was obtained. When whole platelets or homogenates were used as the enzyme source with 20:4, 12-L-hydroxy acid (HETE) was detected as the end product (Fig. 3). Lipoxygenation and prostaglandin synthesis were inhibited by albumin, whereas prostaglandin formation but not lipoxygenation was inhibited by indomethacin.

In direct contrast, Ho, Walters, and Sullivan (149) observed arachidonic acid lipoxygenase activity in particulate preparations derived from fresh and stored human platelets. They could not recover enzymatic activity in the supernatant when their platelet microsomal preparations were frozen and thawed. This discrepancy may be related to differences in methodology, or to differences in distribution of the enzyme in human and bovine platelets.

Turner, Tainer, and Lynn (45) have shown that HETE produced during platelet aggregation is a mediator of neutrophilic leukocyte chemotaxis (45). A soluble lipoxygenase fraction from human platelets was prepared (147) and incubated with arachidonic acid. Chemotactic activity for leukocytes was measured, and samples from the incubation mixture were removed, acidified, and extracted with diethyl ether. The ether extracts also contained chemotactic activity and HETE was identified by TLC, GLC, and mass spectrometry. The same material was present when indomethacin was included in the incubation system, indicating that the products were not derived from the action of cyclo-oxygenase. Interestingly, HETE could also be generated nonenzymatically by ultraviolet photo-oxidation of arachidonic acid. The relationship between this system and the platelet-derived chemotactic activity in serum described by Weksler and Coupal (44) (which acts on the fifth component of complement) is yet to be established.

The lipoxygenase pathway of 20:4 oxygenation has been implicated as the source of slow-reacting substance (SRS) (150). SRS is a polar acidic lipid(s) of unknown structure possessing contractile properties toward bronchial and ileal smooth muscle. Release of SRS from leukocytes and other tissues occurs during IgE-type allergic reactions, or after exposure to calcium ionophore A23187. Jakschik et al. have shown that addition of 20:4 to rat basophilic leukemia cells that have been stimulated by A23187 induces a fivefold increase in SRS production (20:4 in the absence of A23187 produced only small amounts of SRS). This response was inhibited by ETYA, 18:2, and 18:3, but not by indomethacin. Studies with radiolabeled arachidonate revealed incorporation into SRS by thin-layer radiochromatography, and the material was distinguishable from all other known 20:4 metabolites. When platelet suspensions were substituted for the leukemic basophils in the incubation system, SRS was not detected. Thus, SRS may be a new metabolite directly derived from 20:4 lipoxygenation, whose structure remains to be elucidated.

Biological activity of endoperoxides (PGG_2 and PGH_2) and thromboxanes (TXA_2 and TXB_2)

In studying the role of prostaglandins in acute inflammation, Kuehl and associates (153) have presented evidence that PGG_2 or hydroxyl radicals ($OH\cdot$), which can form during the former's conversion to PGH_2 (154), are the major mediators of inflammatory reactions derived from arachidonic acid oxygenation. Experiments on the effects of nonsteroidal anti-inflammatory agents (indomethacin, ASA, phenylbutazone) on the enzymatic oxygenation of 20:4 consistently showed inhibition of PGG_2 formation (153). Observations on an experimental anti-inflammatory drug (MK-447) indicated a requirement for a free phenolic group for its action. Phenol is known to promote prostaglandin synthesis by stimulating uptake of O_2 by cyclo-oxygenase and by protecting it from inactivation. Phenol also enhances conversion of PGG_2 to PGH_2, during which it acts as a free radical scavenger. Experimentally the anti-inflammatory properties of both phenol and MK-447 could be related to their ability to scavenge free radicals. Thus, the component of 20:4 oxygenation that promotes inflammation could be hydroxyl radicals, which can form during conversion of PGG_2 to PGH_2. This concept may fit with the proposal by van der Ouderaa et al. (139) that PGG_2 may not accumulate in tissues to a signifi-

cant extent because of the presence (at least in vitro) of peroxidase activity in purified cyclo-oxygenase preparations. In a recent study Williams and Peck (155) challenged the contention of Kuehl et al. (153) that PGG_2 was a pivotal mediator of the inflammatory response. They maintain that nonsteroidal inflammatory compounds reduce inflammatory edema as a consequence of suppression of vasodilatation (155). It is of interest that free radicals such as superoxide (O_2^-) can aggregate platelets and lower the threshold for the action of other aggregating agents (156), and superoxide radicals are produced by metabolizing platelets (37).

In addition to the original techniques described for preparing PGG_2 or PGH_2 (85, 99), other investigators have recently published methods for preparing these endoperoxides in good yield (157, 158). Studies in our own laboratory have confirmed these methods. Acetone–pentane powders prepared from microsomes derived from sheep seminal vesicles remain the starting material of choice as a source of cyclo-oxygenase activity (143). The glands are best obtained from uncastrated sheep since atrophied seminal vesicles have low enzyme activity.

Synthetic endoperoxides, such as 9,11-azo-prostanoid III (azo analogue III) (151), as well as the methyl ester of PGH_2 (152), are gradually becoming available and will be useful in in vitro test systems since they are stable and presumably not metabolized to thromboxanes. Thus, azo analogue III is 7.9 times more active than PGG_2 in inducing aggregation in PRP and 6 times more potent in promoting serotonin release. Isolated rabbit aorta strip is also sensitive to this analogue. The use of such compounds may help answer the question of whether endoperoxides per se can induce aggregation or whether they must be converted to thromboxane in order to do so. However, as Smith, Ingerman, and Silver (159) have pointed out, natural endoperoxides and their synthetic analogues might mimic thromboxanes as aggregating agents to different degrees. These differences may eventually be explained when the relationship between three-dimensional structure and pharmacologic activity is elucidated (160).

Charo et al. (89) used the endoperoxide analogue U-46619 to study aggregation and release simultaneously. At high concentrations (2.2 μM) the analogue induced aggregation and release, but at low concentrations (0.5 μM) aggregation occurred in the absence of detectable secretion. Intermediate concentrations (1.2 μM) induced a biphasic response, in which release occurred during the second wave of aggregation. This was in contrast to the effect of PGG_2 or PGH_2, wherein intermediate concentrations induced an unusual response—reversible aggregation accompanied by secretion. These results suggest that synthetic analogues may not always produce the same biological effect as natural endoperoxides.

At present the relative roles of endoperoxides and thromboxanes as aggregating agents, as well as their precise mechanisms of action, are not clear. Needleman, Minkes, and Raz (161) have maintained that the ability of endoperoxides to induce platelet aggregation and their conversion to thromboxane can be dissociated. For example, when they added PGH_2 to PRP, aggregation occurred but virtually no TXA_2 was detected (161). This is in contrast to washed platelets and has also been observed by Smith et al. (159), who found that PGH_2 could form PGE_2 and PGD_2 in platelet-rich plasma or platelet-free plasma. However, since PGD_2 is inhibitory, the aggregation observed was probably due to PGH_2 itself before it isomerized (159). Thus, thromboxane may not have formed in these experiments. Needleman et al. also incubated PGH_3 (synthesized from 20:5) with indomethacin-treated platelet microsomes and obtained thromboxane A_3, which possessed strong vasoconstrictor activity. However, PGG_3, PGH_3, and TXA_3 did not induce an aggregation response (161).

Using imidazole (100 μg/ml) as a selective thromboxane synthetase inhibitor, Needleman and colleagues (162) showed that endogenous or exogenous endoperoxides could induce aggregation in washed platelets without transformation to thromboxane A_2. They concluded that imidazole was inhibiting thromboxane synthetase, and this led to the accumulation of biologically active endoperoxides that induced aggregation. This hypothesis was extended in a recent study (160) wherein structural features of endoperoxides could be correlated with their aggregation-inducing properties. Conversion of endoperoxides to thromboxanes was not a prerequisite for induction of platelet aggregation. Vasoconstrictor properties of certain thromboxanes were not associated with their capacity to induce an aggregation response (160).

Studies with azo analog I (9,11-azo-prosta-5,13-dienoic acid) by Gorman and co-workers have led to somewhat different conclusions (113). This compound differs from azo analog III of Corey et al. (151) in that the 15-*S*-hydroxyl group is absent. Azo analog I inhibited platelet thromboxane synthetase activity and aggregation. Preincubation of PRP with azo analog I blocked subsequent aggregation induced by PGH_2 and high concentrations of 20:4 and sharply reduced the aggregation response to collagen. Also inhibited were the second wave of ADP- and epinephrine-induced aggregation. Azo I prevented synthesis of TXB_2 from 20:4 or PGH_2 when the latter

were incubated with platelet microsomes. The inhibitory effect of azo I was not associated with an increase in cyclic AMP in platelets. In the presence of azo I, synthesis of platelet PGE_2 and PGD_2 from PGH_2 increased. Gorman et al. (113) have concluded that arachidonic acid or PGH_2 will not induce platelet aggregation in PRP or washed platelets unless it is converted to TXA_2. They also propose that collagen-induced aggregation and the second wave of ADP and epinephrine responses depend on formation of TXA_2. Recent data presented by Charo, Feinman, and Detwiler (90) are in agreement with the ADP and epinephrine experiments of Gorman's group, but Charo and associates maintain that collagen induces secretion and aggregation simultaneously, and the response cannot be blocked by indomethacin. Clearly there is room for compromise, since several investigators believe that collagen can act by *either* the ADP or the arachidonic acid oxygenation pathway (48, 49, 107). Furthermore, Claesson and Malmsten (63) have re-emphasized that the effects of PGG_2 on platelets in PRP are mediated mainly via the rapid release of ADP induced by this endoperoxide.

Needleman et al. (163) reported the presence of thromboxane synthetase activity in horse and human platelet microsomes[4] by incubating the latter with PGG_2 and demonstrating generation of RCS activity. Ellis and co-workers (164) noted the same RCS activity from thrombin-treated platelets on porcine coronary arteries. Detailed studies of the biochemical properties of thromboxane synthetase from bovine and human platelet microsomes have now been reported (165–167). Solubilization of the system, as recently accomplished (141, 168), should prove valuable for further research. Production of thromboxane A_2 activity during phagocytosis has also been described (169).

Smith, Ingerman, and Silver (170) have shown that after incubation of PRP with arachidonic acid, TXA_2 activity reached a maximum in 3 min but remained detectable for 10 min. Controls indicated that the material present at 10 min was not endoperoxide, and when the same experiment was carried out in a washed platelet system (67), TXA_2 reached a maximum in 30 sec and was essentially absent in 3 min. Thus, a component of plasma (or serum) stabilized TXA_2 and preserved its activity. This component was not albumin or a constituent of eight Cohn fractions tested.[5] It should be mentioned that 1 ml samples of PRP were incubated with 1 mM arachidonic acid (plus ^{14}C-labeled 20:4) (170). Persistence of TXA_2-like material following treatment of PRP with other stimuli has not as yet been reported, and the effect may be unique for arachidonic acid. Of interest in this regard is a report (171) that plasma and serum contain a protein associated with the haptoglobin fraction that has an inhibitory effect on the prostaglandin synthetase system. It therefore appears that a constituent(s) of plasma and serum can both block prostaglandin synthesis and at the same time preserve the activity of one of its end products from platelets exposed to 20:4.

Malmsten et al. studied thromboxane synthesis (radioimmunoassay for TXB_2) and ADP release in several platelet diseases (172). The results were commensurate with current concepts of the role of arachidonic acid oxygenation in platelet aggregation and could be correlated with the pathogenesis of the disease in question. If the patient's platelets were congenitally deficient in stored ADP (Hermansky-Pudlak syndrome), addition of this nucleotide normalized the aggregation response; but stimulation with PGG_2 or collagen resulted in diminished aggregation. The ADP defect had no influence on TXB_2 formation. In Glanzmann's disease (thrombasthenia), wherein the subject's platelets do not aggregate in response to ADP, PGG_2 and 20:4 evoked a poor aggregation response (172).[6]

In 1972 White and Witkop (173) reported an interesting observation which can now be explained biochemically. Platelets from patients with the Hermansky-Pudlak syndrome and platelets from subjects who had ingested aspirin were mutually corrective in an in vitro aggregation test system. The aspirin-treated platelets had an inactivated cyclo-oxygenase but normal storage pool of ADP, whereas the Hermansky-Pudlak platelets lacked a storage pool but their cyclo-oxygenase was intact. This cross-correction technique can thus be used to distinguish patients who have ingested aspirin or who may be deficient in cyclo-oxygenase from those with storage pool defects. The approach has recently been employed to identify a possible congenital deficiency in thromboxane synthetase (174). Platelets from the propositus ag-

[4] 'Microsomes' is an operational term used to describe a pellet obtained by ultracentrifugation of the supernatant after nuclei and mitochondria from a cell homogenate have been spun out. In nucleated cells it contains RNA and fragments of endoplasmic reticulum. Strictly speaking, platelets do not contain a microsomal fraction if this implies the presence of endoplasmic reticulum and RNA. In most prostaglandin studies, platelets have not been homogenized according to techniques appropriate for platelets, so that microsomal preparations derived therefrom must be regarded as poorly defined.

[5] Folco and associates have recently reported that albumin can stabilize thromboxane A_2 (1977. *FEBS Lett.* **82:** 321–324).

[6] In collaboration with Dr. Marjorie Zucker we confirmed these observations using biosynthesized PGH_2 in two patients with thrombasthenia. No aggregation occurred with PGH_2 or 20:4.

gregated when mixed with platelets from a subject who ingested aspirin, or from a patient with possible cyclo-oxygenase deficiency. However, they did not aggregate when exposed to PGG_2.

Prostaglandin D_2 and platelet function

Early reports indicated that exogenously added PGD_2 had little effect on platelet aggregation in rabbit and rat PRP. In 1974 Smith et al. (175) showed that PGD_2 (15 nM) inhibited aggregation in human platelets induced by either collagen or ADP. The effect was twice that of PGE_1, which was previously thought to be the strongest inhibitor of platelet aggregation. Bressler, Broekman, and Marcus (107) have found that PGD_2 abolishes the oxygen burst and aggregation induced by collagen in washed platelet suspensions.

Subsequently Smith, Ingerman, and Silver (159, 176) demonstrated PGD_2 production during PGH_2-induced aggregation. After incubation of PGH_2 in PRP (37°C, 3 min), PGD_2 (as well as PGE_2 and PGA_2) could be recovered. However, the same phenomenon occurred in platelet-free plasma, indicating that this represented spontaneous isomerization of PGH_2 to PGD_2, a process known to be enhanced by serum albumin (177). It was further shown that PGD_2 forming from PGH_2 in PRP was capable of inhibiting ADP-induced aggregation.

The above information on exogenously produced PGD_2 was supplanted by the demonstration of PGD_2 production by platelets in PRP within seconds of stimulation by thrombin, collagen, epinephrine, and 20:4 (178). The quantity of PGD_2 formed from activated platelets (5.5–28.3 nM) is sufficient to inhibit aggregation induced by virtually all known stimuli (178). It is therefore possible that PGD_2 in PRP may play a role in feedback inhibition of platelet aggregation, since we already know that thromboxane A_2 activity persists for up to 10 min in this medium (170). Ali et al. (179) have also reported synthesis of PGD_2 (and TXB_2) by human platelets stimulated with collagen or 20:4. Synthesis was increased in frozen and thawed platelet preparations, which may have permitted greater access to PGH_2 by PGD_2 isomerase.

Cyclic nucleotides and 20:4 oxygenation

As already mentioned, agents that increase levels of cyclic AMP in platelets inhibit aggregation. The increase can be achieved by adenylate cyclase activation or inhibition of cAMP phosphodiesterase. Hidaka and Asano (180) have shown that thrombin and ionophore A23187 induce secretion of cAMP and cyclic guanosine monophosphate (cGMP) phosphodiesterases into the surrounding medium. A proposed consequence of the latter was accumulation of intracellular cyclic AMP via calcium mobilization. However, the increase in intra-platelet cAMP may have resulted from rather than caused aggregation (180). This concept is novel and interesting.

Malmsten, Granström, and Samuelsson (181) reported that dibutyryl cyclic AMP (dBcAMP) inhibited aggregation and 5-HT release when collagen and 20:4 were the stimuli but not when PGG_2 was the aggregating agent. They therefore concluded that elevated cAMP levels blocked platelet aggregation by inhibiting cyclo-oxygenase activity. In a subsequent paper Claesson and Malmsten (63) proposed that effects of elevated cAMP were not only on cyclo-oxygenase but on other reactions involved in the induction of aggregation and release, including hydrolysis of 20:4 from platelet phospholipids. They also reiterated their previous contention (106) that aggregation induced by PGG_2 occurs mainly via release of ADP, which in turn lowers platelet cAMP levels. Addition of PGG_2 to PRP in the presence of creatine phosphate/creatine phosphokinase (which removes released ADP from the system) resulted in inhibition of the aggregation response (63).

In contrast, Minkes and associates (64) found that preincubation of platelet suspensions with dBcAMP had no effect on 20:4- or PGH_2-induced RCS, TXA_2, or MDA formation. However, RCS, TXB_2, and MDA production induced by thrombin were indeed inhibited. They proposed that cAMP does not directly affect cyclo-oxygenase or thromboxane synthetase but blocks hydrolysis of 20:4 from platelet phospholipid by phospholipase A_2 following the addition of thrombin. These investigators also showed that dBcAMP could block the subsequent aggregation response and ADP release induced by 20:4 or PGH_2 (or thrombin) in the setting of normal RCS, TXB_2, and MDA formation stimulated by 20:4 or PGH_2 (however, inspection of the figures in the publication indicates that thrombin did induce slight aggregation and release). This reinforces the concept that inhibition of aggregation and release by cAMP and its modulation of PLA_2 activity may be separate regulatory entities.

Gerrard et al. (127) arrived at similar conclusions, i.e., that direct or indirect elevations of cAMP inhibited 20:4-induced aggregation, but TXB_2 formed normally. In contrast to the findings of Minkes et al. (64), the aggregation response to thrombin was not inhibited by an increase in platelet cAMP levels. Thus, when platelets were preincubated with 1 mM dibutyryl cyclic AMP, normal thrombin-induced aggregation ensued but very little free 20:4 was hydrolyzed, nor was HETE, HHT, or TXB_2 de-

tected. These results support the hypothesis that thrombin can aggregate platelets via more than one pathway (48, 49).

Miller, Johnson, and Gorman (65) and Gorman and Miller (62) have shown that PGH_2 and TXA_2 do not alter basal cAMP levels when they induce platelet aggregation. In order to show an effect of PGH_2 or TXA_2, cAMP levels must initially be stimulated by PGE_1. Under these conditions PGH_2 and TXA_2 inhibited PGE_1-stimulated cAMP accumulation, with TXA_2 being the more effective. Of interest is the recent report of Fitzpatrick and Gorman (182) which shows that when PGH_2 is added to PRP, thromboxane A_2 forms prior to the onset of irreversible aggregation. They have concluded that TXA_2 formation is essential during 20:4-mediated aggregation. This observation extends previous work by this group (113), and may explain why Needleman et al. (161) did not detect TXA_2 in platelet-rich plasma following aggregation induced by PGH_2. Fitzpatrick and Gorman found that thromboxane formation after PGH_2 addition could only be detected during the first 20 sec of incubation (182). No changes were noted in cyclic GMP levels, although it has been shown (183) that peroxidized unsaturated fatty acids can stimulate guanylate cyclase in human platelet homogenates. In agreement with others, Miller et al. (65) proposed that PGH_2 or TXA_2 may act on adenylate cyclase indirectly by inducing release of calcium from storage sites. The elevated intracellular calcium subsequently lowers adenylate cyclase levels and promotes aggregation and release. White and Gerrard (136) and White, Rao, and Gerrard (184) have proposed that an elevation in cytoplasmic calcium concentration is the critical event in initiation of platelet 'contraction' and release. They believe that contractile phenomena predominate in the platelet responses to aggregating agents. The increase in cytoplasmic calcium concentration is blocked by cAMP (136, 184).

In contrast to others (63, 65), Glass and colleagues (185) reported that arachidonic acid-, collagen-, and PGG_2-induced platelet aggregation was accompanied by an increase in cyclic GMP levels. This occurred in the setting of no significant change in cAMP. The 20:4 and collagen effect was blocked by prior treatment of the washed platelets with ASA or indomethacin, but the cGMP response to PGG_2 was not. The elevation in cGMP was found to occur after PGG_2/PGH_2 had formed, and it was therefore concluded that endoperoxide formation was a prerequisite for cGMP accumulation. Since other investigators (63, 65) have been unable to detect changes in cGMP levels under comparable conditions of stimulation, these findings merit further investigation. Haslam (59) has recently reviewed the role of cGMP in platelet function.

Mills and Macfarlane (186) demonstrated stimulation of platelet adenylate cyclase and accumulation of cyclic AMP by PGD_2. The phosphodiesterase inhibitor RA233 potentiated both stimulation of cAMP accumulation by PGD_2 and its inhibitory effect on aggregation. PGD_2 was a more potent inhibitor than PGE_1, and PGD_2 did not inhibit platelet phosphodiesterase. There were differences in the response of platelets to PGD_2 and PGE_1, suggesting that there might be two prostaglandin receptors on the platelet surface that influence the same adenylate cyclase (186).

PROSTACYCLIN (PGI_2)

In October of 1976 Moncada et al. (18) described an enzyme in blood vessel microsomes that transformed PGG_2/PGH_2 into an unstable substance that relaxed certain blood vessels and inhibited platelet aggregation. When animal aortic microsomes were incubated with endoperoxides for 15–30 sec, the vasoconstrictor effects of PGG_2/PGH_2 disappeared. In their place was a transient substance with new biological properties whose appearance was not accompanied by MDA formation. At this time the material was arbitrarily referred to as PGX.

Addition of PGX to PRP 30–60 sec prior to induction of aggregation resulted in complete inhibition. The residue from an ether extract of the microsomal–endoperoxide incubate (1–2 min at 22°C) also inhibited arachidonic acid-induced aggregation in PRP.

PGX was 20–30 times more potent than PGE_1 and 5–10 times more potent than PGD_2 as an inhibitor of platelet aggregation (187). The anti-aggregation effect disappeared gradually after 20 min (22°C) or after boiling for 15 sec. Activity was stable at 4°C for 2 hr or in dry acetone at −20°C for several days. In some samples of PRP, aortic microsomes alone were inhibitory. This might be related to a comparable effect of aortic extracts previously reported by Heyns et al. (188).

It could be shown that PGX was not identical to any previously described product of 20:4 oxygenation or endoperoxide transformation. For example, formation of PGD_2 was ruled out because the isomerase catalyzing conversion of PGH_2 to PGD_2 is in the supernatant of the microsomal pellet and requires glutathione as a cofactor. Thus blood vessel microsomes transform endoperoxides to PGX while platelet microsomes convert PGG_2/PGH_2 to TXA_2, a deriva-

tive with opposing effects. Herein seemed to be the first explanation for the lack of platelet adhesion to intact endothelium in blood vessels (189, 190). Shortly thereafter PGX activity was also observed in human arterial and venous tissues obtained from fresh surgical specimens (19).

Microsomes prepared from rat stomach fundus also generated PGX from PGH_2, and this effect was blocked by the monoamine oxidase inhibitor, tranylcypromine, as well as 15-hydroperoxy arachidonic acid (15-HPAA)[7] (191). Platelet aggregation induced by ADP, PGG_2, PGH_2, collagen, epinephrine, and 5-HT was prevented by PGX. Furthermore, an aggregation response that had progressed to completion could also be reversed upon the addition of PGX (187). In some cases aortic microsomes alone were observed to induce aggregation in PRP (which was attributed to possible contamination with collagen), but this was reversed by subsequent addition of PGG_2/PGH_2. Reversal by PGG_2/PGH_2 was blocked by preincubation with 15-HPAA (187). When microsomal preparations also displayed anti-aggregatory activity, such activity was stable to boiling and was not blocked by 15-HPAA. This observation may be similar to those of Heyns and co-workers (188).

Initially, when arachidonic acid was added to aortic microsomes there was no conversion to PGX (18, 191), but later addition of 20:4 to arterial rings was seen to result in low transformation (0.5–1%), which was prevented by indomethacin or 15-HPAA (192). When PGG_2/PGH_2 were added to aortic microsomes or arterial rings, conversion to PGX was 80–90% complete and was blocked by 15-HPAA but not by indomethacin (192).

Concomitantly, while studying the role of prostaglandins in regulation of vascular tone, Needleman, Kulkarni, and Raz (193) and Raz and associates (20) noted that arachidonic acid induced a relaxation response when added to bovine or human coronary arterial strips. Furthermore, PGG_2/PGH_2 also caused relaxation of bovine coronary arterial strips. This paradoxical effect was correctly attributed to development of a labile metabolic product of arachidonic acid that was an intermediate between PGH_2 and 6-keto-$PGF_{1\alpha}$ and was a potent vasodilator (21). In these experiments bovine coronary strips could continuously generate the vasodilator from 20:4, and the authors proposed that this pathway in bovine coronary arteries could function independently of endoperoxides released from platelets (20). Therefore, Needleman's group was also studying PGX. Comparable experiments were later reported with prostacyclin by Dusting, Moncada, and Vane (194).

Structure and synthesis of PGI_2

A collaborative study between Johnson et al. of the Upjohn Company and Vane and colleagues at the Wellcome Research Laboratories resulted in elucidation of the structure of PGX (17). Using microsomal preparations from pig aorta, as well as ^{14}C-labeled and ^{3}H-labeled PGH_2, they generated unstable and acid-labile biological activity. The end product was identified as 6-keto-$PGF_{1\alpha}$ and the enol-ether intermediate in its formation from PGH_2 was 9-deoxy-6,9α-epoxy-Δ^5-$PGF_{1\alpha}$, i.e., PGX. During conversion of ^{3}H-labeled PGH_2 to PGX, one tritium was lost from the molecule, which was consistent with a cyclization between C_6 and C_9 (Figs. 2, 3). Thus the trivial name prostacyclin was proposed (17), and later the abbreviation PGI_2 was introduced. The original publication (17) is recommended to the reader as a superb example of biochemical research. It is of great interest that in 1971 Pace-Asciak and Wolfe (16) proposed what is now considered to be PGI_2 as a minor intermediate in the formation of 6(9)oxy-Δ^7-$PGF_{1\alpha}$ in rat stomach fundus. Pace-Asciak also isolated 6-keto-$PGF_{1\alpha}$ from rat stomach and confirmed its formation from PGG_2/PGH_2 (see reference 195 for a review of this work). The structures of prostacyclin and 6-keto-$PGF_{1\alpha}$ are depicted in Fig. 3.

PGI_2 and derivatives thereof have now been synthesized (196–198) and have been used for in vitro studies of its action. The parent compound is difficult to work with because it spontaneously hydrolyzes to 6-keto-$PGF_{1\alpha}$, especially in pH ranges below 7. Thus, many investigators verify the presence of PGI_2 by TLC radiochromatography of 6-keto-$PGF_{1\alpha}$ or by measuring biological activity of PGI_2.[8] However, synthesis of stable analogues of PGI_2 should prove valuable for more detailed studies of its biological effects (199).

PGI_2 and cyclic nucleotides

Ho et al. (200) showed that reversal of platelet aggregation by aortic microsomes (which were presumably synthesizing PGI_2) was due to an effect on platelet metabolism and not on TXA_2 directly. This was borne out when Gorman and colleagues (201) and Tateson and associates (61) reported that PGI_2 was the most potent inducer of cAMP accumulation

[7] This is in contrast to the hydroperoxy acid formed by the platelet lipoxygenase pathway (HPETE), which is a 12-hydroperoxy acid. In subsequent studies Salmon et al. showed that other hydroperoxy fatty acids can inhibit prostacyclin synthetase (1978. *Biochem. Biophys. Acta* **523:** 250–262).

[8] A radioimmunoassay for 6-keto-$PGF_{1\alpha}$ has recently been reported (1978. Salmon, J. A. *Prostaglandins* **15:** 383–397).

in platelets yet tested. When added to either PRP or platelet microsomes, prostacyclin was at least 10 times more potent than PGE_1 in raising platelet cAMP (201). An additional property of PGI_2-induced cAMP elevation was its persistence for almost 300 sec, which is in contrast to the effects of PGE_1 or PGD_2 (61, 201). In platelet microsomes PGI_2 was shown to stimulate adenylate cyclase activity directly (201). Similar results were reported by Best and colleagues (202), although the time course for elevation of cyclic AMP levels by PGI_2 was not as sustained as reported by others (201, 61). It is possible that the material measured by Best et al. (202) may have also contained PGD_2. Bressler et al. (107) showed that both PGI_2 and PGD_2 abolished the oxygen burst and aggregation response induced by collagen in washed platelet suspensions.

Lapetina et al. (121) found that PGI_2 (0.01–0.1 μg/ml) prevented thrombin-induced deacylation of 20:4-radiolabeled horse platelet phospholipids. The same effect was observed when platelet suspensions were preincubated with dibutyryl cAMP prior to thrombin addition. This was in agreement with the results of Minkes and associates (64). However, it is probable that inhibition of platelet aggregation by dibutyryl cAMP and PGI_2 occurs by other mechanisms in addition to inhibition of phospholipase A_2 activity.

Sites of production of PGI_2

Moncada et al. (203) studied prostacyclin formation in different layers of rabbit aorta. Intimal cells, internal elastic lamina, media, and adventitia were dissected with a scalpel blade and identified by light microscopy. Samples from each layer were evaluated for their ability to generate PGI_2. The assay system was PRP with 20:4 as the aggregating agent. Inhibition of aggregation was compared to a standard curve constructed with synthetic PGI_2 (197). Prostacyclin generation was highest in intimal cell suspensions, and activity decreased as layers toward the adventitial surface were examined. As reported in other cell types (146), prostacyclin activity was detected during preparation of the tissue, although it gradually declined. Cell suspensions from indomethacin-treated animals showed little or no basal PGI_2 production; but when they were incubated with PRP 1–10 min prior to the addition of 20:4, platelet aggregation was blocked. Incubation of indomethacin-treated intimal suspensions with PGH_2 for 3 min resulted in formation of prostacyclin. When intimal cell suspensions from indomethacin-treated animals were incubated with 15-HPAA, there was no inhibition of 20:4-induced aggregation and no conversion of PGH_2 to prostacyclin. These studies indicated that intimal cell suspensions could utilize endoperoxides released by platelets aggregating in response to stimulation by 20:4.

Results of these and other experiments indicated that the endothelial lining of blood vessels was capable of transforming endoperoxides released by platelets into prostacyclin.[9] In addition, intimal surfaces could convert arachidonic acid to PGI_2 (19, 192), and it is plausible that PGI_2 synthesis from free endogenous 20:4 in the endothelial cell can take place continuously (203).

Weksler, Marcus, and Jaffe (22) studied inhibition of platelet aggregation by cultured endothelial cells from human umbilical veins and bovine aorta. Intact endothelial cells inhibited platelet aggregation and release induced by a wide variety of aggregating agents. Preincubation of the cells with tranylcypromine (an inhibitor of PGI_2 synthetase) resulted in a normal aggregation response to 20:4. Supernates from disrupted endothelial cells required less preincubation with PRP for induction of inhibition than did intact cells. Microsomes from endothelial cells cultured with indomethacin were also inhibitory. When endothelial cells were incubated with 20:4, the supernates derived therefrom inhibited platelet aggregation, as did ether extracts of the arachidonic acid incubate. The inhibitory activity was lost after boiling for 30 sec or by acidification to pH 4 (22).

[^{3}H]Arachidonate was incubated with human or bovine endothelial cell microsomes (10 min), after which unlabeled PGI_2 standard (196) was added. Following ether extraction, a two-step radio-TLC system was employed for the separation and identification of PGI_2 and 6-keto-$PGF_{1\alpha}$. This was done in order to eliminate large quantities of uncoverted 20:4 and to separate prostacyclin from PGE_2, which is a major prostaglandin produced by endothelial cells. Prior to the second radio-TLC separation, it was necessary to prepare methyl esters of the products eluted from the first TLC plate. Some PGI_2 passed into the aqueous phase during ether extraction and could be recovered by slight acidification (pH 6.5) and rapid extraction with ethyl acetate. Thus it was demonstrated that endothelial cells, which completely blocked platelet aggregation, were synthesizing PGI_2.[10]

Significance of PGI_2 in occlusive vascular disease

Fig. 4 is a schematic outline of arachidonic acid oxygenation in platelets and endothelial cells. It

[9] The concept that endoperoxides can be transformed into PGI_2 by intact blood vessel surfaces has recently been challenged by Needleman and colleagues. (1978. *J. Clin. Invest.* **61:** 839–849).

[10] MacIntyre, Pearson, and Gordon have recently reported synthesis of PGI_2 by cultured porcine endothelial cells after exposure to stimulated platelets. In addition, these authors showed that platelet-free plasma could stimulate synthesis of PGI_2 as well. (1978. *Nature* **271:** 549–551).

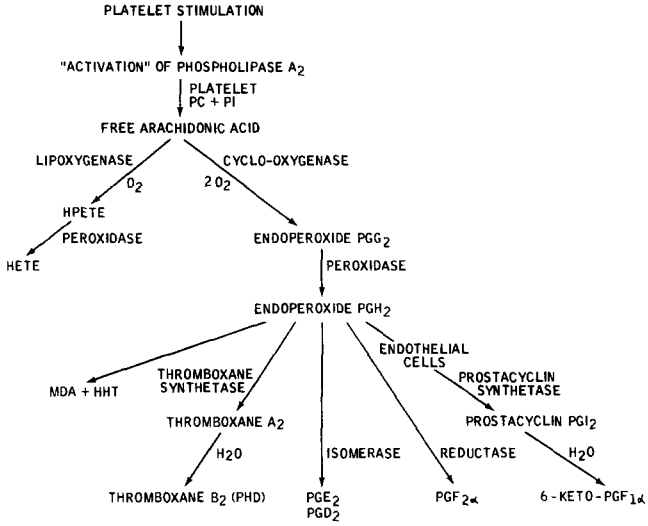

Fig. 4. The essential fatty acid, 20:4, plays an important role in the regulation of platelet and endothelial cell function. This figure depicts the step-wise transformation of arachidonic acid in platelets and endothelial cells. Platelet stimulation leads to hydrolysis of arachidonate from phospholipid by the putative phospholipase A_2. The subcellular location of the phospholipid donor of 20:4 in the platelet is not known. There is evidence for specificity toward 20:4 by the phospholipase in platelets (118) and in rabbit heart under certain conditions (231). One oxygen molecule is added to carbons 9 and 11, and a second molecule oxygenates carbon 15. Cyclization then occurs with formation of a 5-membered ring by carbons 8–12. The reaction is catalyzed by lipoxygenase and cyclo-oxygenase. Both enzymes are inhibited by ETYA and cyclo-oxygenase is inhibited by aspirin (acetylation) and indomethacin (98, 85, 104). All oxygenated and transformed products of the cyclo-oxygenase pathway must pass through the endoperoxide stage. It is now apparent that the end products are qualitatively and quantitatively different in each tissue and organ in which they are formed (162, 195). This phenomenon should be taken into consideration when the use of cyclo-oxygenase inhibitors is anticipated (206). The major end product in platelets is thromboxane B_2 (formed from the biologically active TXA_2), whereas PGE_2 and $PGF_{2\alpha}$ are mainly synthesized in the kidney (195). The specific determinant of end product synthesis from PGG_2/PGH_2 in a given tissue is not known but is of critical importance. The biological activity of endoperoxides themselves is also a major consideration. For example, Needleman et al. (162) contend that further conversion of PGG_2/PGH_2 in platelets to TXA_2 is not necessary for the achievement of an aggregation response, whereas Fitzpatrick and Gorman disagree (182). (The differences may be methodological.) Endothelial cells transform endoperoxides to PGI_2, which opposes the effects of TXA_2. The cells can utilize endoperoxides released from stimulated platelets or endogenous 20:4 for the synthesis of PGI_2 (203). The use of inhibitors for evaluating the biological activity of endoperoxides and their end products can be fraught with problems of specificity (232). Thus, formation of biologically active oxygenated products is now a well-established function of essential fatty acids. However, 1 mg of prostaglandin metabolites is excreted in the urine every 24 hr, whereas 10 g of essential fatty acids are ingested (221). What other biological functions will eventually be attributable to essential fatty acids? It will also be important to discern which transformations are enzymatic and which are spontaneous.

may well be superseded by a different sequence in the future. However, even in its present form some current concepts may require reappraisal. The metabolic pathway of arachidonic acid transformation in one tissue may serve an entirely different function than the same pathway in another tissue. Platelets convert PGG_2/PGH_2 mainly to thromboxane A_2 and B_2. Presumably TXA_2 is normally utilized for hemostasis and may be abnormally involved in thrombosis. Other tissues that mainly biosynthesize TXA_2 may use it for regulation of local perfusion and oxygen utilization (162). Blood vessels in different tissues may oxygenate 20:4 principally to PGI_2 for hemodynamic functions such as vasodilatation, and for physiological functions like repulsion of stimulated platelets (203–205). Therefore a cyclo-oxygenase inhibitor like aspirin might eliminate synthesis of a potentially useful product of 20:4 oxygenation, such as PGI_2 (162, 206).

The question of reducing PGI_2 synthesis with ASA has already arisen in connection with a recent report in which patients were given aspirin for prevention of postoperative embolism following hip surgery (206, 207). The patients were shown to benefit from aspirin administration, as evidenced by a reduction in thrombi in the calves and legs (207). On the basis of recent reports (145, 146), it may be presumed at this time that there is a difference in cyclo-oxygenase sensitivity between platelets and blood vessels. Thus, platelet cyclo-oxygenase is quite sensitive to ASA, whereas the enzyme in other tissues may require larger quantities of aspirin for inactivation. Furthermore, other tissues may be capable of resynthesizing cyclo-oxygenase, whereas platelets cannot synthesize protein, and cyclo-oxygenase inactivation is permanent. However, the issue should not be considered closed until we have more information on the quantitative aspects of aspirin inhibition of cyclo-oxygenase in other tissues, such as carotid and coronary arteries.

Another aspect of Fig. 4 is worthy of mention. This concerns synthesis of prostaglandins E_2 and $F_{2\alpha}$ by platelets. Hamberg and Samuelsson (67, 104–106) have already emphasized that classical prostaglandin synthesis in platelets is inconsequential, and that thromboxanes are the most important products of 20:4 oxygenation. Thus, reports of increased production of prostaglandin E-like material and increased metabolism of 20:4 to E-like substances in disease states, such as diabetes mellitus, should be interpreted accordingly (208). These findings may indeed represent an overall increase in sensitivity of the prostaglandin synthesizing pathway, but they do not provide information on production of TXA_2. As Mustard and Packham (209) have pointed out, an increase in platelet sensitivity may be secondary to another event that increases platelet turnover and results in a population of more responsive platelets. It can also be speculated that an increased propensity to 20:4 oxygenation represents a more generalized compensatory mech-

anism for increasing PGI_2 production in the vascular system. It may in fact be desirable for diabetic patients or patients with type IIa hyperbetalipoproteinemia (210) to synthesize PGI_2 in their blood vessels in maximal amounts. If this conjecture is true, then cyclo-oxygenase inhibitors might not be beneficial for these individuals.[11]

ROLE OF PLATELETS IN BLOOD COAGULATION

Background

The intrinsic and extrinsic pathways to fibrin formation are summarized in **Fig. 5**. Since coagulation research is a rapidly advancing discipline, the diagram should be regarded as tentative. Several scholarly reviews, each with a different emphasis, are currently available (211–213).

Knowledge of the surface contact and binding phase of coagulation has increased exponentially (214, 215). Hageman factor (factor XII) presumably binds to damaged or disrupted blood vessel surfaces in vivo, thus initiating the contact phase of coagulation. Prekallikrein and high molecular weight kininogen are proteins that serve to amplify the contact phase and render it more efficient. It can also be seen (Fig. 5) that, in addition to the intrinsic coagulation pathway, the contact phase initiates fibrinolysis and formation of vasoactive peptides such as bradykinin (214, 215).

The long-held view that the intrinsic and extrinsic systems were independent of each other may require revision. Radcliffe et al. (216) and others have demonstrated that factor VII, a component of the extrinsic system, can be activated by Hageman factor fragments. Finally, Miletich and colleagues (217, 218) have shown that the receptor site for activated factor X on the platelet surface is coagulation factor V, and that phospholipids are a relatively poor substitute for the platelet surface in this reaction.

Primary and secondary hemostasis require blood vessel and platelet surfaces for maximal effectiveness. In both mechanisms the platelet and fibrin mass must overcome the afferent pressure exerted by circulating blood. In quantitative or qualitative platelet disorders the bleeding time, which measures platelet plug formation, is prolonged. In disorders of fibrin formation the bleeding time is normal, but frequently rebleeding is noted at a later time due to lack of reinforcement of the platelet plug by fibrin strands (23–25). Thrombosis may represent a pathological aberration of primary and secondary hemostasis, wherein platelet plugs and fibrin masses form at undesirable and life-threatening locations in the vascular tree. This is the basis for anticoagulant therapy and research for pharmacologic agents that might inhibit platelet stimulation. Fig. 5 does not depict the important contribution of natural plasma protease inhibitors, such as $alpha_2$-macroglobulin and antithrombin III, which serve to regulate the hemostatic process. The physiology of these inhibitors has recently been reviewed (219).

Platelets and fibrin formation

The contribution of platelets to the coagulation process can be illustrated by a simple experiment (25). If platelets are removed from plasma by centrifugation prior to recalcification, the coagulation time is prolonged. The defect is correctable by the addition of platelets or certain phospholipids (25). When clot-promoting phospholipids were isolated from platelets (1, 12), rapid coagulation times could be achieved with their use; but when compared quantitatively to total lipid extracts or whole platelets, their in vitro biological activity was less than expected (1, 12). Additional insight into this apparent discrepancy was obtained when isolated platelet membranes were studied in in vitro coagulation systems (27). Whereas 500 μg of extracted platelet membrane phospholipid gave an 11-sec clotting time, only 22.5 μg were required when membrane vesicles were substituted for the extracted phospholipids (27). This was interpreted to mean that the term platelet factor 3 should be reserved for the clot-promoting activity of the platelet lipoprotein surface, and that it was not synonymous with the term "platelet phospholipid" (15, 27, 220). Such an interpretation was also in agreement with the generally accepted view that lipids are not present in the free state in biological systems but are combined with protein and/or carbohydrate (221).

The important studies of Walsh (36) have indicated that primary and secondary hemostasis are strongly linked via a pathway involving factors XII, XI, and ADP, as well as via another pathway that includes factor XI and collagen but not factor XII. Walsh has also proposed that at least two reactions occur on the stimulated platelet surface that protect activated coagulation factors such as X_a from inactivation (36).

Receptor site for the factor X_a on platelets

New and exciting information demonstrating that more than platelet phospholipid is required in the intrinsic prothrombin activation step has been provided by Miletich and associates (217, 218). ^{125}I-labeled factor X_a was incubated with washed platelets in the presence of purified prothrombin and calcium. A small quantity of thrombin formed, followed by the

[11] Recent research suggests that PGI_2 may be a circulating hormone. See Dollery and Hensby. 1978. *Nature* **273:** 706.

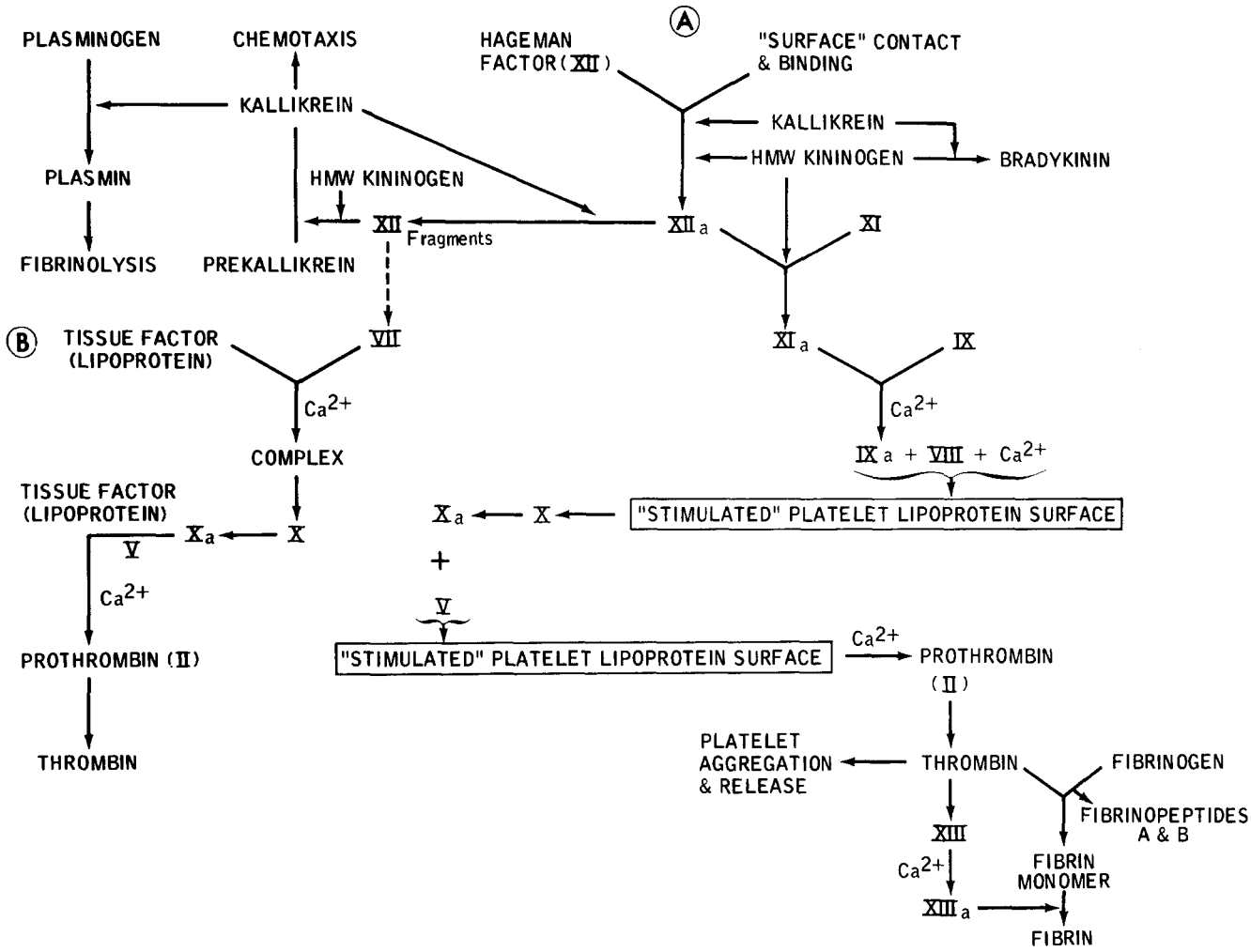

Fig. 5. Outline of the intrinsic (*A*) and extrinsic (*B*) pathways of fibrin formation. These interactions, which represent 'secondary hemostasis,' occur simultaneously with development of the hemostatic platelet plug, i.e., primary hemostasis. The subendothelial blood vessel surface exposed by vascular damage or severance serves as a nidus for platelet adhesion and stimulation. Factor XII (Hageman factor) also binds to the subendothelium and in so doing is converted from its precursor (zymogen) form to an activated molecule (XII_a). This interaction by itself (known as the contact phase) is relatively prolonged and inefficient (211). Amplification and enhancement occur by virtue of participation of prekallikrein and high molecular weight kininogen (214, 215). The contact phase is also involved in initiation of fibrinolysis, kinin generation, and chemotaxis (233). Subsequent stages of intrinsic and extrinsic coagulation may be conceptualized as a biphasic catalytic system (223) in which activated zymogens such as IX_a form a complex with factor VIII (211). In the presence of calcium the complex catalyzes activation of factor X on the platelet lipoprotein surface. The X_a receptor site on the platelet surface is known to be coagulation factor V (217, 218) (not phospholipid). Tissue factor lipoprotein (*B*) probably functions in a manner analogous to the stimulated platelet membrane. During activation of prothrombin by the X_a–V surface complex, prothrombin is bound via a calcium-mediated interaction. Three pairs of adjacent γ-carboxyglutamic acid residues are present on the prothrombin molecule, and each pair binds a calcium ion (211, 212). In the absence of vitamin K an abnormal prothrombin molecule is synthesized containing glutamic but not gamma-carboxyglutamic acid. Thus, calcium-mediated binding of prothrombin to the X_a–V–platelet lipoprotein complex is defective (these reactions have not as yet been studied with platelets or platelet membranes, and the last statement is an assumption). Thrombin, a two-chain serine protease, cleaves arginine–glycine bonds of fibrinogen. One chain of thrombin (the B chain) closely resembles the serine proteases produced in the pancreas. Thrombin in concentrations that do not interact with fibrinogen induces platelet aggregation and release. The stimulatory effect of the platelet surface may not be confined to coagulation proteins per se. It has been shown that tissue factor-type activity generated by leukocytes in the presence of endotoxin is enhanced by platelet membranes (234).

release reaction and binding of X_a to the platelet surface. The binding was specific, saturable, and reversible and resulted in a 50,000-fold increase in the activity of factor X_a. This was in sharp contrast to a 50-fold increase in X_a activity when phospholipid was substituted for platelets in the incubation mixture. The receptor for X_a on the platelet surface was shown to be coagulation factor V, which was previously known to be associated with platelets (217, 218).

When platelets from two patients with congenital

factor V deficiency were studied, there were no detectable X_a receptor sites in one patient and the other had a markedly reduced number of sites, although the affinity of the sites for X_a was normal. Furthermore, when an antibody to factor V was added to the incubation system prior to the addition of ^{125}I-labeled X_a, binding and thrombin formation did not occur (218).

Phospholipids in in vitro coagulation studies

The recent review of Suttie and Jackson (212) provides a critical summary of this research. Appropriately charged, well-characterized phospholipid micelles can provide a clot-promoting surface which approximates that of the platelet membrane. Useful and significant information on the role of phospholipids in blood coagulation has been obtained by this technique (10, 212). There do not appear to be major differences in the clotting activity of phospholipids isolated from platelets and those derived from other tissues. Studies of the interactions of purified coagulation proteins and platelet membrane vesicles obtained by the nitrogen decompression technique of Broekman et al. (30) might represent a fruitful approach for future research.

Papahadjopoulos and Hanahan (222) were among the first investigators to study binding of coagulation proteins to phospholipid vesicles. They showed that binding of X_a and prothrombin to phospholipid required calcium, and also showed that the presence of a net negative charge on the phosphatide under study was more important than the structure of the phospholipid itself. Binding of calcium by prothrombin is now known to be related to the presence of gamma-carboxyglutamic acid in the prothrombin molecule (212). Barton (223) suggested that protein–protein interactions taking place during blood coagulation occur at the phospholipid–water interface. The phospholipid serves to orient the protein at the lipid–water interface (heterogeneous catalysis), thus catalyzing the reaction. Steps in the sequence involve diffusion of reactants to the surface, adsorption, surface reaction, desorption, and diffusion of the products from the surface (223). Since the orientation of phospholipids in the platelet membrane is now known (129), studies of the clot-promoting properties of liposomes of similar composition have been reported (224). The most active coagulant phospholipid, phosphatidylserine, is located on the inner surface of the platelet membrane. This finding has two possible interpretations: *a*) it represents a safety mechanism to protect the circulation from possible hypercoagulability and thrombosis (224); or *b*) the arrangement of platelet membrane phospholipids has little importance for platelet function since other cell membranes are similarly organized. I incline toward the latter and doubt that platelet membrane lipids per se play a role in the coagulation process. These lipids are probably not in direct contact with the external milieu of the platelet in vitro or in vivo. In my opinion, platelet phospholipids play only an indirect structural role in coagulation. The specificity and reactivity of platelets in clotting reflect the architecture of the lipoproteins on the surface following stimulation by agents such as collagen, thrombin, and ADP. Details of this architecture are not yet known.

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