

## HISTORICAL SKETCH

# The early discoveries of collagen–platelet interaction and studies on its role in hemostatic plug formation

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In the recently published book on *Platelets in Thrombotic and Non-Thrombotic Disorders* it is stated in Chapter 11 [1] that ‘collagen–platelet interaction is fundamental to normal hemostasis and pathological thrombotic incidents’. Nice, then, to recall that more than 40 years ago we contributed a small stone to the foundation of a large construction which has proved to be very complicated.

It so happened that Helge Stormorken, who had been my classmate in the medical studies, contacted me, and asked if I was interested in starting a scientific project. He was a veterinarian before he started his medical studies, and had recently been appointed as professor in physiology at the Veterinary College of Norway. He was already an established scientist from the Owren group at Rikshospitalet where he later returned as head of the institute. He made great efforts in building up research activities in different fields at his Department of Physiology, and followed my work with great interest. I left my job in internal medicine at Rikshospitalet and began the studies on the circulation and the hemostatic process in rabbit mesenterium in 1960. Hugues [2,3] had in 1959 published studies on hemostatic plug formation in rabbit mesenterium. Stormorken and Professor Peter Hjort had become interested in these studies and after a visit to the Liege group, they wanted to pursue a similar approach.

The Department of Physiology was then located in an old building and the experimental conditions were not as good as they are today, but we started up and created a similar setup to what they had in Liege. The exteriorized mesenterium in chloralose-urethan anesthetized rabbits were continuously irrigated with Zweifach’s solution kept at 38 °C, and the vessels were observed with a binocular microscope with a camera attachment. When cutting the vessels with a razor blade, the rapid adhesion of platelets to the vessel lips could be observed, followed by formation of the hemostatic plug which became impermeable within 3–4 min. The plug formation was studied under various conditions, but, unfortunately, these

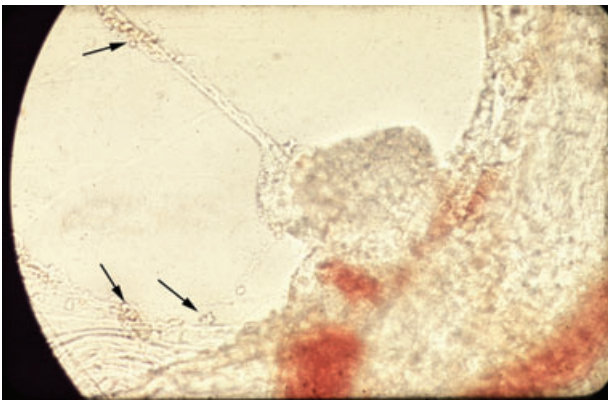
results were not published. Figure 1 shows a light microscopic picture of a hemostatic plug from these *in vivo* studies. As seen in the picture, we were able to observe platelet-like structures adhering to strands of mesenteric tissue as observed by the Liege group [2].

At that time the term viscous metamorphosis was much used about clots, hemostatic plugs and thrombi [3,4]. This term was defined as aggregation and fusion of platelets with loss of their individual outline, resulting in a structureless confluence. The term is not very precise, and in order to analyze the composition of the plug in more detail, we decided to carry out electron microscopic studies. A Siemens electron microscope was just installed at the Department of Pathology in Rikshospitalet, where Dr A. Kjaerheim and S. Lystad taught me the use of the instrument and participated with great interest in the first study.

The platelet plugs were fixated *in situ* while still bleeding or after hemostasis had occurred, by dripping 2% buffered osmium tetroxide directly on the bleeding site. At that time we were not familiar with the use of glutaraldehyde as the primary fixative. Stormorken provided equipment and technical help. All the preparatory work and the sectioning of the embedded material was performed at the Department of Physiology.

As demonstrated in Fig. 2A,B, a hemostatic plug, 5 min after vessel transection, is composed of platelets with varying degrees of morphological change, but with a preserved membrane surrounding each platelet and with a distance between neighboring platelets of about 20–40 nm. Thus, as seen from our ultrastructural studies, the term viscous metamorphosis did not seem justified. The platelets close to collagen fibrils of the vessel wall (Fig. 2B) were swollen and had more or less completely lost their internal structure, but  $\alpha$ -granules and mitochondria could occasionally be seen. In this region there were breaks in the surrounding platelet membrane. In the central regions of the plug numerous pseudopods were seen, increasing the contact area between the platelets. The platelet organelles were partly present in this part of the plug. At the periphery both rather well-preserved and empty platelets were observed. Fibrin was present at the plug surface, but usually in small amounts, probably due to the continuous superfusion of the mesenteric tissue. Leukocytes (Fig. 2A) were only rarely seen in association with the plug. Already 45 s after transection, the ultrastructural picture of the

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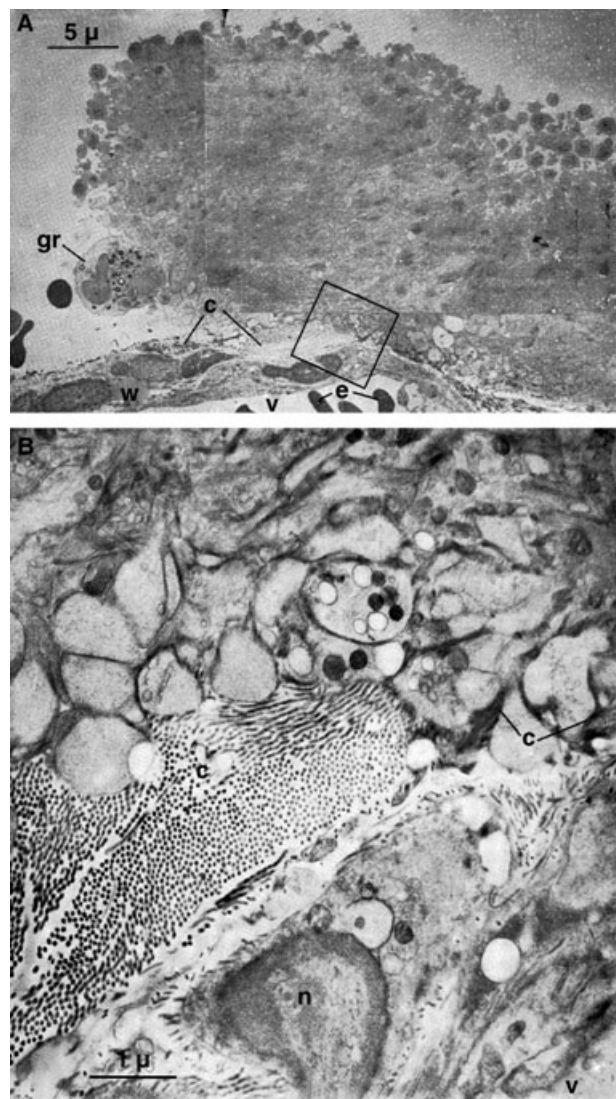
**Fig. 1.** *In vivo* photograph of a hemostatic platelet plug in rabbit mesenterium, 5 min after vessel transection. Note the tiny platelet structures adhering to tissue strands (arrows).

plug was similar to that described above, but the platelet changes were less pronounced.

These findings were first presented at a congress in Prague in 1961, and published in 1962 [5]. At that time we could not afford to go by plane from Oslo to Prague, so we had to use the train. The only complication with train travel was that during our stay in Prague, the Berlin wall had been built, which made a difficult barrier on our way home.

As can be seen in Fig. 2A, platelets were closely attached to collagen fibrils of the vessel wall and, as far as we knew, ultrastructural observations of this phenomenon had not previously been described. Of course, these findings stimulated the interest in evaluating more closely the interaction between collagen and platelets. As connective tissue contains other substances than collagen, such as cells, proteoglycans and other proteins, we wanted to explore platelet adhesion to single collagen fibrils [6,7]. At that time we knew the ultrastructure of collagen type I and III with the characteristic cross-striated fibrils. Rabbit tendons contain both collagen I and III, mostly type I. We therefore made a preparation from rabbit tendon tissue by disintegrating the tissue in 0.9% NaCl in a blender, further shaking it in a shaking machine for 15 min and then centrifuging for 15 min at  $1800 \times g$  at room temperature. The supernatant was clear and faintly yellow, and because it seemed so clear I named it 'extract'. By using negative staining technique and electron microscopic analysis, however, we found collagen fibrils and subfilaments (Fig. 3A). The fibrils showed characteristic cross-striation with a periodicity of 67–70 nm. Thus, we had a preparation suitable for the aggregation studies. At that time we performed the aggregation studies by shaking the tube with rabbit citrated platelet-rich plasma (PRP) after adding the agonist, and the aggregation was observed both visually and by phase contrast microscopy.

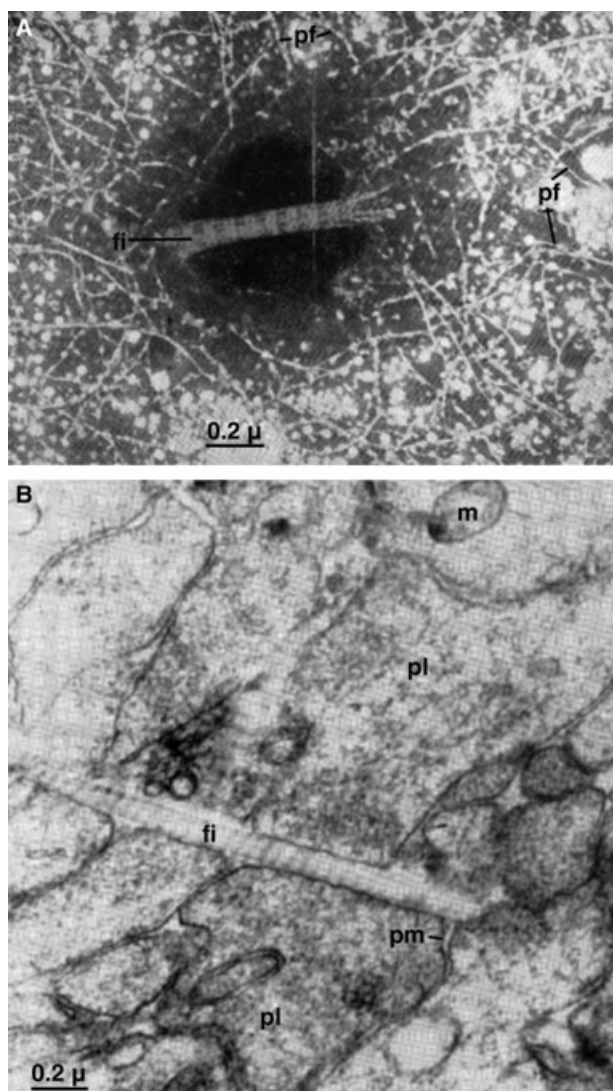
With the standard 'extract' preparation platelet aggregation was induced after about 60 s at room temperature. The aggregation time was influenced by final 'extract' concentration and the optimal temperature for aggregation was 37 °C. When the 'extract' was centrifuged at  $10\,000 \times g$  for 30 min, the supernatant showed no aggregating activity. When tendon



**Fig. 2.** (A) Low-power transmission electron micrograph of a platelet plug 5 min after vessel transection. The vessel wall (w) with collagen (c) and vessel lumen (v) with erythrocytes (e) at lower part of the picture. A neutrophil granulocyte (gr) at the plug periphery ([5]). (B) Detail revealing close contact between the platelets and collagen fibrils of the vessel wall (c) with loss of platelet internal structure, but preserved surrounding membrane. Nucleus (n), vessel lumen (v) ([5]).

tissue was extracted with 0.05% acetic acid and the extract dialyzed against 0.9% saline, precipitation occurred, and the precipitates induced platelet aggregation when added to citrate-PRP. When the tissue 'extract', containing 1 mm calcium chloride, was incubated with collagenase for 1 h at 37 °C, the aggregating activity was completely lost. We measured the hydroxyproline content of the 'extract' and concluded that 0.15–0.5 μg to 0.5 mL citrated rabbit PRP was sufficient to induce platelet aggregation.

Thus, we were confident that the aggregating activity was associated with the collagen fibrils themselves. By the use of transmission electron microscopy we observed adhesion of platelets to such fibrils (Fig. 3B) and loss of platelet organelles



**Fig. 3.** (A) Electron micrograph of a collagen fibril (fi) and subfilaments (pf) as shown with negative staining technique ([10]). (B) Platelets (pl) adhering to a single collagen fibril (fi). Platelet membrane (pm), mitochondrion (m) ([10]).

occurred. Just before these observations were published, however, Zucker and Borrelli [8] reported their observation of the platelet clumping effect of connective tissue suspensions and collagen, but they did not report ultrastructural studies.

The next question was obvious: what was the mechanism behind the aggregating effect of collagen? One night I suddenly woke up and the answer seemed clear to me. It might be ADP released from the platelets themselves! Their ultrastructure was altered with loss of internal structure after contact with collagen and this would mean possible loss of aggregating substances. The findings of Hellem [9] and Gaarder *et al.* [10] from the Owren group, that the platelet aggregating factor R from red blood cells was identified as ADP, was certainly at work in my mind during sleep. In the morning I told my wife (also MD) about the dream, and it was certainly not the last time we spoke about it.

We did not hesitate to start the experiments for testing the idea. After inducing platelet aggregation with addition of the collagen 'extract' to citrated PRP, the aggregates were centrifuged for 3 min at  $1800 \times g$ . The supernatant (supernatant A) was then added to citrate-PRP at room temperature, and after about 15 s aggregation occurred. Thus, a new principle was established: the platelets themselves were the source of a platelet-aggregating substance, released by contact with collagen [11].

The next step now was to identify the platelet aggregating principle. The aggregation was temperature dependent with optimum at  $37^\circ\text{C}$  and the activity was reduced by storage. After centrifugation of supernatant A for 20 min at  $100\,000 \times g$ , the aggregating activity was still present in the new supernatant and thus seemed to be a small molecular substance. When adenosine monophosphate (AMP) was added to citrated PRP before the supernatant A, aggregation was markedly inhibited. Thus, it seemed reasonable to assume that the aggregating principle might be ADP. At this time Holm Holmsen, who still was a student in biochemistry, had joined the staff in the department. He became interested in the platelet work, and did not hesitate in helping to explore the possibility that ADP might be the aggregating substance. I think this was the beginning of his outstanding career in platelet work. By separation of supernatant A and platelet-poor plasma (PPP) containing  $200\ \mu\text{g}$  of  $\text{ADP mL}^{-1}$  by Sephadex filtration, we could show that the maximal aggregating effect of the eluate from supernatant A appeared just at the step of elution which contained ADP. Other experiments also indicated that ADP might be the active principle, but this had still to be proved. Holmsen composed a micromethod based on the ability of the firefly enzyme luciferase to emit strong light on contact with ATP. Since ADP can specifically and quantitatively be converted to ATP by a pyruvate kinase system, a sensitive and reproducible assay was possible. Using this system we could confirm that the aggregating principle released by collagen-platelet interaction really was ADP! [11].

When relating these findings to our observations of the close contact between platelets and collagen fibrils in the platelet plug, we realized that the findings could be essential for the understanding of the hemostatic process, but we were not really aware of the relevance also for the mechanisms of thrombosis.

The next step was to explore the effects of calcium and magnesium on the platelet aggregation and ADP release to collagen. These studies were inspired and followed by the late Assistant Professor Karl Halse, who was the biochemist in the department and an expert on calcium metabolism. While EDTA has been widely used as a chelating agent in hematological research, little attention has been paid to another complexing agent, ethyleneglycol diaminoethyl tetraacetate (EGTA). In contrast to EDTA, EGTA forms a much more stable complex with calcium than with magnesium. We therefore tried to make use of this difference in the analysis of the effects of calcium and magnesium on the collagen-platelet interaction. Calcium was estimated according to a micromodification by Halse (K. Halse, personal communication) of the

method described by Ringbom *et al.* [12]. The studies were carried out with both PRP and suspensions of washed platelets, and ADP, collagen and thrombin were used as agonists. I can not go into the details of these rather complicated studies here, but refer to the original article [13].

Briefly, I will mention that  $Mg^{2+}$  can liberate considerable amounts of  $Ca^{2+}$  in a calcium-EDTA solution, whereas the exchange reaction is negligible in the presence of EGTA. When comparing the effects of collagen and ADP on heparinized or non-heparinized EDTA-PRP and EGTA-PRP, we found that aggregation was inhibited in both when the chelators were present in excess, and the aggregating ability was restored after recalcification. Magnesium could replace calcium in EDTA-PRP, but not in EGTA-PRP unless a surplus of calcium was present. Dialyzed or cation exchanged plasma contained calcium concentrations of about 0.15–0.25 mM. When washed platelets were resuspended in such plasma, aggregation could not be produced with collagen or ADP unless the calcium concentration was increased or magnesium was added. The platelet adhesion to collagen seemed reduced, but was still present both in EDTA-PRP and EGTA-PRP as demonstrated by electron microscopy, but aggregates were not observed. Collagen did not induce release of ADP in EDTA-PRP or EGTA-PRP.

The conclusion from the above-mentioned studies was that ionized calcium is a necessary factor for collagen- and ADP-induced aggregation of platelets and for the collagen release of platelet ADP. Magnesium can not replace calcium unless small amounts of calcium are present. The chelators did not prevent platelet adhesion to collagen fibrils.

At this point, with great gratitude to Stormorken and staff, I left my work at the Veterinary College of Norway and Rikshospitalet in Oslo and moved to the University of Toronto, where I was invited by J. F. Mustard to spend a year in 1964–1965. Again I experienced a situation where part of the work was carried out in a Department of Physiological Sciences in a Veterinary College, but this time in Guelph, where the veterinarian, Professor Harry C. Rowsell, was an excellent organizer of studies on dogs and pigs. The other part of the work was carried out at the Blood and Vascular Diseases Research Unit in Toronto. In addition to Mustard and Dr M. A. Packham, Dr W. J. Dodds participated in the work with her expert knowledge on dogs with congenital coagulation defects. Dr Leif Jørgensen from Oslo arrived in Toronto during my stay there, and also took part. The *in vitro* studies on collagen-platelet reaction and hemostatic plug formation were expanded in Toronto, but here I will mention only two of the reports. I must point out that the papers were not finished before I left Toronto, and this in part explains the delay in publication of the results.

In a series of experiments we studied the hemostatic process in normal dogs and in dogs with coagulation defects in factor (F)VII, factor (F)IX, and factor (F)VIII [14]. Furthermore, we wanted to explore the effects of heparin and dicumarol, and a pyrazole compound, possibly interfering with the collagen-platelet interaction [15]. We also discussed treating dogs with

acetylsalicylic acid, which unfortunately we decided not to do. Later, Mustard's group published studies on the effect of acetylsalicylic acid on platelet function [16]. Our set-up was essentially similar to that described above for the rabbit experiments [5], although it was more complicated to work with dogs. The formation of the hemostatic plugs was observed by direct microscopy *in vivo*, and the plugs with the surrounding tissue were removed at intervals between 10 and 30 min following vessel transection and prepared for light and electron microscopy.

Platelet plugs were formed in all cases, and in the normal and FVII-deficient dogs the bleeding time was normal. When the central stream of blood was nearly arrested, one or more new side channels were formed before the bleeding stopped at about 3 min after transection. In the FIX-deficient dogs the bleeding time was not clearly prolonged, but later rebleedings occurred. In association with the rebleedings, new platelets were attached to the plug, which often became larger than normal. Fragments of the plug were often observed to break off. The bleeding time in the FVIII-deficient dogs was clearly prolonged (mean > 7 min) and in a few cases the bleeding did not stop during the observation period (20–30 min).

The administration of dicumarol (reducing the FVII level to 2% or lower) caused only a slight prolongation of the bleeding in FIX-deficient dogs. When heparin was given to FVII-deficient dogs, hemostasis was impaired with a mean bleeding time of about 11 min. Whereas no effect of phenylbutazone was found on normal dog hemostasis, the drug prolonged the bleeding in FIX-deficient dogs.

The ultrastructural appearance of the plugs in the normal dogs was similar to that previously described for rabbits [5]. The plugs of the FVII-deficient dogs showed a similar picture. In the FIX-deficient dogs the platelets were as closely packed to collagen as in normal dogs. There were, however, channels containing red blood cells and a few leukocytes, but little or no fibrin. A similar picture was found in the FVIII-deficient dogs, where the plugs contained even less fibrin, and at the surface many loosely packed platelets could be seen. Ultrastructural examination of the plugs from FVII-deficient dogs given heparin did not show evidence of fibrin. Platelets were in close contact with collagen, where, as usual, they showed pronounced morphological changes. Similar findings were made in the phenylbutazone-treated FIX-deficient dogs, where the plugs contained many channels and regions with rather well-preserved platelets.

Using the standard collagen suspensions, we were not able to demonstrate platelet aggregation in citrated PRP from the dogs when using the standard turbidimetric technique. By winding collagen fragments around the stirbar, however, aggregation was produced in the usual way. When comparing the aggregation reaction induced by collagen, we did not find any difference between normal dogs and those with coagulation defects.

From these studies we concluded that in the presence of a marked congenital coagulation defect in the intrinsic pathway, platelets accumulated at the end of a transected vessel in a normal manner in the initial stages. The close contact between

collagen of the vessel wall and the platelets was present in all cases and it seemed that the initial platelet interaction with the vessel wall was independent of coagulation. This was also true for situations in which both the extrinsic and intrinsic pathways of coagulation were defective. But the intrinsic pathway of coagulation was found to be important for the formation of a stable non-permeable hemostatic plug.

We also carried out some morphological studies on the transformation of hemostatic plugs after 20 h in normal and FIX-deficient dogs [17]. During this period of time, the normal plug underwent marked structural changes with shrunken platelets showing loss of internal structure and partly disintegration, leaving open spaces. The meshwork of fibrin was extensive throughout the plugs, especially in areas close to the vessel wall and at the surface. The plugs from FIX-deficient dogs showed similar platelet changes in some regions, but there were still open channels in the plugs with rather well-preserved platelets near the channels. The amount of fibrin had increased in these plugs, but the increase was less than in normals, leaving areas with loosely packed platelet remnants and little or no fibrin. We concluded that adequate formation of fibrin in the hemostatic plugs with time is essential for permanent hemostasis. In hemophiliacs with loosening of the platelet packing by time and insufficient fibrin formation in the plugs, new channels are formed and rebleeding occurs.

As mentioned, we did not test the effects of acetylsalicylic acid in these experiments. Such studies would have been interesting in demonstrating the combined effects of the drug and congenital coagulation defects on hemostasis. A few years later several reports on this topic were published as reviewed by Weiss [18]. I had no such data in my pocket when returning to Oslo in 1965, but I tried to get clinical studies started with the use of aspirin in coronary disease. I was not able to convince my colleagues about the value of such a study, however, and failed to get it through.

Back in Oslo, Stormorken and I started a study on bleeding time wounds from patients with von Willebrand's disease compared with wounds from normal individuals [19]. At that time the mechanisms of the disease were not clear. Jorgensen and Borchgrevink [20] had published a light microscopic study on patients where biopsy of a bleeding time wound was taken in one patient after 6 min of bleeding and in two after 45 min. The authors found no hemostatic plugs in any of the biopsies, and they concluded that the initial adhesion to the endothelial cells was disturbed, possibly due to lack of ADP.

In our study we did light and electron microscopic examination of wounds from three normals and eight von Willebrand patients. Furthermore, three patients were studied before and after transfusion with PPP. The bleeding time (Ivy method) was normal for the controls, but more than 30 min for all patients. Biopsies of the wounds were taken 4 and 15 min after the cut had been made, and prepared for morphological studies. In the 4-min biopsies there was a striking difference between normals and the patients, as virtually no plugs were seen in the patient material. Instead, the ultrastructural examination revealed naked vessel ends that were curved and

markedly contracted. Small platelet clumps were occasionally observed attached to collagen fibrils. After 15 min of bleeding, however, light microscopy revealed large platelet masses in the gap of the wound and at the cut surface. Ultrastructurally, some of the vessels, especially at the ends of arterioles, appeared sealed by plugs, but with less densely packed platelets than normals. At the periphery platelets were swollen and strands of fibrin were present. Plasma transfusions in the patients mostly restored the plug formation, but some vessels were still naked.

We concluded from these patient studies that the hemostatic defect was not related to disturbed vessel contraction which had been suggested [21]. Whereas the platelet-aggregating stimulus at the wound surface, where platelets accumulated and aggregated, was obviously adequate, this was not the case at the naked transected vessel ends. The attachment to collagen and subsequent aggregation, thus, was related to the speed at which the blood stream passed. The normalizing effects of plasma transfusions confirmed previous observations that the functional platelet defect was related to lack of a plasma factor.

It is so strange to write about these findings now, about 40 years later, when so much new knowledge has accumulated about platelets and coagulation, and when related scientific disciplines such as immunology and molecular biology have been developed and opened up so many new aspects. The findings were exciting then, but seem elementary today. However, there still seems to be some disagreement about the complex processes of collagen–platelet interaction, and there is obviously more work to be done. I am grateful that I had the opportunity to participate in this research in its early days.

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