HISTORICAL SKETCH

Platelet membrane glycoproteins: historical perspectives

A. T. NURDEN,* D. R. PHILLIPS† and J. N. GEORGE‡

*Institut Fédératif de Recherche 4, CHU Bordeaux, Pessac, France; †Portola Pharmaceuticals Inc., South San Francisco, CA, USA; and ‡Hematology-Oncology Section, Department of Medicine, The University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA

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The function of blood platelets to prevent blood loss at injured sites in the vasculature depends largely on their capacity to adhere, aggregate, promote coagulation and secrete factors that induce vasoconstriction and recruit inflammatory cells. Produced in large numbers from megakaryocytes, platelets enter the circulation for 7–10 days primed to fulfill their hemostatic function. By 1970, thoughts were becoming well formulated concerning the involvement of platelets not only in hemostasis, but also in the pathogenesis of arterial thrombosis. By this timeframe, pioneering studies had also established many of the basic concepts of platelet biology. Physiologic agonists of platelet aggregation such as ADP, collagen and thrombin had already been identified. Protein cofactors including von Willebrand factor (VWF) and fibrinogen were known to be involved in platelet aggregation [1]. However, in the background of these basic studies, the elegant morphological characterizations of platelets by investigators such as Jim White and Dorothea Zucker-Franklin were teaching us that that the two primary activities of platelets in thrombosis and hemostasis, that is adhesion and aggregation, were dependent upon as yet undescribed elements on the platelet membrane surface. It was also clear that this surface had procoagulant activity. While important studies had been initiated to study platelet membrane proteins, for example, the work in Graham Jamieson's laboratory to characterize peptide fragments of membrane proteins released by proteases [2–5], and the contributions of Ralph Nachman's laboratory on platelet membrane protein content [6,7], further progress in our understanding of thrombosis and hemostasis required defining the protein content of the platelet membrane surface and identifying specific proteins that were involved in platelet function.

What follows is a story of collaborative research that began 30 years ago and has lasted across our careers. The ability to label platelet surface glycoproteins with radioisotopes and to solubilize them in sodium dodecyl sulfate (SDS) and separate them by polyacrylamide gel electrophoresis (PAGE) was an important advance that provided the basis for each of our projects. Using these techniques, each of us began with different perspectives and different goals. Our ideas all came together in 1975 at the meeting of the International Society for Thrombosis and Haemostasis (ISTH) in Paris. By that time we appreciated the complexity of the platelet membrane glycoproteins and understood that they had important roles in platelet function, documented by their abnormalities in the congenital disorders, Glanzmann thrombasthenia and Bernard–Soulier syndrome. Many of the other early investigators in this field are to be found in Fig. 1, a group photograph taken at a meeting held in San Antonio, Texas in 1983 to develop our book, *Platelet Membrane Glycoproteins* (Fig. 2) [8]. But to tell the story of how the three of us arrived at this point requires that we begin again, each of us on our own.

Correspondence: James N. George, The University of Oklahoma Health Sciences Center, Hematology-Oncology Section, PO Box 26901, Oklahoma City, OK 73190, USA.
Tel.: +1 405 271 4222; fax: +1 405 271 6444; e-mail: james-george@ouhsc.edu

Alan T. Nurden

My story began in 1968 as an assistant at the Sir William Dunn School of Pathology in the University of Oxford. The then head of Pathology, Dr John French, presented me with a copy of a recently published paper by Olav Behnke, an electron microscopist, who had used histochemical techniques to locate a carbohydrate-rich coat on the surface of platelets [9]. John French, himself a well-known electron microscopist, asked my research director, Dr Eric Adams, and myself to identify the components of this coat as they were surely 'the key components of platelet aggregation and adhesion'. Our initial attempts in Oxford were to isolate the components liberated from platelets by proteases such as papain and trypsin, according to the methods of Jamieson's group (2,3) and to compare them with those from endothelial cells stripped from the surface of bovine aortae. Dissecting the latter at 7:00 AM on a winter's day in the Oxford abbatoir proved my love for research. I rapidly learned how to analyze sugars by gas-liquid chromatography but in vain, as the unexpected and untimely death of John French meant that I was to be quickly left with the task of finding a new laboratory.

With the benefit of the imagination of youth, I wrote a project to compare the surface structure of platelets of a range of mammals. Thanks to a grant from the British Heart Foundation, I was able to perform this in the Nuffield Institute of Comparative Medicine in London, in the laboratory of

Platelet membrane glycoprotein symposium
October, 1983 San Antonio, Texas

Fig. 1. Investigators at the San Antonio meeting to develop the book [8] shown on Fig. 2. *Back row* (left to right): Avner Rotman, Nils Olav Solum, Kent Gartner, Alan Nurden, Tom Kunicki, Rod McEver *Middle row*: David Phillips, Sharron Pfueller, Inger Hagen, Jim George, Ken Clemetson, Larry Leung, Deane Mosher, Shirley Levine. *Front row*: Ralph Albrecht, Joel Bennett, Barry Coller, Ernst Lüscher, Isaac Cohen, Joan Fox.

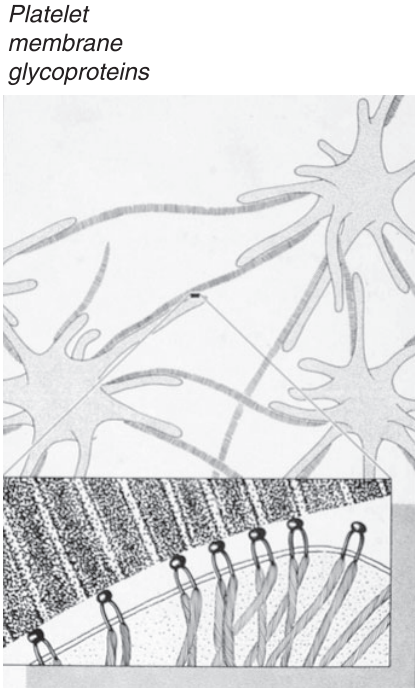

Fig. 2. The book that was written by the investigators shown in Fig. 1. (Reproduced with permission of Plenum Press, New York).

Dr Christine Hawkey. Platelets of all species aggregate, and the object was to identify those surface components common to the platelets of all mammals. At around this time, procedures were developed allowing the separation of proteins by PAGE. Recalling the publication of Behnke [9], I used histochemical procedures to identify glycopeptides released from the surface of platelets by trypsin and separated by PAGE and I presented my early data at the ISTH Congress in Oslo in 1971. Alcian blue proved most effective and differences in the migration of large acidic glycopeptides (subsequently shown to come from GPIb) were apparent when the patterns for human platelets were compared with those of platelets of different species of primates. This work formed the basis of my first publication and was accepted as a letter to *Nature* [10]. After adding SDS-PAGE to these procedures, I was able over a period of 2 years to examine the glycoprotein composition of as wide a range of mammalian species as the London Zoo could offer [11]. Glycoproteins were indeed common to the surface of platelets of all mammals.

Soon after that I encountered Professor Jacques Caen, who was already established as a leading authority on inherited disorders of platelet function. It seemed important to both of us to examine the membrane glycoproteins of platelets from patients with Glanzmann thrombasthenia where aggregation was lacking [12]. And so in 1973, I found myself flying into Paris one Sunday evening to begin a 3 month stay. The first patient duly came and the SDS-PAGE gels looked different when colored for carbohydrate. The first band was clearly present, but the two others? After the difference was seen for the third unrelated patient, I was convinced and our description of an altered membrane glycoprotein composition of platelets in Glanzmann thrombasthenia was published early in 1974 (Fig. 3) [13]. Soon, I was again in Paris, this time to study the giant platelets of patients with the Bernard–Soulier syndrome, where a platelet adhesion defect was suspected. This time, it

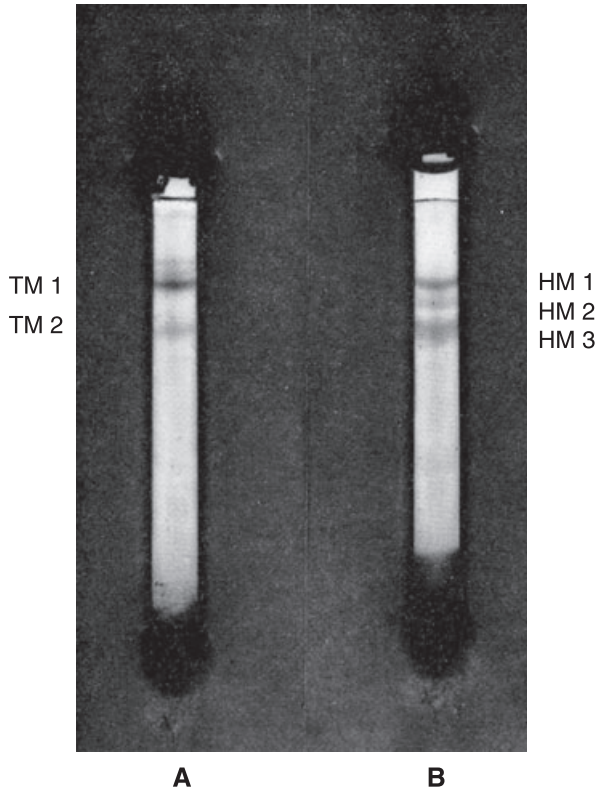

Fig. 3. The original description of an altered membrane glycoprotein composition in Glanzmann thrombasthenia platelets. Shown are the results of polyacrylamide gel electrophoresis in the presence of SDS of (A) solubilized thrombasthenic platelet membranes (TM) and (B) normal human platelet membranes (HM). Glycoprotein was located by staining of the tube gels for carbohydrate by the periodate-Schiff reaction. (Reproduced from *Br J Haematol* 1974; 28: 253–260 with permission.).

was the first of the colored bands that was missing. Not only this, but trypsin failed to release the acidic glycopeptides as located by alcian blue. A 'GPI' defect was hypothesized and our paper in *Nature* in 1975 highlighted the specific roles of platelet membrane glycoproteins in platelet function (Fig. 4) [14].

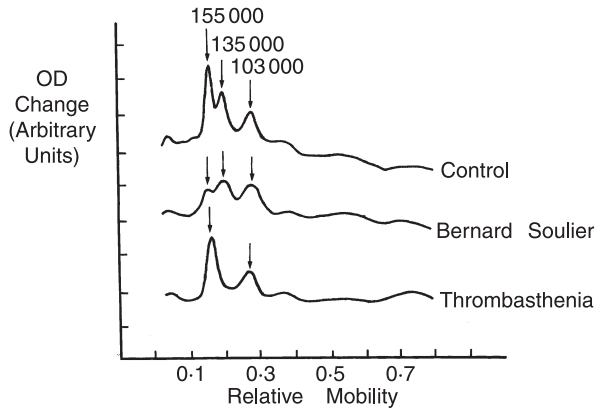

Fig. 4. Polyacrylamide gel electrophoresis in the presence of SDS showing the distribution of glycoprotein in the membranes obtained from control human platelets (A); Bernard-Soulier platelets (B), and thrombasthenic platelets (C). Carbohydrate staining was followed by densitometric scanning. (Adapted from *Nature* 1975; 255: 720–722 with permission.).

I subsequently obtained a permanent position in Paris and my work and friendship with Jacques Caen has continued ever since. Our characterization of the membrane glycoprotein deficiencies in Glanzmann thrombasthenia and Bernard–Soulier syndrome continued and was marked by two major collaborations that I would like to acknowledge. The first was the arrival in Paris for a sabbatical year of Inger Hagen from Oslo. Inger used crossed immunoelectrophoresis (CIE) to confirm that the glycoprotein deficiencies in Glanzmann thrombasthenia and Bernard–Soulier syndrome were indeed distinct (Fig. 5) [15]. The next to arrive was Thomas Kunicki from Milwaukee. Tom was able to show that GPIIb and GPIIIa were present as a non-covalently bound, Ca^{2+}-dependent complex [16]. Without perhaps knowing it, we were on the integrin trail. My close friendship and collaboration with Tom has continued and strengthened as the years have gone by. It was during this period that a series of young collaborators emerged in my group, including Dominique Pidard, Jean-Philippe Rosa, Nelly Kieffer, and Martine Jandrot-Perrus, who all contributed in a major way to the work that was to emerge.

David R. Phillips

In early 1969, I began a postdoctoral fellowship with Dr Martin Morrison at St Jude Hospital in Memphis, TN. Marty was an expert in peroxidases and, based on work by Gladys Bayse in his lab [17], correctly deduced that lactoperoxidase-catalyzed iodination of tyrosine, under certain conditions, occurred *via* an enzyme-substrate complex. My job was to determine whether this reaction would enable specific labeling of membrane-surface proteins. At this time, descriptions of cell surface protein 'signatures' was in its infancy, and it was readily apparent that the development of such a method would enable this to be achieved on most cell types that could be prepared in a viable, intact and homogeneous fashion. As conditions were already established for the iodination of proteins by lactoperoxidase, it was readily apparent that we had two technical difficulties to overcome. First, we had to prove the directionality of membrane labeling. My initial efforts, lasting almost a year, were frustrating. Dean Danner in Marty's lab had experience in studying reactions in oxidative phosphorylation, so I attempted initially to establish the method using rat liver mitochondria. However, the data were ambiguous, as my tracking of enzyme markers showed that I was unsuccessful in isolating inner mitochondrial membranes free of outer mitochondrial membranes – and *vice versa*. Accordingly, I could not demonstrate that the proteins labeled were, in fact, exposed on the outer surface of this organelle. When I switched to erythrocytes, however, the data set was complete within weeks. Hemoglobin was not labeled on intact cells; and, the proteins on the inner aspect of the membrane (primarily spectrin, previously shown by Ted Steck to be resistant to proteases on intact cells [18] were only labeled following cell lysis. So, with proper selection of cell type, we were quickly able not only to establish a new method to determine membrane protein

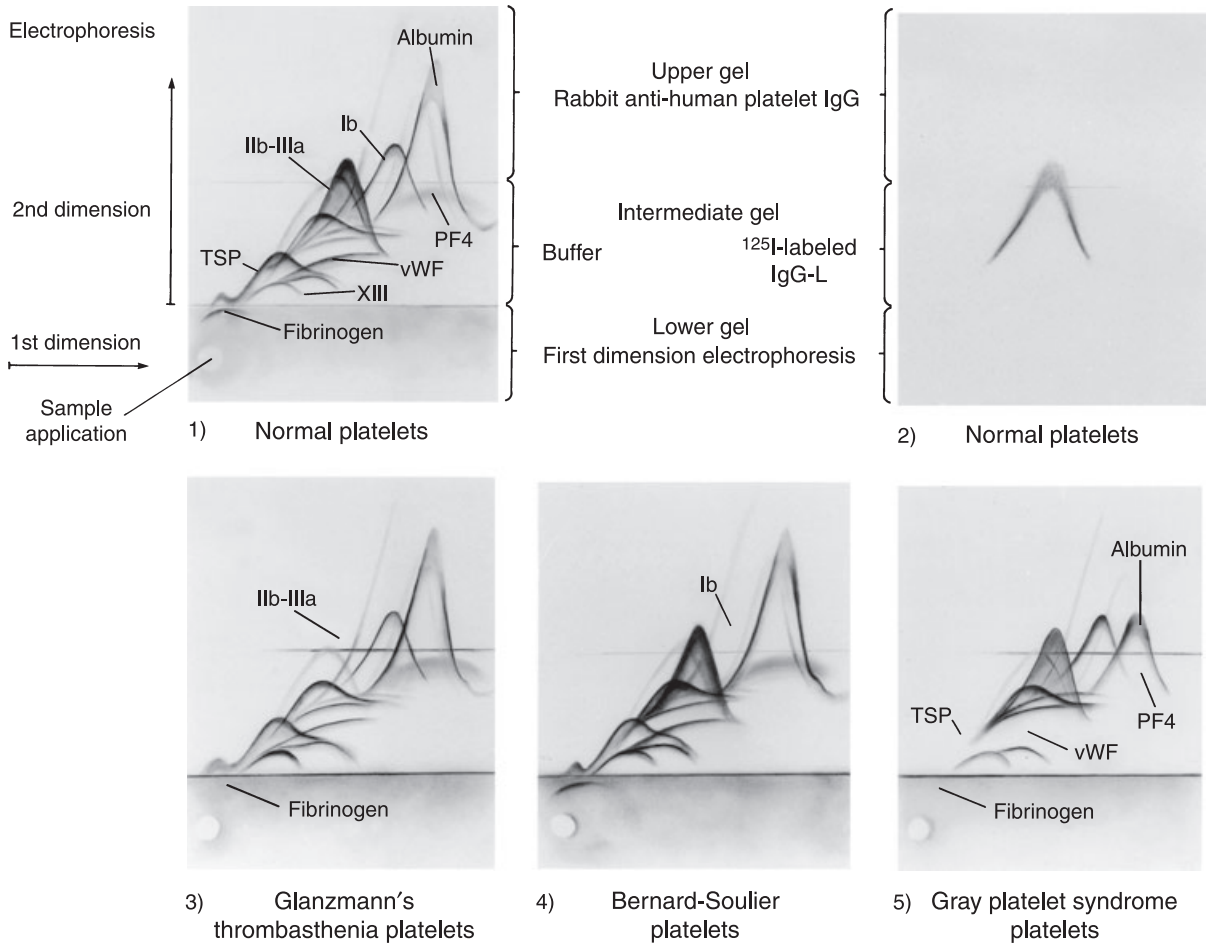

Fig. 5. Analysis of platelet proteins and antiplatelet antibodies by two-dimensional crossed immunoelectrophoresis. The figure is a drawing made from original material to allow a clearer, more consistent presentation of typical analyses performed in our laboratories. Platelet proteins were separated by first-dimension electrophoresis in the lower gel sections (left to right). An intermediate gel section contained only buffer, except in Plate 2, where a trace of ^{125}I-labeled IgG-L (the alloantibody to glycoprotein IIb-IIIa isolated from a patient with Glanzmann's thrombasthenia who had received multiple transfusions) was added. The upper gel section in each plate contained precipitating amounts of rabbit IgG isolated from antiserum to whole human platelets. Second-dimension electrophoresis moved the separated platelet proteins into the antibody-containing gel sections (bottom to top). After electrophoresis, the plates were stained for protein with Coomassie blue, and in Plate 2, ^{125}I activity was then detected by autoradiography. Normal platelets were used in Plates 1 and 2, and platelets from patients were used in Plates 3 through 5 as indicated. The major immunoprecipitation arcs are identified in Plate 1, and the missing or abnormal arcs are noted in Plates 3 through 5. The radiolabeled arc in Plate 2 represents glycoprotein IIb-IIIa. TSP denotes thrombospondin, PF4 platelet factor 4, and von Willebrand factor (VWF). (Reproduced with permission from *The New Eng J Med*, 1984; 311:1084–1098).

sidedness but also to identify the disposition of several of the membrane proteins known to exist in the erythrocyte plasma membrane [19].

When Marty asked me to continue on at St Jude, two events converged that compelled me to begin work on platelets. First, while Marty was a joy to work with, the youthful desire for independence dictated that I find another membrane to work on. Second, Jim White had developed a provocative slide show using morphology to document a plethora of cell surface reactions on platelets. I heard his talk at a chance attendance at a meeting of the New York Academy of Science and was therefore introduced to a wide spectrum of cell surface unknowns on the platelet. This was a cell surface-labeler's dream: a membrane surface rich in biology but with a protein composition that was largely unexplored. I recall using one of the old-style glass slides for my appointment seminar at St Jude to indicate that once the cell surface proteins on platelets were identified, one could go on to specifically identify those involved in aggregation, adhesion and in the formation of thrombin. Little did I know how ambitious such a statement was as 35 years later it is still possible for a young investigator to initiate a career with the same objectives!

I, like many investigators new to platelets, had a somewhat trying time getting a washed platelet preparation that I was comfortable with. This was much different than working with erythrocytes. Remarkably, the lactoperoxidase-catalyzed iodination technique worked beautifully on platelets. I believe the only change I had to make was to reduce the amount of hydrogen peroxide added, as higher concentrations almost always caused me to lose my preparation of platelets. It was immediately obvious from the initial labeling experiments that two glycoproteins, GPII and GPIII, were the primary proteins

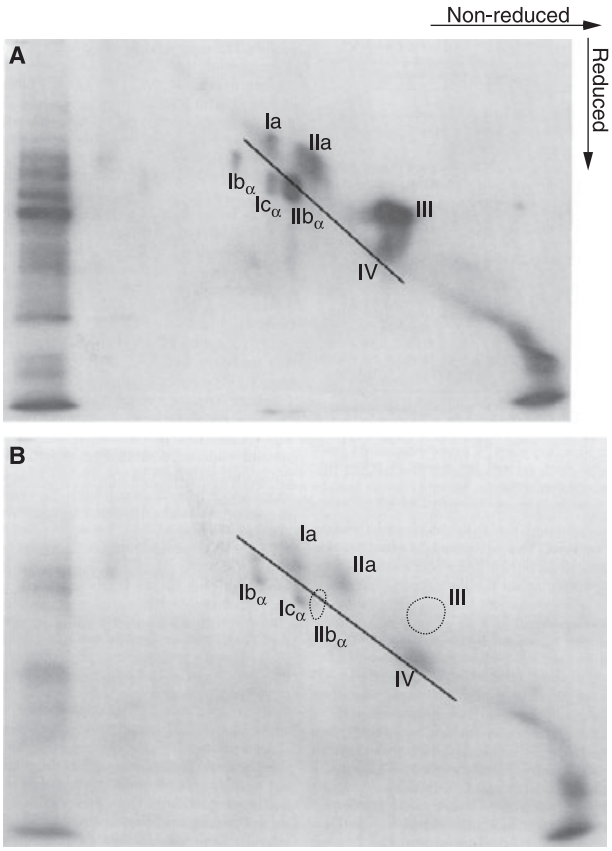

Fig. 6. Separation of iodinated polypeptides of normal (A) and thrombasthenic (B) platelets by non-reduced reduced two-dimensional electrophoresis. Thrombasthenic and normal platelets were washed, labeled by lactoperoxidase-catalyzed iodination, solubilized anaerobically in SDS, and subjected to two-dimensional analysis. The samples were first electrophoresed without reduction (disulfide bonds intact). Polypeptides resolved by this separation were then reduced with 10% β-mercaptoethanol to cleave disulfide bonds and electrophoresed in the second dimension. The figures are autoradiograms of the dried gels. In B, the dotted circles indicate the positions of IIb$_\alpha$ and III in gels of normal samples. Slight labeling by IIb$_\alpha$ and III could be seen if the autoradiograms were overexposed. The gel to the left of each diagonal is a one-dimensional separation of the reduced sample. (Reproduced with permission from *The Journal of Clinical Investigation*, 1977; 60:535–545).

labeled and I somehow felt that this should be the focus of subsequent work [20]. The nomenclature, with the addition of subsequent a's, b's and c's to accommodate new glycoproteins with similar molecular weights, continues to this day, with the exception of the insertion of the integrin nomenclature by biologists but not cardiologists. Figure 6 illustrates this point using 2-dimensional gels to fractionate iodinated proteins from control and thrombasthenic platelets [21,22]. In this example, carried out in collaboration with Patty Agin, we could (i) identify disulfide-linked platelet surface proteins, and (ii) bring clarity to the affected glycoproteins in Glanzman's thrombasthenia, which sometimes was not apparent when stained, 1-dimensional tube gels were examined [13].

My first presentation on platelets was at the ISTH meeting in Washington, DC in 1973. I recall being a lonely voice, but became familiar with other investigators studying platelet biochemistry, including Professor Ernst Lüscher, Director of the Theodor Kocher Institute in Berne, Switzerland. I made a mental note, and on my next trip to Europe, visited him in Berne and arranged to spend a year in his lab. This was time well spent as I not only acquired valuable techniques which we use to this day in my lab, but also had the joy of working with Ernst.

James N. George

Like David Phillips and other investigators who have devoted their careers to the study of platelets, I began my training with studies on red cells. My fellowship project at the University of Rochester in 1968 with Bob Weed and Claude Reed was to measure red cell adhesiveness [23]. When I moved to my first faculty position in San Antonio in 1970, it seemed appropriate to exploit my methodology in a new, independent direction, to study mechanisms of platelet adhesion. For studies of cell adhesion, platelets seemed more appropriate than red cells, as adhesion is one of their principal functions. Also, platelets were much less studied than red cells at that time, so opportunities for new observations were greater.

To study platelet adhesion required an understanding of platelet surface proteins. At that time Graham Jamieson's group had described a technique to isolate platelet plasma membranes [4]. My goal was to combine Jamieson's technique with a novel radioisotope label for surface proteins that had been synthesized and characterized for studies on red cells by my colleague in San Antonio, David Sears. This compound, diazotized diiodosulfanilic acid (DDISA), could be iodinated with ^{125}I and would react covalently and selectively with proteins on the cell surface, being unable to penetrate the plasma membrane [24]. My goal was to show that the ^{125}I-DDISA-labeled platelet surface proteins participated in platelet contact interactions. For this story, two things happened in 1973. I attended the first Gordon Conference on Hemostasis and I was awed by the power of the new technique of SDS-PAGE to characterize membrane proteins. But this knowledge could not have helped me without the arrival in my laboratory of a freshmen medical student, Ray Potterf, who had just completed his PhD in biochemistry at Baylor College of Medicine in Houston. Ray had experience with SDS-PAGE and was eager to apply his experience to platelet membrane proteins. This is when I became familiar with David Phillips' publication in 1972, in which he had identified the three major platelet membrane glycoproteins and designated them GPI, GPII, and GPIII, in order of decreasing molecular size [20]. Although our results were similar to David's data, the ^{125}I-DDISA label had different properties from David's ^{125}I-lactoperoxidase labeling technique. His label reacted predominantly with GP III [20], whereas ^{125}I-DDISA reacted equally with both GP III and GP II, as well as having some reactivity with GP I [25]. We also noted other differences from David's data that were puzzling to me. Ray Potterf was an imaginative student who wanted to try different concentrations of acrylamide, both with and without disulfide reducing agents,

to look for different qualities of resolution. These experiments revealed multiple different periodic acid-Schiff (PAS)-positive bands that reacted with ^{125}I-DDISA. My goal had been to reproduce David's work; these results were different; different results may be exciting but they are also discomforting. When we submitted our first papers on platelet membrane glycoproteins in April, 1975, we designated our first novel observation of a new glycoprotein in the GP II region as GP IIa. These data, along with data on the loss of membrane glycoproteins from circulating rabbit platelets [26], were the material for our poster at the Paris 1975 ISTH meeting.

In the summer of 1975, I began a sabbatical year with Ernst Lüscher. My first 2 months overlapped with the last 2 months of David Phillips' sabbatical year. Both of us attended the ISTH meeting in Paris that July. There I met Alan Nurden for the first time. That summer, the common interests of the three of us became apparent and a lasting friendship and continuing collaboration began.

My year with Professor Lüscher was a valuable immersion into the techniques for studying platelets. Studies on platelets were still new; techniques for isolation and washing of platelets were complex; artefacts were rampant. No group knew these pitfalls and could overcome them better than the Lüscher laboratory. When I returned from Switzerland, I was prepared to do the definitive studies to document the relationships among the platelet membranes glycoproteins. We developed techniques to quantify the intensity of the PAS reaction and to localize and quantify the ^{125}I-DDISA labeling of each PAS-positive band on the gels [27]. Using these techniques with different SDS-PAGE concentrations, we identified many different platelet membrane glycoprotein, using the designations of a, b, and c to expand David's original nomenclature [27].

Concluding remarks

Following our first acquaintance in Paris in 1975, we had frequent visits and discussions. Out of these came the idea to collaborate on a project that could summarize the work that we had carried out, each from our individual perspective, and also summarize the new observations on platelet structure and function. Our idea was to do a book – perhaps the misguided ambition of all young investigators. The incentive for this idea was to bring together our colleagues and friends, not only to contribute to this book but also to gather in San Antonio and enjoy the ambience of South Texas. This was a wonderful experience, and not only for the science. Many years later, Nils Olav Solum (Fig. 1) still reminisced about throwing horseshoes, of all things, in San Antonio. The book was published (Fig. 2) [8], and also we worked together to document this area of investigation for a larger audience in *The New England Journal of Medicine* [28].

The 1970s saw the emergence of a platelet membrane glycoprotein 'community' that persists to this day. It has been fascinating to both witness and participate in the unfolding stories concerning the cloning and characterization of glycoprotein complexes in platelet membranes, the interaction of cognate ligands for individual glycoproteins that affect platelet function, the signaling reactions of the various membrane glycoproteins, and the development of drugs to platelet membrane glycoproteins that impact platelet-directed pathologies.

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