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Studies on fibrin polymerization and fibrin structure—a retrospective

Marsha D. Bale Oenick*

Ortho Clinical Diagnostics, 100 Indigo Creek Drive, Rochester, NY 14626, United States

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Nature provides an elegant set of checks and balances to preserve life in the presence of injury through blood coagulation, focused at the site of injury, followed by lysis of the clot and subsequent wound healing. Over the course of several decades, John Ferry focused on a specific subset of this phenomenon: polymerization of the soluble circulating monomer precursor, fibrinogen, into a viscoelastic network of fibrin capable of withstanding substantial stress. I had the good fortune to be Ferry's last graduate student prior to his retirement in 1982.[1] The years I was a graduate student and the subsequent 2 years while I was a post-doctoral fellow in Deane Mosher's laboratory in the Departments of Physiological Chemistry and Medicine (literally across the street from Ferry's laboratories) allowed me to participate in a very active period of research leading to a greater understanding of the fibrinogen to fibrin conversion and the nature of the fibrin structure.

When I arrived in Ferry's laboratory in 1979, there were multiple areas of research on fibrinogen and fibrin and well as investigations of the viscoelastic properties of synthetic polymer melts and films. Consistent with Ferry's entire career, multiple techniques were used for these investigations including birefringence, small angle X-ray scattering, sedimentation in the ultra centrifuge, and viscoelastic measurement techniques developed in Ferry's laboratory for the study of polymer films [1], polymer melts (Plazek torsion pendulum) [2] and dilute solutions (Birnboim-Schrag multiple-lumped resonator [3]). The biochemical technique of gel electrophoresis had recently been added to the laboratory [4] to examine biochemical characteristics of fibrin gels as well as to characterize fibrin oligomers that had previously [5] been identified during delayed polymerization of fibrin in the presence of high pH and more recently studied following sonication of fibrin clots [6]. During the years of my tenure, Ferry's various graduate students and post-doctoral fellows enhanced the dilute solution and bulk viscoelastic techniques available in the laboratory and the laboratory collaborated with others at the university and elsewhere to add the techniques of quasi-elastic light scattering, electron microscopy of deposits of solutions, high-voltage electron microscopy of bulk fibrin gels and films, and large-angle X-ray scattering, to examine the fibrinogen–fibrin conversion and properties of fibrin and other biological molecules at the molecular and bulk level. Ferry's collaborative skills also afforded the laboratory access to precious enzymes and peptides that were critical to the progress made on our understanding of the fibrin network. In addition to our own internal group meetings and discussions, the laboratory participated in several discussion and seminar groups. Laboratory members focused on the fibrinogen and fibrin problems participated in the Macromolecular Seminar series involving the chemistry department groups of John Ferry, Thomas Record, John Schrag, and Hyuk Yu; and a Madison/Milwaukee group meeting involving the groups of John Ferry, Deane Mosher (University of Wisconsin) and Michael Mosesson (The Blood Center of Southeastern Wisconsin). We also had active discussions regarding our work on fibrinogen and fibrin with numerous visitors to the university including Harold Scheraga of Cornell University and T.A.J. Payens from the Netherlands.

By the mid-1970s, much detailed biochemical and biophysical information about the fibrinogen molecule

* Tel.: +1 585 453 5760; fax: +1 585 453 6084.
E-mail address: moenick@ocdus.jnj.com.

[1] Ferry was the primary thesis advisor for an additional student, Craig Carriere, following his retirement with Schrag serving as major.

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and fibrin was available [7]. In gross summary, the enzyme thrombin (resulting from activation of the proenzyme, prothrombin, through a complicated cascade of enzymatic events) was known to release two sets of peptide pairs (two A and two B peptides) from the center "E" nodule of the trinodular fibrinogen molecule, revealing A and B binding sites on this nodule. The A binding sites on the E nodule could then form non-covalent bonds with pre-existing "a" binding sites on the end "D" nodules. Polymerization of the activated fibrin monomers proceeds with formation of a gel structure as the ultimate outcome, the optical and physical properties of which depend on the polymerization conditions including enzyme and substrate concentration as well as pH and ionic strength of the polymerization media. The latter aspects were first shown in early experiments conducted by Ferry while at the Harvard School of Medicine. Early results from Ferry's laboratory after he joined the chemistry department of the University of Wisconsin provided evidence for the now well-established staggered overlapped structure of linear oligomers or protofibrils. At physiological pH and ionic strength, lateral aggregation of protofibrils can occur, leading to formation of thicker fibrils. Branching within the fibrin gel thus could occur via lateral association of growing fibrils. Gels formed under these conditions are readily synersed. Fibrin film, the original "product" of Ferry's work, is formed when such a "coarse" gel is compacted and fluid expressed from it. As the pH or ionic strength is raised, the optical and mechanical properties of the gel change substantially, due to suppression of lateral aggregation compared with protofibril lengthwise growth during polymerization. Under "fine" clotting conditions, pH 8.5 and ionic strength 0.45, a nearly clear gel is formed. By suppressing lateral aggregation during polymerization, the primary mechanism for "crosslinking" via branching is also minimized or eliminated and the structure of the fibrin gel was originally hypothesized to be a collection of interpenetrating nearly rigid rods.

Information was also available [7] about the results of other enzymatic reactions occurring presumably simultaneously during naturally occurring clotting. Thrombin also releases a pair of B peptide from the central E nodule of the fibrinogen molecule revealing "B" binding sites which have "b" binding site partners, also on the end "D" nodules. However, release of B peptides is neither required for protofibril nor for fibril formation, since apparently normal fibrin can be generated with ancrod, a thrombin-like protease purified from the venom of the Malaysian pit viper, *Agkistrondon rhodostoma*, known to release A but not B peptides from fibrinogen. Another blood coagulation proenzyme, Factor XIII also known as fibrin stabilizing factor and fibrinoligase, was also known to be activated to an enzymatic form, Factor XIIIa, by thrombin. Factor XIIIa covalently ligates together adjacent D-nodules of fibrin monomer via enzymatically catalyzed formation of a lysine-glutamyl bond. Factor XIIIa also ligates together alpha chains extending from the end nodules. The latter occurs (at least measurably) only in coarse gels. While by the mid-1970s, it was already clear that ligation had a substantial impact on the mechanical properties of fibrin, it was not clear what, if any, impact ligation had on the fibrin polymerization process.

During my tenure in Ferry's laboratory, we had the good fortune to gain access to both materials (enzymes and peptides) and techniques (electrophoresis, quasi-elastic light scattering, electron microscopy, high-voltage electron microscopy, and novel viscoelastic techniques) that helped reveal how the biochemical reactions that occur during and after polymerization of fibrin monomers into a gel structure drive the properties of that gel.

Since the monomer precursor and monomer in the fibrin polymerization reaction are nearly identical and polymers in their own account (340,000 molecular weight), and monomers are held together only by non-covalent bonds, study of the fibrin polymerization reaction is complicated. Nelb et al. [4] introduced the method of Morio et al. [8] into Ferry's lab. Polymerization could be studied by quenching a reaction mixture of fibrinogen, thrombin, and Factor XIIIa quenched with a high concentration of urea and sodium dodecyl sulfate (SDS). The oligomers ligated together by Factor XIIIa were stable in the presence of the denaturing agent and could be separated by molecular weight via gel electrophoresis through a 2% agarose media. The distinct oligomer bands were visualized with Coomassie Brilliant Blue stain and the weight fraction of each oligomer was quantified via scanning spectrophometry. Gary Nelb and Greg Kamykowski showed that fibrin oligomers, formed in the presence of Factor XIIIa before the gelation time, showed a much higher concentration of large oligomers (n>=10) than would be predicted by random polymerization of fully activated monomers. However, the relative rates of activation of fibrinogen and Factor XIII by thrombin, polymerization of fibrin monomers, and ligation of fibrin by Factor XIIIa were at that point unclear.

As an entering graduate student, I was assigned to extend Gary and Greg's work in an effort to more completely understand the early polymerization process. Meanwhile, Paul Janmey, another graduate student in Ferry's laboratory, became intrigued with the interesting distribution of oligomers revealed by the electrophoresis technique. He developed a theory [9] to predict the distribution based on the relative concentrations of monofunctional (one A peptide released from the E nodule) and difunctional (both A peptides released form the E nodule) polymerizable units, which in turn is driven by the relative rates of release of the first and second A peptides from the E nodule. High concentrations of difunctional units, due to a more rapid cleavage of the second peptide compared with the first, would lead to a higher concentration of long oligomers at earlier times than if the peptides were released at similar rates. Thus, we worked to sort out how to manage the relative contributions of the following reactions: fibrinopep-

tide release; assembly; ligation so that this theory could be tested [10].

To ensure ligation by Factor XIIIa was not rate limiting, the proenzyme had to be converted completely to the active form before initiation of the fibrin polymerization reaction as well as be at the right concentration. Activation was accomplished by separate incubation with thrombin prior to addition of an aliquot of this mixture to a solution of fibrinogen. Since this Factor XIIIa/thrombin/Ca^{2+} preactivation mixture would provide all of the thrombin provided to the final reaction mixture, it was also important to find activation conditions so that the thrombin concentration would be low enough to ensure fibrinopeptide A release was the gating factor. Thus, the concentration of thrombin, Factor XIII, and incubation time became important factors. We utilized a thrombin inhibitor, *p*-nitrophenyl-*p*′ -guanidinobenzoate (NPGB) to selectively inhibit A peptide release reaction while assembly or ligation could continue. Paul's quasi-elastic light scattering experiments tracking assembly found conditions for which assembly was faster than fibrinopeptide release. My electrophoresis experiments revealed conditions where ligation kept pace with assembly.

Fig. 1 shows the characteristic behavior of fibrin polymerization when gated by fibrinopeptide release. Large oligomers show up very quickly and there is little difference in the weight fraction for each of the visible oligomers for $n>4$. Reasonable agreement was achieved between Paul's theory and experimental findings for specific oligomer distribution as well as number and weight average molecular weight when the ratio of release of the second peptide to that for the first was set to approximately 16 [10].

Paul arranged collaboration with Lorne Erdile, a graduate student in the Molecular Biology department who was expert in electron microscopy preparation techniques [11]. Polymerization mixtures were analyzed by both the electrophoresis technique and manual counting of oligomers of different lengths visualized by electron microscopy. Electron microscopy analysis showed that the oligomers formed early in polymerization were straight and long, regardless of whether the reaction was carried out under conditions leading to fine gels (pH 8.5, ionic strength 0.45) or more physiological conditions (pH 7.5, ionic strength 0.15). The

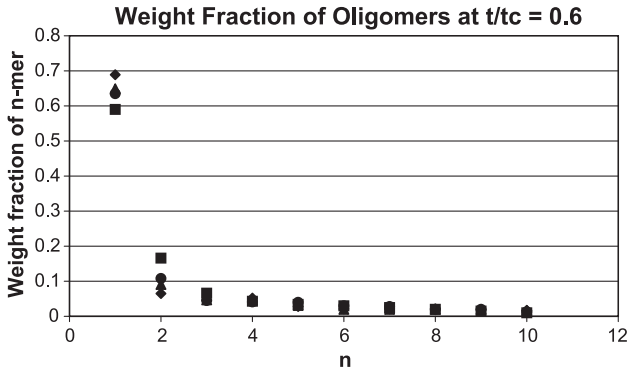

Fig. 1. Weight fraction of fibrin oligomers prior to clotting.

distribution obtained via electron microscopy agreed qualitatively with the electrophoresis data in that very long oligomers are obtained very quickly after initiation of polymerization. In fact, even longer oligomers were revealed via electron microscopy than could be readily resolved via electrophoresis. These results confirmed that fibrin polymerization is characterized by very rapid lengthwise growth of protofibrils, even when lateral association has not been inhibited by pH or ionic strength.

The remaining question regarding the early stages of polymerization was whether or not the presence of Factor XIIIa and ligation of developing oligomers influenced the distribution of oligomers. We addressed this question [12] by comparing the distribution of oligomers prepared in the presence and absence of Factor XIIIa . For the latter case, ligation was initiated following inhibition of fibrinopeptide A release with the thrombin inhibitor NPGB. The distributions agreed. Additional experiments with both agarose and polyacrylamide gels also allowed more detailed examination of the ligation reaction. Results showed that the reaction was first order in the concentration of ligatable sites and the initial velocity of reaction was proportional to the enzyme concentration. The size distribution of ligated oligomers as a function of time was consistent with random insertion of ligations.

The viscoelastic behavior of fibrin gels was very intriguing to Ferry since the source(s) of elasticity and the relaxation mechanisms available were very different for fibrin gels compared with traditional synthetic materials. Starting in the early 1970s and continuing until Ferry's "true" retirement from experimental studies in the later 1980s, a large number of experiments were conducted with exquisitely sensitive and remarkably simple instrumentation. The early experiments [1,2,13–15] established the basic features of fibrin's viscoelastic behavior: (1) nearly perfectly elastic behavior at small deformations; (2) slow creep under constant stress for unligated gels while maintaining a nearly constant initial modulus; (3) incomplete recovery of strain for unligated gels following the release of stress but recovery was predicted by the Boltzman superposition theory (indicating no decay of structure); (4) creep was essentially eliminated for both fine and coarse gels prepared in the presence of Factor XIIIa and Ca^{2+} with immediate return of strain to essentially zero when stress was removed. Roska and Ferry's [1] stress relaxation experiments with fibrin films prepared from coarse clots in combination with bifringement of the stressed and relaxed films gave strong evidence of orientation of fibrils in the deformed state. This was later substantiated with direct visualization via high voltage electron microscopy by Muller et al. [16]. Elastic modulus for both coarse and fine gels was explained by bending of fibril bundles or protofibrils, respectively. Stress relaxation of coarse fibrin films under large strains and creep of coarse fibrin gels under stress were proposed to be due to slippage of protofibrils past each other within fibril bundles, a mech-

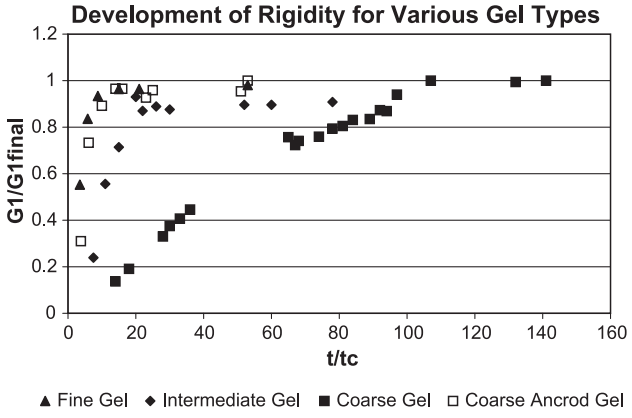

Fig. 2. Development of fibrin structure as measured by the fraction of initial modulus for the fully developed structure.

anism thought not to be available within fine fibrin structures and which would be eliminated in ligated coarse gels. The small but non-zero creep of unligated fine fibrin gels was postulated to correspond to sliding of rod-like protofibrils through a network of interpenetrating rods devoid of interactions other than steric hindrance. The elimination of creep for ligated fine fibrin gels was not completely explained by this model. Work in this period also noted that the fibrin structure continued some development, as measured by initial modulus, for some time after the gelation, or clotting, time was achieved. Viscoelastic experiments conducted on incompletely developed structures were interesting, but complicated.

In the early 1980s, Michael Muller joined Ferry's laboratory as a post-doctoral fellow. His doctoral thesis work under Walter Burchard included light scattering experiments on polymerizing fibrin, the results of which were interpreted as providing evidence for the development of trifunctional branch points during the polymerization of protofibrils [17,18]. When he joined Ferry's lab, Michael collaborated with Hans Ris of the Department of Zoology to utilize electron microscopy to directly visualize the structure of fine and coarse fibrin gels and films [16]. He utilized critical point drying of fibrin gels as well as embedding and sectioning techniques on fibrin gels and films to prepare samples for examination via high-voltage electron microscopy. His results provided direct evidence that the fine fibrin structure was not as simple as a network of interpenetrating rods, but rather the network also contained junctions where individual protofibrils came together, twisted around each other and then diverged again. The coarse fibrin structure demonstrated the expected larger scale of twisting and anastomosis of larger fibrils.

Bulk property experiments I conducted during the early 1980s [19] by using simple spectroscopy and the Plazek torsion pendulum with fibrin gels focused on understanding the structural implications of the rate of fibrin polymerization and of the noncovalent interactions made possible by release of B peptides. Interpretation of the experiments was aided dramatically by Michael's electron microscopy experiments and by his experiments using stress relaxation and large angle X-ray scattering on fibrin films produced with thrombin and ancrod. Varying the thrombin concentration to change the relative rates of protofibril extension and lateral aggregation, as measured spectrophotometrically, generated gels of varying coarseness. Due to limits of practicality, the "finest" structure was created by the more classical method of higher pH and ionic strength. In addition, fine and varyingly coarse gels were prepared using ancrod, the thrombin-line protease that releases only A peptides. By measuring opacity and the initial modulus (Fig. 2) as functions of time, it was shown that the rate of structural development slows as the coarseness of the gel increases if both A and B peptides have been released while the initial modulus of coarse ancrod gels (only A peptides release) developed at a very similar rate to that of a fine thrombin gel. These results suggested that the formation of B–b noncovalent bonds developed slowly under these conditions and were important in defining the viscoelastic properties of the structure. In fact, the initial extent of deformation under constant stress, J1, the rate of creep, and the amount of unrecoverable deformation following removal of stress were all dependent both on the coarseness of the gel and on whether or not B peptides had been released as shown in Figs. 2 and 3. The impact of B peptide release (and presumed B–b noncovalent interactions achieved) was extreme for the fine fibrin structure (Fig. 4). Fine ancrod gels deformed so rapidly that experiments were substantially truncated. However, the overall density of structure remained constant while under stress, even for fine ancrod gels, as shown by adherence to the Boltzman superposition principle and by constant initial modulus measured at different times in the experiment. Given the new evidence of junctions of twisted protofibrils in fine gel structure, the possibility of slippage of these twisted protofibrils past one another was a mechanism available to fine gel structures as well as for coarse gels. For coarse gels, the presence of multiple protofibrils within a twisted fibril may provide

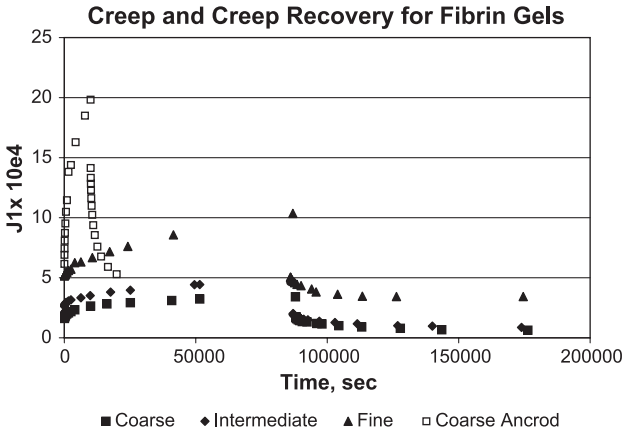

Fig. 3. Creep and creep recovery for various fibrin gels. Note that creep recovery was initiated for the coarse ancrod gel at 10,000 s vs. approximately 86,000 s for the other gels.

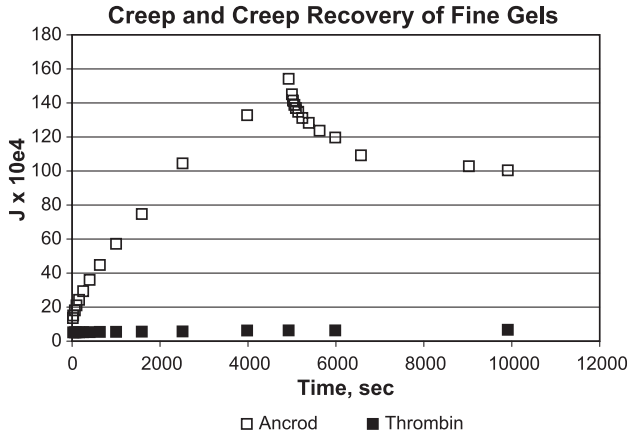

Fig. 4. Creep and creep recovery (ancrod gel only) for fine fibrin. Note the differences in x and y scales from previous figures.

increasing stability against this slippage, especially if B–b interactions have developed completely. Inhibition of creep for ligated gels may be due to increased rigidity of the protofibril, hence reducing the looseness of the twisted junction. However, ligation also may eliminate an alternative mechanism of protofibril rupture. While this mechanism may not occur at low strains, it probably does occur at high strains as observed in experiments [20,21] not discussed in this summary.

Following graduation, I took a postdoctoral fellowship in Deane Mosher's lab to study the interactions of polymerizing fibrin with thrombospondin, a platelet protein released from the alpha granules of activated platelets. We discovered that thrombospondin could modulate the structure of fibrin [22–24]. Gels formed in the presence of thrombospondin developed less optical density and the optical density developed more rapidly than in the absence of the platelet protein. Experiments with 125-I labeled fibrinogen showed that fibrin polymerization was complete—thrombospondin in the reaction mixture did not create a soluble fibrin fraction. This suggested that thrombospondin could change the coarseness of the fibrin gel. The results were confirmed with high-voltage electron microscopy of critical point-dried gels formed in the presence of varying amounts of thrombospondin. There were no differences in fiber structure except for width of the fibers. Increasing concentration of thrombospondin in the reaction mixture led to a larger number of thinner fibers in the final network. Experiments with 125-I thrombospondin showed that the protein was specifically incorporated into the network and could be covalently bonded to it in the presence of Factor XIIIa. Given that thrombospondin is composed of three identical polypeptide chains, it is intriguing to propose that thrombospondin forms a trifunctional branching unit thus promoting formation of more numerous growing fibrils at an earlier time in fibrin polymerization that would occur otherwise. The impact of additional branching points within the fibrin gel on its mechanical properties would be interesting to determine. Unfortunately, we were unable to obtain sufficient quantities of purified protein to complete these experiments.

I continued collaborating with Ferry's laboratory during my postdoctoral fellowship. Working with Michael Muller and Gunther Schindlauer, another post-doctoral fellow, we studied the interaction of the peptide Gly-pro-arg-pro (GPAP) with developing fibrin protofibrils [25] and with fully developed fibrin gels [26]. Laudano and Doolittle [27] showed that the tetrapeptide Gly-pro-arg-pro binds to fibrinogen and inhibits the polymerization of fibrin monomer. This peptide is the analog of the amino termini of the alpha chain of fibrin monomer on the central E nodule following release of the A peptide by thrombin. This region is presumably part of the A binding site which binds to the "a" binding site on the terminal D nodules. Using the agarose gel electrophoresis to visual the distribution of soluble oligomers following their ligation by Factor XIIIa, we established that GPAP could successfully compete with A binding sites in protofibrils and dissociate preformed protofibrils. To study the influence of the peptide on fully formed gels, we introduced a solution of the peptide around the fine gel while in the torsion pendulum and allowed it to diffuse into the gel structure. The modulus slowly decreased with time of diffusion and the decrease was more rapid with increasing concentration of the peptide. At sufficiently high concentrations, the fine gel was completely liquefied. These results established that the GPAP peptide could exchange with the A binding site within the protofibril structure leading to protofibril severance. Fine gels with infused peptide showed substantially more creep under stress and more irrecoverable strain upon removal of the stress. However, the overall structure appears to continuously reestablish during the creep experiment since the initial modulus remained relatively constant throughout the experiment. Ligation of the gamma chains of protofibrils in the fine gel structure prior to introduction of GPAP eliminated its effect on mechanical properties giving support to the proposal that severance of protofibrils was a mechanism of structural rearrangement during creep experiments in the presence of this peptide.

The tetrapeptide Gly-His-Arg-Pro (GHRP), the analog of the amino termini of the beta chain following release of the B peptide [27], was studied in a similar manner. This peptide had much less influence on the mechanical properties of fine fibrin gels than the GPAP peptide. In addition, although increasing the amount of creep exhibited under stress by normal thrombin fine gels, fine ancrod gels (having intact B peptides) showed substantially greater creep under stress as well as unrecovered strain when stress was released. These results suggest the GHRP peptide was not particularly effective in competing with the B binding site in B–b interactions, consistent with a lower binding constant between this peptide and fibrinogen compared with the GPAP peptide.

In summary, I was fortunate to have participated in a very active and productive period of Ferry's work focused on

understanding the molecular basis of fibrin's interesting and critical mechanical properties. We learned that these properties are exquisitely sensitive to the relative rates of protofibril extension and lateral aggregation of protofibrils into larger fibers, which, under physiological conditions, is dependent on the thrombin concentration available to activate the fibrinogen precursor. Our studies on the early stages of polymerization established that by enabling more rapid release of the second A peptide from the fibrinogen molecule by thrombin compared with the first A peptide, there is a predisposition to very rapid protofibril extension. Thrombospondin, when released in the vicinity of a growing fibrin gel structure, can also alter the ultimate structure, although its specific impact on the mechanical properties are not currently known. We did learn, however, that fibrin's mechanical properties are modulated substantially by the presence of non-covalent B–b binding. When coupled with the previous information that gamma–gamma chain ligation of fibrin monomers within individual protofibrils or alpha chain ligation presumably between protofibrils within larger fibrils greatly impacts the mechanical properties of fibrin (in addition to increasing their resistance to fibrinolysis [7]), it is apparent that nature has provided several mechanisms for naturally modulating the mechanical properties of fibrin.

The discoveries we made during this period were a direct consequence of the collaborative spirit Ferry fostered. He provided the catalyst necessary to bring together the critical combination of people, materials, and techniques I have discussed.

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