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Reflections

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A Brief Historical Review of the Waterfall/Cascade of Blood Coagulation

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This article explores some of the events and people involved in unraveling the basic mechanisms leading to the clotting of blood. It also brings to focus the important role that my teachers and colleagues had on my career in research. When I entered college majoring in chemistry I had little idea that I would have an opportunity to become a professor of biochemistry at a major university. This was clearly the result of the excellent advice and encouragement that I received, particularly from my teachers early in my career.

As a senior at the University of Washington, I took a course in biochemistry to complete my credit requirements for a B.S. in chemistry. The biochemistry course was taught by Donald Hanahan, an excellent lipid biochemist who understood the importance of a quantitative measurement. I was fascinated by the biochemistry class that Hanahan taught, as well as by a course dealing with the chemistry of natural products. When Hanahan invited me to work in his laboratory on a senior project, I got my first real experience of what it was like to do laboratory research and I enjoyed it. In the fall of 1950, I entered graduate school at the University of Washington and decided to do my thesis research with Hans Neurath to learn something about protein structure and function. Neurath was an excellent teacher, a distinguished protein chemist, and a leader in his field (Fig. 1). He also had high standards and was very demanding of his students. Neurath was originally trained as a colloid chemist at the University of Vienna. Following his postdoctoral training at the University of London in the chemistry department headed by Frederick Donnan, he immigrated to the United States in 1935, where his research interests shifted to protein structure. He then held positions at the University of Minnesota, Cornell University, and Duke University, and in 1950, he joined the University of Washington School of Medicine as chairman of a newly established Department of Biochemistry. His research interest then focused almost entirely on proteases, particularly pancreatic trypsin, chymotrypsin, and carboxypeptidase, because these proteins were available in sizable amounts and could be prepared in high purity. Much of his career then dealt with their structure and function, including their active sites, substrate specificity, their kinetics, amino acid sequence, interaction with inhibitors, and their mechanism of activation. Over the years, Neurath also became well known for his leadership in the publication of scientific literature, having founded and served as Editor-in-Chief of *Biochemistry* for 30 years. In 1990, he founded another new journal, *Protein Science*, as well as editing several excellent volumes, such as *The Proteins* initially with Kenneth Bailey and later with Robert Hill.

For my Ph.D. thesis research, Neurath suggested that I should compare trypsinogen and trypsin to gain some insight as to the mechanism of zymogen activation. Little difference between the two proteins was anticipated because Cunningham and other postdoctoral fellows in Neurath's laboratory had shown that trypsinogen and trypsin had essentially identical molecular weights of 23,800 as measured in the ultracentrifuge (1). In my initial studies, I was unable to detect any difference between the two proteins at their carboxyl-terminal end. This suggested that no peptides or amino acids were removed or peptide bonds cleaved at the

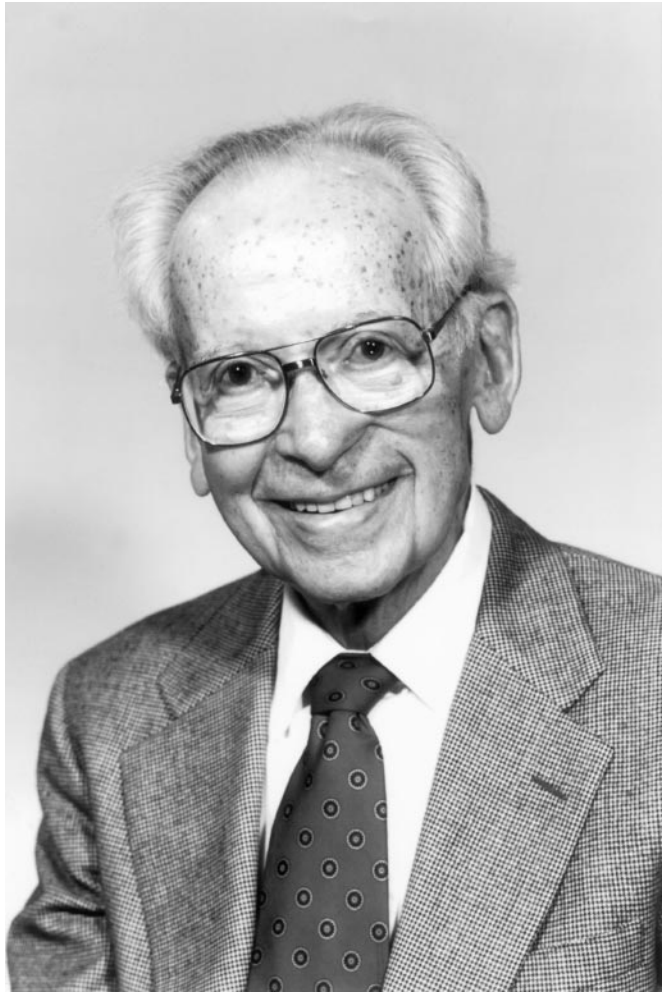

FIG. 1. **Professor Hans Neurath (1909–2002).**

carboxyl end of trypsinogen during its conversion to trypsin. However, in France Rovery and co-workers (2) found that trypsinogen contained an amino-terminal valine, whereas trypsin contained an amino-terminal isoleucine. Shortly thereafter, I isolated an acidic peptide (Val-$(Asp)_4$-Lys) that was generated by limited proteolysis during the activation of trypsinogen (3). Most importantly, the appearance of this peptide correlated exactly with the formation of the enzymatic activity of trypsin. This finding was consistent with the loss of a small amino-terminal valyl peptide from trypsinogen as well as a slight increase in the isoionic point of the protein during its conversion to trypsin (9.3–10.01). As a graduate student, I was very excited by the fact that we were the first to make this observation. Little did we realize at the time that "limited proteolysis" would be a common mechanism seen over and over again in biological systems. This mechanism included the activation of other proteases such as those participating in blood coagulation (4), fibrinolysis (5), and complement activation (6). Furthermore, limited proteolysis is now known to occur in a wide range of biological reactions such as the processing of prohormones (7), the activation of cells via their protease-activated receptors (PARs) (8), the cleavage of signal peptides from proteins destined for secretion (9, 10), and the cleavage of ubiquitin from proteins on their way to endosomes or proteosomes (11). Additional examples are the removal of small or large fragments from proteins such as fibrinogen prior to polymerization (12) and the removal of small propeptides that are signals for protein modification such as carboxylation (13). Also, regulated intramembrane proteolysis (RIP) plays an important role in determining the level of cholesterol in membranes via the sterol regulatory element-binding proteins (SREBPs) (14). Other examples include signaling receptors such as Notch, a single transmembrane protein that is activated by proteolytic cleavage at three specific sites generating an intracellular domain of the protein (15). Notch is then translocated to the nucleus where it binds to transcription factors that play a role in devel-

FIG. 2. **Dr. Bert L. Vallee, Edgar M. Bronfman Distinguished Senior Professor (1919–).**

opment. Thus, proteases have many functions in addition to the digestion of proteins in the gut.

Being in the Neurath laboratory gave me an excellent chance to interact with his other graduate students and postdoctoral fellows. Individuals such as Leon Cunningham gave me considerable guidance during those early years. I also had the chance to meet and visit with many outstanding visitors and collaborators of Hans Neurath. Visitors such as Fred Sanger presented the amino acid sequence of the two chains of insulin, a milestone in amino acid sequence analysis (16).

Another distinguished visitor was Bert Vallee from Boston (Fig. 2). His visit to Seattle led to the discovery of zinc in carboxypeptidase (17). Vallee received his early education at the University of Berne and then immigrated to the United States in 1938 where he entered New York University School of Medicine. In 1946, he moved to Boston for his studies at MIT and Harvard. Vallee developed a highly sensitive flame and spark emission spectrometer for the detection of trace metals in proteins, and this technique led to the development of the field of metalloproteins in his laboratory. Following the discovery of carboxypeptidase A as a zinc metalloenzyme, he then identified dozens of other zinc-containing enzymes employing atomic absorption spectroscopy (18). These enzymes participate in lipid, protein, carbohydrate, and nucleic acid metabolism as well as gene transcription, cell division, and development. Zinc plays a structural as well as a catalytic or cocatalytic role in these proteins. When zinc is associated with DNA-binding proteins, it is bound to three distinct motifs referred to as zinc fingers, twists, and clusters (19). More than 300 metalloenzymes have now been identified and include metallothionein, a small protein that Vallee found in horse kidney (20). This small protein has a unique structure, amino acid composition, and metal binding characteristics. Vallee made many other important contributions such as the discovery and role of angiogenin in blood vessel formation. These visitors made the Neurath laboratory a very stimulating place for a graduate student.

When I received my Ph.D. in 1954, Neurath recommended Fritz Lipmann's laboratory in Boston for postdoctoral study (see "Hitler's Gift and the Era of Biosynthesis" by E. P. Kennedy (21)). Lipmann's laboratory was another very exciting place to work because Mary Ellen Jones and Leonard Spector were close to identifying carbamoyl phosphate as an intermediate in the formation of citrulline (22), while Helmuth Hilz and Phil Robbins were characterizing "active sulfate" (3′-phosphoadenosyl 5′-phosphosulfate) (23, 24), an intermediate employed in the biosynthesis of tyrosine sulfate in proteins and other molecules such as chondroitin sulfate. Lipmann suggested that I should try to isolate one of the amino acid carboxyl-activating enzymes that were thought to be involved in protein biosynthesis (25). Hopefully a pure activating enzyme might provide an approach to finding the natural acceptor for this family of enzymes, something equivalent to the role that CoA played in the activation of acetate. At the time, the biological acceptor tRNA was not known. These studies were carried out with Victor Koningsberg, a postdoctoral fellow from The Netherlands. We employed beef pancreas as an enzyme source because this tissue was very active in protein biosynthesis. These studies led to the isolation and characterization of a tryptophanyl carboxyl-activating enzyme that catalyzed the exchange of pyrophosphate into ATP in the presence of tryptophan and the formation of tryptophan hydroxymate when hydroxylamine was added to the reaction mixture (26).

After two very enjoyable years in Lipmann's laboratory, I learned about a faculty position at Western Reserve University in Cleveland, Ohio in Harland Wood's Department of Biochemistry. Wood had built a first rate department with an outstanding reputation for its research in intermediary metabolism. The biochemistry faculty included distinguished scientists such as Mert Utter, Robert Greenberg, Warwick Sakami, and many others. Wood had talked to Lipmann about this position, and Lipmann had suggested me for the job. At the time, academic positions were rarely advertised as is presently done.

Harland Wood was a very talented and productive scientist who was a giant in the field of intermediary metabolism (Fig. 3). He received his undergraduate training in chemistry and bacteriology at MacAlaster College in Minnesota. In 1931, he became a Ph.D. student in microbiology in the laboratory of Charles Werkman at Iowa State University. As a graduate student, he made the monumental discovery that carbon dioxide fixation occurred in heterotrophic bacteria (27). At the time, CO_2 was thought to be an end product for all cells except for photosynthetic autotrophs. The fixation of CO_2 was discovered long before its role was established in other important reactions such as a building block for purines, amino acids, and fatty acids. The reaction in which pyruvate and CO_2 would generate oxalacetate in bacteria soon became known as the "Wood-Werkman reaction." In collaboration with Alfred Nier and co-workers at the University of Minnesota, Wood was able to show that $^{13}CO_2$ was incorporated into succinate in bacteria (28). This was in complete agreement with the proposal that CO_2 and pyruvate would generate oxalacetate followed by reduction to succinate.

In 1946 Harland Wood moved to Western Reserve University where he was appointed chairman of the Department of Biochemistry. In Cleveland, he continued his studies with ^{13}C to study metabolic pathways employing a mass spectrometer that he built from scratch (29). He then turned much of his efforts to enzyme isolation and characterization. One of his favorite enzymes was a biotin-containing transcarboxylase from *Propionibacterium shermani* (29). This very large protein (M_r ~1,200,000) forms propionyl-CoA and oxalacetate from methylmalonyl-CoA and pyruvate. It consists of a hexameric central subunit attached to 12 biotinyl subunits and 6 dimeric outer subunits. Wood referred to this enzyme as the "Mickey Mouse" enzyme because of its appearance in the electron microscope and its multiple subunits.

Wood was a wonderful boss and I admired him a great deal. He had phenomenal physical stamina and loved to work at the bench throughout his life. Furthermore, he had a strong passion for good science and was very supportive of the research programs of his faculty. He was also unusually frank and honest with his colleagues. When I first visited Cleveland for an interview for a faculty position, he informed me that there was only one tenure position in the department and that was his as Chairman. He assured me, however, that tenure wasn't too important anyway because if he and I didn't get along, one of us would have to leave and it wouldn't be him. This was not a threat, however—just a simple and honest fact of life.

My initial studies in Cleveland dealt with the isolation and identification of aminoacyl adenylates that had been proposed as intermediates by Hoagland and co-workers (25) and DeMoss and coworkers (30) in the carboxyl activation of amino acids. These intermediates, however, had not been isolated from an enzymatic reaction. They were analogous, however, to

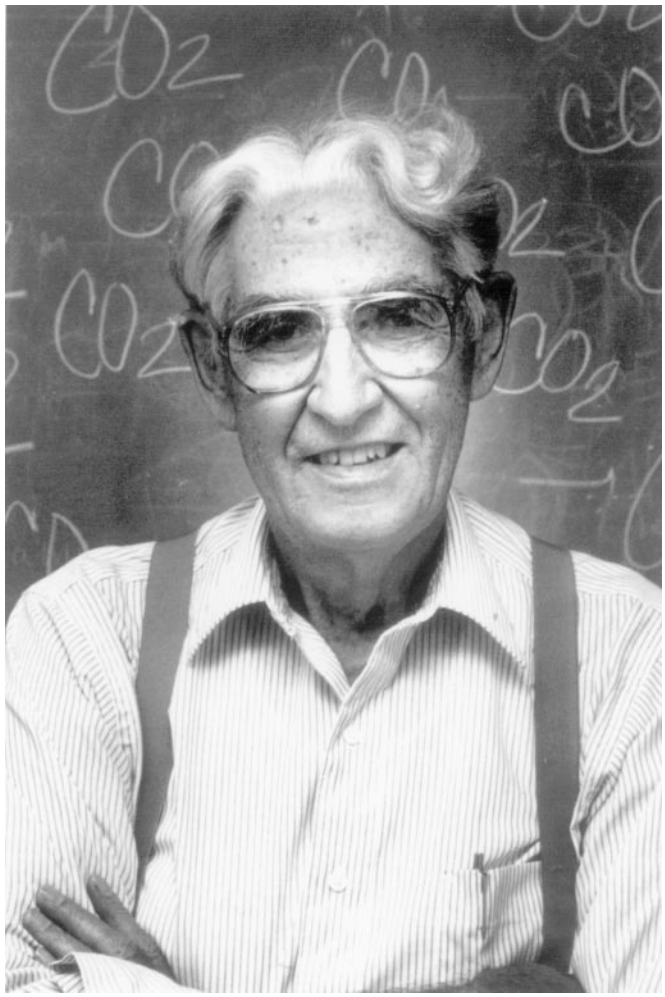

FIG. 3. **Professor Harland G. Wood (1907–1991).** Photograph kindly provided by The Plain Dealer Publishing Co., Cleveland, OH and reprinted with permission.

adenylyl acetate suggested earlier by Berg in the formation of acetyl-CoA (31). Experiments carried out in our laboratory with Henry Kingdon and Les Webster in Cleveland led to the isolation and characterization of adenylyl tryptophan and adenylyl serine and demonstrated that these elusive intermediates could be identified in reactions that included amino acid, ATP, Mg^{2+}, activating enzyme, as well as pyrophosphatase to shift the equilibrium of the reaction toward the enzyme-bound intermediate (32, 33).

In 1957, Wood introduced me to Oscar Ratnoff, a distinguished Professor of Medicine at Western Reserve University (Fig. 4). This introduction to Ratnoff started much of my career in a new direction, mainly research in blood coagulation. Ratnoff received his medical training from Columbia University College of Physicians and Surgeons and then worked with C. Lockard Conley and Robert Hartmann in the Department of Hematology at Johns Hopkins University in Baltimore (34). He joined the Department of Medicine at Western Reserve University in Cleveland in 1953 and soon developed an outstanding reputation in blood coagulation. Working on a project in collaboration with Ratnoff seemed like a good idea because he was well known for his great intellect, hard work, and an uncanny ability to relate a large pool of clinical information to basic research. Furthermore, I had developed a few skills in protein chemistry, and it was quite clear that they would be helpful to unravel some of the complex reactions leading to fibrin formation.

One of the most unusual and remarkable properties of blood is its ability to solidify or clot. In humans, this physical change is initiated by tissue injury and destruction and involves plasma proteins, platelets, and tissue components. In invertebrates, the clotting reaction is primarily because of cell aggregation and agglutination. In higher organisms, however, the vascular pressures are high and this increases the risk of bleeding. Thus, the mechanisms for initiating and regulating blood coagulation in humans are far more complex and include the following three general processes: (*a*) the immediate contraction of blood vessels at the site of

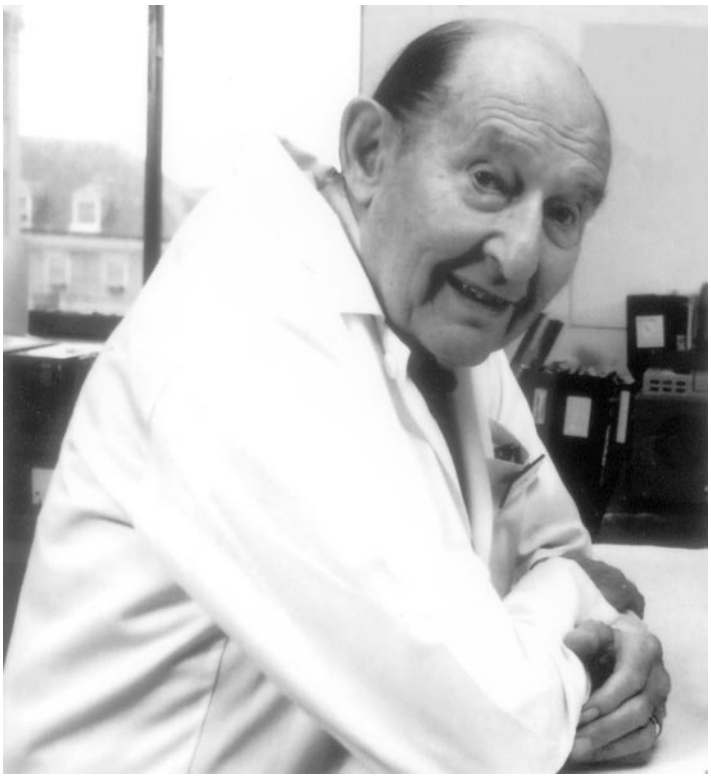

FIG. 4. **Professor Oscar D. Ratnoff (1916–).**

vascular injury, (*b*) formation of a platelet plug, and (*c*) the generation of a fibrin clot to stabilize the platelet plug. The latter reaction results from the interaction of tissue and plasma proteins in a series of reactions that occur primarily on the surface of the activated platelets and other cells. These cells provide phosphatidylserine, a membrane phospholipid that is essential for clotting and becomes exposed when cells are activated or damaged.

Ratnoff had many interesting patients who had abnormal blood coagulation. One of Ratnoff's most unusual patients was John Hageman, who had a rather strange clotting abnormality in that his blood didn't clot when added to a glass test tube (35). This could be corrected, however, by the addition of a small amount of plasma or serum from normal individuals or from patients with other known coagulation disorders such as hemophilia. Surprisingly, however, John Hageman had not experienced any bleeding tendency. Ratnoff inquired as to whether I might be able to help him in the isolation of this plasma protein that he called Hageman factor. I suggested that column chromatography on DEAE or carboxymethylcellulose (CMC) might be useful because Peterson and Sober had just published a novel method by which one could separate plasma or serum proteins with these resins (36). Because these reagents were not available commercially, I prepared small amounts and started the purification of Hageman factor from normal pooled human plasma. In these studies, plasma was separated into 50 or 60 fractions, and these were given to Ratnoff for assay of their clot accelerating activity. A few days later he informed me that all the clotting activity was present in a few tubes that contained little or no detectable protein.

It was clear from the earlier studies of Ratnoff and Rosenblum that Hageman factor (now called factor XII) was present in plasma in an inactive form and was activated in a test tube when bound to a glass surface or crushed glass or kaolin (37). This was consistent with the idea that Hageman factor activation could trigger fibrin formation in blood collected in a glass container. In contrast, blood collected in paraffin-lined or silicone-coated bottles would clot very slowly or not at all. After several purification steps, we were able to show that Hageman factor was a plasma protein capable of initiating blood coagulation in the test tube and did so in the absence of tissue extracts (38). Thus, it was participating in the intrinsic pathway of blood coagulation in contrast to the extrinsic pathway that also required tissue extracts. Little was known, however, about the mechanism by which Hageman factor could activate the clotting process. Ratnoff and I then had a number of discussions about the early phases of blood coagulation because I had little knowledge of the field other than the conversion of

prothrombin to thrombin by thrombokinase, and fibrinogen to fibrin by thrombin in reactions requiring calcium. This pathway had been proposed in the early 1900s by Morawitz, who described thrombokinase as a coagulant activity from platelets or damaged tissue (39). Howell (40) called the clot-accelerating activity from tissue, thromboplastin, a complex that converted prothrombin to thrombin. Some investigators, however, thought the activity from tissue was because of phospholipid or lipoprotein. Purification was then carried out in a number of laboratories, including Chargaff and co-workers (41), Williams and Norris (42), and Nemerson and Pitlick (43) who identified thromboplastin as a combination of clotting activities. One was a protein now known as tissue factor present in most tissues except platelets and the other as phospholipid. Since then, tissue factor has been purified extensively from bovine (44) and human (45) tissue.

Over the years a number of clinicians identified many patients with different coagulation disorders. In 1936, Patek and Stetson (46) had found that patients with hemophilia were lacking a factor present in normal plasma. The following year it was partially enriched by Patek and Taylor who called it anti-hemophilic factor (AHF) or anti-hemophilic globulin (AHG) (47). This deficiency now called hemophilia A (or factor VIII deficiency) is found almost exclusively in males and is one of the most common of the hereditary coagulation disorders. This clotting abnormality was corrected by the addition of a small sample of normal plasma to the hemophilic plasma restoring the generation of a fibrin clot in the presence of calcium. Hemophilia has been of considerable historical interest since the son of Tsar Nicholas II of Russia had hemophilia and his care occupied much of the time and effort of his parents distracting them from the political problems that were developing (48). Consequently, hemophilia played a significant role in the Bolshevik Revolution in 1917 that led to the execution of Nicholas, his wife, and their children following the October revolution.

In 1944, Robbins (49) made the interesting observation that fibrin formed in the presence of a plasma protein and calcium became rather insoluble in urea. In further studies, Laki and Lorand (50) partially purified this plasma protein that became known as the Laki-Lorand factor, fibrin-stabilizing factor, and presently as factor XIII. Years later, Chen and Doolittle (51) showed that activated factor XIII cross-links fibrin monomers by forming $\epsilon(\gamma$-glutamyl) lysine bonds between two adjacent fibrin molecules. In 1947, shortly after World War II, Owren in Norway (52) described another hemorrhagic disease in a young woman lacking a plasma protein that was called proaccelerin. This disease, referred to as parahemophilia (factor V deficiency), was a rare disorder resulting in bruising and bleeding after minor lacerations or dental extraction. In 1949 Alexander and co-workers (53) described another factor in serum that accelerated the conversion of prothrombin to thrombin. This factor was called serum prothrombin conversion accelerator (SPCA), and the defect was named factor VII deficiency. Another clotting disorder, called hemophilia B, was described in 1952 by Aggeler and co-workers (54), Biggs and co-workers (55), and Schulmann and Smith (56). The protein lacking in these patients was known as plasma thromboplastin component (PTC), Christmas factor, or factor IX. Patients with hemophilia B had clinical symptoms essentially identical to those with classic hemophilia. Furthermore, like classic hemophilia, the disease occurred only in males.

In 1953 Rosenthal and co-workers (57) described a clotting abnormality that they called plasma thromboplastin antecedent (PTA) deficiency or factor XI deficiency. Individuals with PTA deficiency had mild or moderate bleeding symptoms that often became evident only after injury or surgery, such as dental extractions and tonsillectomies. This coagulation deficiency found mainly in Ashkenazi Jews was readily corrected by the addition of small amounts of normal plasma or plasma from patients with Hageman factor deficiency or hemophilia A. Mixing or complementation experiments have been very useful for identifying new coagulation proteins, and this has been nicely illustrated in a recent article by Graham (58). Additional experiments with PTA-deficient plasma were consistent with the concept that patients with this deficiency lack a protein that participates in an early phase of the coagulation pathway. The concept that clotting might involve enzymes or proteins other than thrombin, however, was not universally accepted. Some investigators, particularly Seegers and co-workers (59), felt that thrombin was the only enzyme involved in clot acceleration and the other clotting activities were primarily degradation products of prothrombin and probably artifacts. The clinical and genetic evidence for additional clotting factors, however, continued to strengthen and expand. Shortly thereafter in 1957, Stuart factor deficiency was described by Hougie and

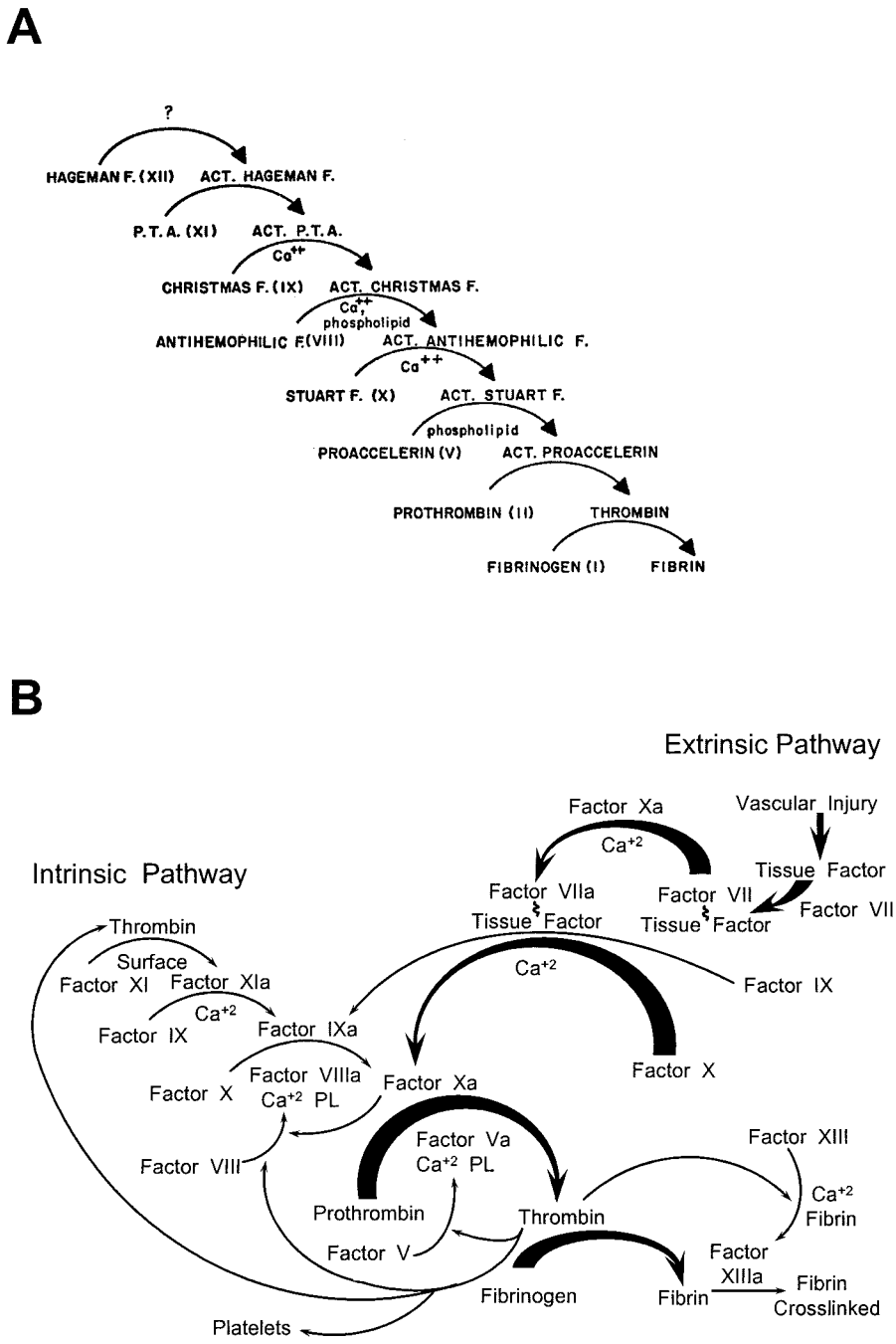

FIG. 5. *A*, "waterfall sequence for intrinsic blood clotting" from Ref. 68. *B*, "coagulation cascade and fibrin formation by the intrinsic and extrinsic pathways" modified from Ref. 4.

co-workers (60). This bleeding disorder occurred in both men and women. Like most other clotting deficiencies, individuals lacking Stuart factor (factor X) fail to readily convert prothrombin to thrombin, resulting in the formation of an abnormal or delayed fibrin clot.

With so many different patients being identified with clotting disorders apparently due to the absence of a functional plasma protein, it was clear that an understanding of how these proteins interacted to generate fibrin would require substantial protein purification. Accordingly, Ratnoff and I continued the purification of Hageman as well as PTA and Christmas factor from normal human plasma or in some cases from human deficient plasma. These studies with partially purified preparations provided clear evidence for the activation of PTA by activated Hageman factor (61), a reaction suggested earlier by Hardisty and Margolis (62). This reaction was followed by the activation of Christmas factor by activated PTA (63) as suggested by Soulier and co-workers (64). These reactions are illustrated in the *top half* of Fig. 5*A*.

An important concept that began to develop in these early studies was that clotting factors were present in blood in an inactive or precursor form and were converted to active enzymes in a step-by-step manner most likely via limited proteolysis. This was supported by the fact that activated PTA was inhibited by diisopropylphosphorofluoridate (DFP), a potent inhibitor of serine proteases (65). With activated PTA, the inhibitor was bound to a serine residue in the active site sequence of -Gly-Asp-Ser-Gly-. These experiments provided good evidence that activated PTA was a trypsin-like serine protease that probably converted Christmas factor to an active enzyme by "limited proteolysis" analogous to our earlier studies on the conversion of trypsinogen to trypsin (3). The activation of Christmas factor also required calcium ions (63). This was the first strong evidence for the role of calcium in the early phases of blood clotting. Thus, the inhibition of clotting by calcium-binding reagents such as oxalate or citrate became clear. The next step in the coagulation pathway was unclear, but it seemed likely that activated Christmas factor could in turn convert anti-hemophilic factor to an active form.

In 1962, I received a letter from Hans Neurath inviting me to join the faculty in the Department of Biochemistry at the University of Washington in Seattle. In just a few years this department had developed a fine reputation for its excellence in protein chemistry and enzymology as well as its superb faculty. Furthermore, Cecil Hougie had joined the medical school faculty in Seattle and was well known in the field of blood coagulation for his studies at Oxford (66), in addition to those at North Carolina previously mentioned (60). It was a difficult decision to leave Harland Wood's department as well as the close collaboration with Ratnoff and the many good friends that I had at Western Reserve. My wife and I finally decided, however, to move back to Seattle because we had grown up in the Pacific Northwest and loved the area.

At the University of Washington, we continued the purification of clotting factors from human and bovine plasma to enrich fractions for Christmas factor, anti-hemophilic factor, and Stuart factor. Our initial studies with Roger Lundblad suggested that activated Christmas factor converted anti-hemophilic factor to an active form, and it in turn converted Stuart factor to an active form in the presence of phospholipid and calcium (67). Similar studies were carried out by Breckenridge and Ratnoff in Cleveland suggesting that activated Stuart factor converted factor V to factor Va, which in turn converted prothrombin to thrombin. This led to a coagulation scheme that Ratnoff and I called a "waterfall sequence for intrinsic blood clotting" (68) (Fig. 5*A*). This proposal that began to take form in late 1962 (63) and early 1963 was submitted to *Science* for publication in early 1964. Unfortunately, it was declined by the editors. Shortly thereafter it was resubmitted to *Science* essentially unchanged and was accepted. About the same time a nearly identical scheme that was called a "coagulation cascade" was published in *Nature* by R. G. MacFarlane from Oxford (69). These two proposals clarified the sequence in which clotting factors interacted and provided concepts that were readily tested in the laboratory. Important additions and modifications were made in these two proposals during the following years. One that was particularly important linked the intrinsic pathway with the extrinsic pathway of blood coagulation at the level of Stuart factor (42, 43, 70, 71). The other significant change related to factor VIII and factor V. These two proteins are readily activated by limited proteolysis particularly by thrombin (72–74), but they participate as cofactors rather than enzymes. Activated factor VIII forms a complex with activated factor IX, an enzyme, whereas activated factor V forms a complex with activated factor X, another enzyme (75–77). Both reactions require calcium and phospholipid (PL), the latter being provided primarily by the activated platelets.

It is now generally accepted that the extrinsic pathway plays the major role in the initiation of blood coagulation following vascular injury and platelet plug formation. This is illustrated in Fig. 5*B* employing the Roman numeral nomenclature for the various clotting factors (4). Tissue factor is an integral membrane glycoprotein located in the tissue adventitia and functions as a receptor for factor VII (or VIIa) circulating in blood (78–81). Factor VII binds to tissue factor only after vascular injury at which time blood comes in contact with the damaged tissue. Tissue factor and factor VII then form a one-to-one complex in the presence of calcium ions. This complex on the damaged cell surface membrane facilitates the conversion of factor VII to factor VIIa by the cleavage of a single internal peptide bond (82). This gives rise to a two-chain factor VIIa held together by a disulfide bond. The active site sequence of -Gly-Asp-Ser-Gly-Gly-Pro- is located in the heavy chain of factor VIIa, a trypsin-like serine protease whose physiological activity occurs only when bound to tissue factor. A number of serine

proteases catalyze the activation of factor VII, but factor VIIa and membrane-bound factor Xa probably play the major role *in vivo*.

The next step in the extrinsic pathway is the activation of factor X by the tissue factor-factor VIIa complex (70, 71, 83, 84) due to the cleavage of a single peptide bond in the heavy chain of the molecule (85). The tissue factor-factor VIIa complex also activates some factor IX by limited proteolysis (86). This latter reaction is particularly significant following minor vascular injury that generates a low level of the tissue factor-factor VIIa complex and reduced factor X activation. Under these conditions, the intrinsic pathway becomes involved via activated factor IXa (87). The formation of factor Xa and factor IXa generates two additional trypsin-like serine proteases. In the next step, it appears likely that factor Xa activates some of the factor V to factor Va and forms a complex with this activated protein in the presence of calcium and cell membrane phospholipid (PL). This complex then converts some prothrombin to thrombin. These reactions are referred to as "the initiation and priming phase" in the cell-based model of coagulation of Roberts and colleagues (88). The extrinsic pathway, however, is quickly inhibited by a multivalent Kunitz-type plasma protease inhibitor called tissue factor pathway inhibitor (89–91). This protein is a factor Xa-dependent inhibitor of the tissue factor-factor VIIa complex and blocks additional thrombin generation by the extrinsic pathway. Present evidence suggests that the small amount of thrombin that is generated, however, activates additional platelets attached to the site of vascular injury and also converts factor XI to factor XIa, factor VIII to factor VIIIa, and factor V to factor Va (Fig. 5*B*). These latter activation reactions all occur by limited proteolysis on the surface of the activated platelets that provides the phospholipid (PL). The activation of factor XI by thrombin triggers the intrinsic pathway of coagulation that also takes place on the surface of the activated platelets (92, 93). Initiation of the intrinsic pathway leads to an increase in the concentration of factor IXa, factor Xa, and a burst in the concentration of thrombin. This is followed by an increase in the conversion of fibrinogen to fibrin and factor XIII to factor XIIIa. Factor XIIIa then cross-links the fibrin monomers forming a tough insoluble clot that stabilizes the platelet plug.

A physiological role for Hageman factor in the coagulation pathway has not been clearly established because individuals lacking this activity have no bleeding complications. Accordingly, Hageman factor has been deleted from most coagulation schemes leading to fibrin formation. A similar situation exists for plasma prekallikrein (94, 95) and high molecular weight kininogen (96–99). Deficiencies of these two plasma proteins do not result in bleeding complications even though the two proteins accelerate blood coagulation *in vitro*.

Important advances in our understanding of the regulation of blood coagulation have also occurred over the years. The generation of fibrin is brought to a halt by plasma inhibitors such as antithrombin III (100, 101), a protease inhibitor that is particularly potent in the presence of heparin. Other plasma inhibitors include α_2-macroglobulin (102, 103) and tissue factor pathway inhibitor recounted earlier (89–91). These plasma proteins form stable inactive complexes with serine proteases such as thrombin. Another important regulatory pathway of the coagulation process results from the inactivation of factor Va (104) and activated factor VIII (105) by activated protein C (106). These reactions also occur by limited proteolysis in the presence of calcium and phospholipid. The inactivation of activated factor V is accelerated by protein S (107), another vitamin K-dependent plasma protein (108). The formation of activated protein C by thrombin (109) requires thrombomodulin, a transmembrane protein discovered by Owen and Esmon (110, 111) that stimulates the activation reaction about 20,000-fold.

Vitamin K was discovered by Dam in 1935 (112), but its biochemical relationship to bleeding became apparent only after the important discovery by Stenflo (113), Nelsestuen (114), and Magnusson (115) and their co-workers of γ-carboxyglutamic acid (Gla) in prothrombin. The Gla residues require vitamin K for their biosynthesis and are clustered in the amino-terminal region of the protein where they bind calcium. Calcium binding leads to a conformational change in the molecule and results in its interaction with phospholipid on the surface of damaged cells or activated platelets (116). The Gla residues are also present in the other vitamin K-dependent plasma proteins including factor VII, factor IX, factor X, protein C, and protein S (117). Coumadin drugs such as dicumarol cause bleeding by interfering with the carboxylation of the Glu residues in the vitamin K-dependent proteins resulting in inactive clotting factors (118). This was discovered in cattle fed fermented sweet clover that was rich in dicumarol (119).

The complete amino acid sequence of all the known coagulation factors has been determined in our laboratory and by others employing amino acid sequence analysis and cloning (120). Thus, the peptide bonds that are cleaved by the various trypsin-like serine proteases when clotting has been initiated are known, and all occur on the carboxyl side of an arginine residue. Furthermore, the structure of many of these proteins has been determined by x-ray diffraction. The three-dimensional structure of fibrinogen by Doolittle (121) and Cohen (122) and their co-workers was a major accomplishment, as well as the structures of thrombin, factor IX, factor X, and protein C by Bode and colleagues (123). In addition, the factor VIIa-tissue factor complex has been determined at 2.0-Å resolution by Banner and co-workers (124) and others (125). These studies have added greatly to our knowledge of these proteins that participate in fibrin formation.

Finally, it is important to mention the fact that the DNA sequences coding for the genes for all the clotting factors have been established in our laboratory (120) and others making it possible to identify the mutations in the clotting factors of thousands of patients with bleeding complications. Indeed, the largest number of mutations occurring in the genes coding for human proteins have been found in factor IX-deficient patients (126). Most importantly, the cloning of the coagulation proteins has made it possible to express and prepare recombinant proteins for the treatment of patients with hemophilia and other clotting disorders without the risk of viral contamination such as HIV and hepatitis (127–129). Last, the availability of recombinant factor VIIa has introduced a new and exciting approach for the treatment of hemophilia patients with inhibitors (antibodies) toward factor VIII, as described by Hedner and Kisiel (130). This recombinant plasma protein has also become extremely important in the treatment of patients undergoing various types of surgery where bleeding becomes life threatening (131–135). Indeed, factor VIIa may be the most important recombinant protein ever developed to assist in the treatment of trauma patients, burn patients, and a vast number of other patients undergoing major surgery.

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Reflections:

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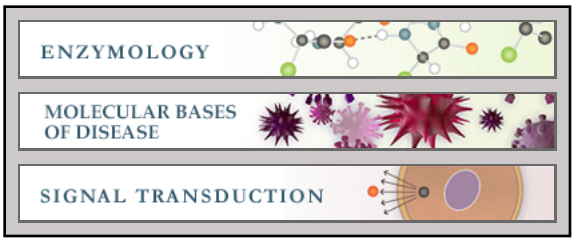

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