FIBRIN ASSEMBLY

Lateral Aggregation and the Role of the Two Pairs of Fibrinopeptides

JOHN W. WEISEL
Department of Anatomy, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

ABSTRACT The structural basis of the wide variability of the physical properties of fibrin clots and the process of assembly of the clot were investigated by electron microscopy of fibers formed under various ionic conditions. In addition, highly specific proteolytic enzymes from different snake venoms were used to remove selectively only the A (batroxobin) or the B (venzyme) fibrinopeptides from fibrinogen, in contrast to thrombin, which removes both pairs. Fibers produced by cleavage of only the B fibrinopeptides displayed a characteristic band pattern indistinguishable from that of fibers formed upon removal of either the A fibrinopeptides alone or of both pairs. Computer modeling studies suggest that there is a unique molecular packing that gives rise to this fibrin band pattern. These findings imply that the release of either fibrinopeptide triggers similar modes of aggregation; the intermolecular binding sites can be localized to particular molecular domains. The diameters of fibers formed with each condition of enzyme, pH, salt concentration, and temperature were measured from electron micrographs. All fibers, except for those produced at both high ionic strength and pH, had about the same average diameter of 85 ± 13 nm. The degree of lateral aggregation of the fibers themselves varied greatly, however; fibers aggregated more readily with cleavage of both pairs of fibrinopeptides and at lower pH and salt concentrations. The formation of such thick fiber bundles increases the stability of the clot and its resistance to proteolytic dissolution.

INTRODUCTION

The fibrin blood clot plays a vital role in many biological and pathological processes, including hemostasis, thrombosis, infection, inflammation, wound healing, and tumor growth. The physical properties of the clot, such as its density, strength, stability, porosity, flexibility, etc., largely determine its interactions with molecules, cells and fluid. Therefore, knowledge of the structural determinants of these properties is necessary for an understanding of the involvement of fibrin in any of the physiological processes just listed. Furthermore, information about molecular events that control the different forms that a biological structure can assume may be applicable to systems in other areas of biophysics as well. Much of the past research on the structure and assembly of fibrin has been focused at the molecular level.

Fibrinogen is the key structural protein in blood clotting. It has a molecular weight of 340,000 and is made up of three pairs of chains, $(A\alpha, B\beta, \gamma)_2$, linked together by disulfide bonds. The complete amino acid sequence of fibrinogen and the pattern of disulfide linkages are now known (for reviews of many aspects of fibrinogen and fibrin, see Mosesson and Doolittle, 1983). A slightly modified bovine fibrinogen has been crystallized, providing the basis for a high resolution structure determination (Tooney and Cohen, 1972, 1977; Cohen and Tooney, 1974; Weisel et al., 1978). Analysis of a variety of crystalline forms by electron microscopy, computer image processing, and x-ray crystallography has yielded a low resolution model for the shape of fibrinogen (Weisel et al., 1981, 1985*a*, *b*; Cohen et al., 1983). The molecule consists of a central globular domain, the disulfide knot where the amino termini of all six chains are linked together, proximal and distal end domains, which comprise the COOH-termini of the β- and γ-chains, respectively, and the α domain, which comprises the COOH-termini of both α-chains. The central and end regions are joined by three-stranded α-helical coiled-coils that are interrupted by a nonhelical segment in the middle. (A simplified representation of the molecule is included as part of Fig. 1.)

Upon cleavage of the A and B fibrinopeptides with thrombin (to form $[\alpha, \beta, \gamma]_2$, sometimes called des-AABB fibrin, fibrin II, or $\alpha\beta$ fibrin), there is a dramatic change in solubility that causes the molecules to aggregate to form fibrin fibers. These fibers have a repeat of 22.5 nm as determined by x-ray diffraction or electron microscopy and a characteristic band pattern as observed by electron microscopy (for a review of fibrin structure, see Weisel et al., 1983). Although the repeat was explained some time ago as arising from 45.0-nm long molecules that are half-staggered, the band pattern itself could not be accounted for until more information about the molecular structure has become available recently (Weisel et al., 1981, 1983; Weisel, J. W., manuscript submitted for publication).

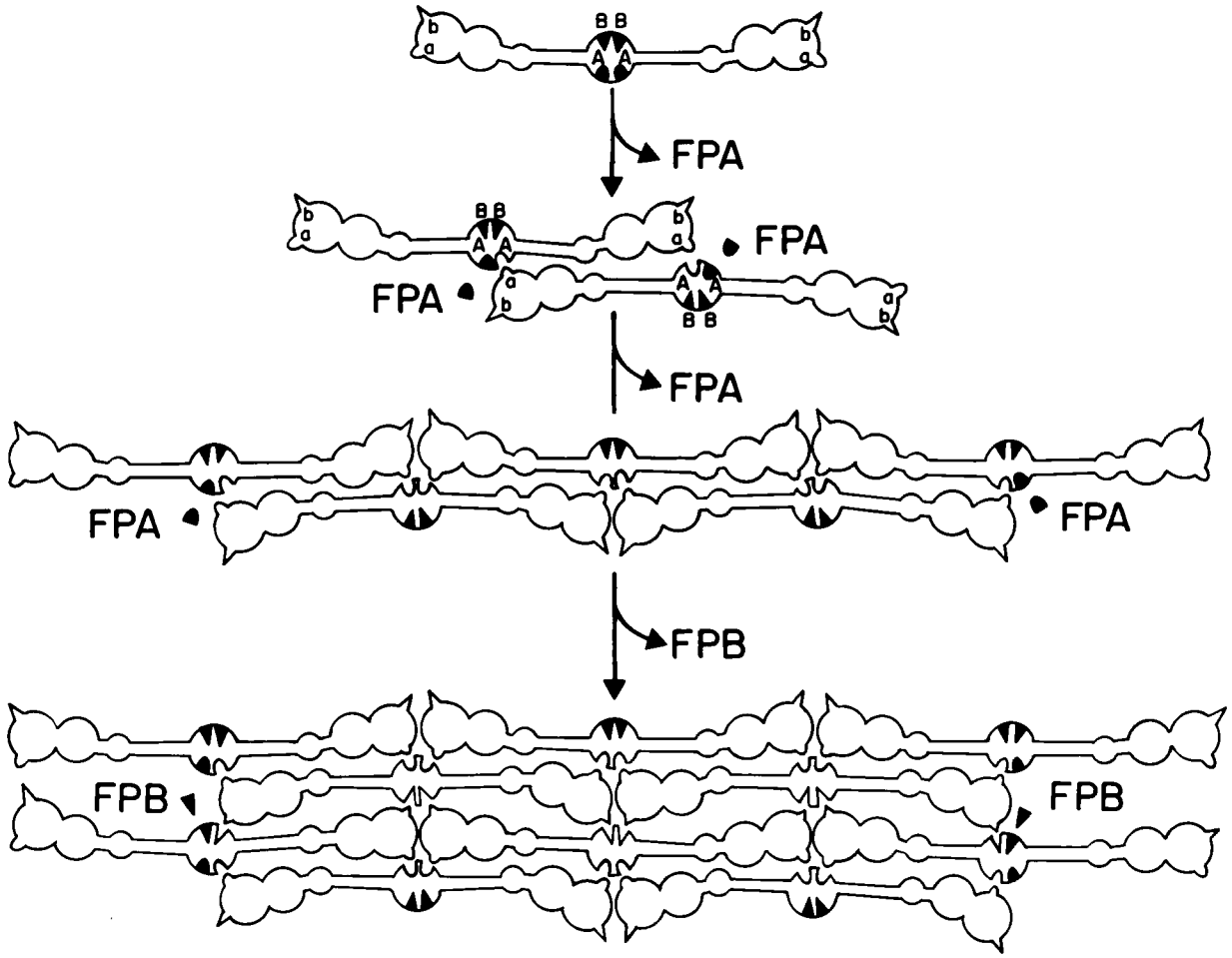

FIGURE 1 A model for fibrin assembly involving two sets of complementary binding sites: one for protofibril formation and another for lateral aggregation of protofibrils. The mechanism depicted is similar to that proposed by Blomback et al. (1978). The molecular shape is that determined by Weisel et al. (1985); but for clarity the α domain is not shown. The globular regions are: central domain (or NH_2-terminal disulfide knot), distal end domain (or γ domain), proximal end domain (β domain), and a small plasmin-sensitive domain that interrupts the rod-like, three chain, α-helical coiled-coil. The *A* fibrinopeptides are shown as black semicircles while the *B* peptides are black triangles. When the fibrinopeptides are removed, binding sites are exposed: *A* sites are semicircular holes, while the *B* sites are triangular holes. The sites complementary to those just described are the knobs that are always present on the distal end domains: *a* sites are semicircular knobs while the *b* sites are triangular knobs. This scheme depicts the removal of the *A* fibrinopeptides to form half-staggered dimers and then a two-stranded protofibril, both through the interaction of the *A*:*a* complementary binding sites. After cleavage of the *B* fibrinopeptides, the *B*:*b* complementary binding sites interact to allow lateral aggregation of protofibrils. The important point here is that each set of sites, *A*:*a* or *B*:*b*, on one molecule is interacting with the complementary sites on a different molecule.

At a higher level of organization, it has been known for some time that the physical properties of clots vary considerably depending upon the conditions under which they are made (Ferry and Morrison, 1947). Extensive characterization (primarily by light-scattering methods) of these different forms of clots and fibrin films has been carried out by Ferry and coworkers and many others (a few examples of recent work are Burchard and Muller, 1980, 1982; Carr et al., 1977; Carr and Hermans, 1978; Cavazza et al., 1981; Hantgan and Hermans, 1979; Hantgan et al., 1980; Muller et al., 1981); recently, some electron microscopic observations of fibrin clots and films in cross-section and in deformed states has been reported (Muller et al., 1984). Probably the most fundamental distinction is between coarse and fine clots. Coarse clots are opaque and are ordinarily formed at low pH and low salt concentration, whereas fine clots are translucent and are produced at higher pH and salt concentration.

Binding sites involved in the formation of the fibrin clot have been identified and one pair has been partially localized in the amino acid sequence. Sites exposed by removal of the A fibrinopeptides in the central domain (called "A" sites) bind to complementary sites (called "a" sites) that are always present in the distal end domain, or γ domain (see the top part of Fig. 1). A peptide consisting of residues 374-396 of the γ-chain binds to the central domain (Olexa and Budzynski, 1981; Horwitz et al., 1984); these and other experiments indicate that this peptide constitutes at least part of the "a" site. Furthermore, another peptide consisting of the residues at the

amino terminus of the α-chain of fibrin (i.e., part of the "A" site exposed by removal of the A fibrinopeptide), Gly-Pro-Arg-X, inhibits the assembly of fibrin monomers to form fibers (Laudano and Doolittle, 1978, 1981; Doolittle and Laudano, 1980; Laudano et al., 1983). This peptide also binds to the D fragment of fibrinogen, which is the end region of the molecule. The important concept that has been supported by the experimental data is that aggregation occurs when the A fibrinopeptides are cleaved, exposing the "A" binding sites (in the central domain) that interact with the "a" sites (in the γ domain), which are always available (Blomback et al., 1978). Although the identified peptides undoubtedly represent only part of complex three-dimensional sites, this scheme of complementary binding sites does account for the half-staggered assembly of molecules in fibrin. The early aggregates in the formation of the clot, termed two-stranded protofibrils, have been observed by electron microscopy in the early stages of clotting (Fowler et al., 1981; Erickson and Fowler, 1983; Williams, 1983; Krakow et al., 1972). The two-stranded protofibril is the expected result of assembly mediated by sets of complementary binding sites (Fig. 1). More detailed structural information about the two-stranded protofibril has also been obtained by computer image analysis of a microcrystalline form of modified fibrinogen in which the molecular packing is similar to that of fibrin (Weisel et al., 1983). The results of this analysis, consistent with the biochemical data, show that the molecules making up the protofibril face each other so that the protofibril is a closed structure.

The role of the release of the B fibrinopeptides in the formation of the clot has been controversial. Blomback first postulated that the A fibrinopeptides are responsible for linear aggregation while the B fibrinopeptides are involved in lateral aggregation (Laurent and Blomback, 1958). This proposal was based on differences in turbidity (in solvent conditions that prevented the formation of fibers) between fibrin with both fibrinopeptides or only the A peptides removed. This idea was later refined by taking into account more data on the chemistry and kinetics of assembly (Blomback et al., 1978). It was proposed that there were binding sites (called "b" sites) that were complementary to those exposed by removal of the B fibrinopeptides (called "B" sites). In other words, this is a scheme like that described above for the A fibrinopeptides, but it was suggested that the B:b sites are involved in aggregation of protofibrils with each other. One version of this proposal is outlined in Fig. 1, modified slightly from the original formulation and using the low resolution shape determined for fibrinogen.

Insight into the precise roles of the fibrinopeptides has been gained by the use of proteolytic enzymes that specifically cleave only the A or B fibrinopeptides. Enzymes such as batroxobin from *Bothrops atrox* remove only the A peptides; batroxobin thus produces the species $(\alpha, B\beta, \gamma)_2$, sometimes called des-AA fibrin, fibrin I, or α-fibrin. A venom from the copperhead snake, *Ancistrodon contortrix,* contains an enzyme, commonly called venzyme, that preferentially removes the B fibrinopeptides (Shainoff and Dardik, 1983); venzyme thus produces the species $(A\alpha, \beta, \gamma)_2$, also called des-BB fibrin or β-fibrin. Thrombin removes both pairs, but the B peptides are cleaved more slowly—primarily, but not entirely, after the assembly process begins. Light-scattering and electron microscopic studies with batroxobin have shown that normal fibers are formed after removal of the A peptides only (Shen et al., 1977; Hantgan and Hermans, 1979; Hantgan et al., 1983). In other words, the cleavage of the B peptides is not required for lateral aggregation of the protofibrils. On the other hand, when the B peptide itself is removed, there is trimerization but no fiber formation at physiological temperature (Shainoff and Dardik, 1983). However, if this reaction is carried out at lower temperatures (e.g., ~20°C or lower), the protein does clot (Shainoff and Dardik, 1979). Aggregation resulting from removal of the B fibrinopeptides is abolished by removal of Bβ 1-42 by plasmin, suggesting that the NH_2-terminal portion of the β-chain of fibrin constitutes an important part of the "B" binding site.

In this paper, I describe investigations into the mechanism of assembly of the fibrin clot and provide new information on how variations in the basic structural plan can lead to clots with different physical properties. In particular, structural studies have confirmed and extended the above findings on the role of the removal of the fibrinopeptides in the formation of fibrin. The band patterns of fibrin formed with the various proteolytic enzymes described above under different environmental conditions were examined by electron microscopy and interpreted in terms of molecular structure and packing. Furthermore, the widths of the fibers made under all of these conditions have been measured. The results indicate that the fiber diameters are relatively uniform, but that the extent of lateral aggregation of the fibers themselves varies.

MATERIALS AND METHODS

Fibrin Formation

Highly purified, plasminogen-free, human fibrinogen, 97–100% clottable, was obtained from Imco Co., Stockholm, Sweden. The fibrinogen, at a concentration of 0.5–2.0 mg/ml, was dialyzed into the appropriate buffer, as described below; the standard fibrinogen buffer was 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4. Salt concentrations were varied from zero to 0.6 M; pH from 5.0 to 9.5; temperature from 0 to 37°C. Some of the most commonly used conditions were 0.05 M NaCl, 0.05 M Tris-HCl, pH 7.4, 0°C; 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, 0°C; 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, 20°C; 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.4, 37°C; 0.40 M NaCl, 0.05 M morpholinoethane sulfonic acid, pH 6.0, 0°C; 0.40 M NaCl, 0.05 M Tris-HCl, pH 7.4, 0°C; 0.40 M NaCl, 0.05 M Tris-HCl, pH 8.5, 0°C. Clotting was initiated by the addition of thrombin to a final concentration of about 2 NIH U/ml. Alternatively, batroxobin (American Diagnostica, Inc., Greenwich, CT; or Pentapharm, Inc., Basel, Switzerland) or venzyme (a generous gift of Dr. John Shainoff, Cleveland Clinic or purchased from Diagnostica Stago, Inc., Paris, France) were

used to selectively cleave either the A or B fibrinopeptides, respectively. A radioimmunoassay system (Mallinkrodt Co., St. Louis, MO) was used for detection of cleavage of fibrinopeptide A. In addition, cleavage of the A or B fibrinopeptides was also followed by sodium dodecyl sulfate gel electrophoresis of reduced samples (Wangh et al., 1983). Gel electrophoresis of dissolved clots showed that there was no ligation by Factor XIII. Under the conditions of the experiments described in this paper, both the A and B fibrinopeptides were removed by thrombin; batroxobin cleaved only the A peptide. Venzyme removed nearly all of the B fibrinopeptide and <5% of the A peptide. Concentrations of enzyme were adjusted so that clotting times were generally <10 min.

Electron Microscopy and Computer Image Processing

Fibrin fibers were applied to 300 or 400 mesh carbon-coated Formvar grids, either by touching a grid to a pre-formed clot, by clotting a thin film of protein on a grid, or by applying a drop of dispersed fibers to the grid. The aim of all of these preparative methods is to obtain a thin enough film on the grid that the electron beam can penetrate for visualization of the structure, while maintaining the integrity of the clot. They succeed to a variable extent. Since all of these methods have their own difficulties, the details of preparation are significant but have previously been fully described elsewhere (Weisel, J. W., manuscript submitted for publication). The most reliable method for visualization of substructure was to add thrombin to fibrinogen in a test tube, and then to disperse the clot with a pasteur pipette just as it began to gel. However, because of concern that the fiber diameters could change after the gelation point, samples were also prepared from gels that had been allowed to stand for at least 100 times the clotting time so that they were fully formed, as well as by producing clots directly on the grids. There were no differences in either the appearances or the diameters of samples prepared in these different ways. The fibers were then negatively contrasted with 1% uranyl acetate and examined in a Philips 400 electron microscope outfitted with a low dose kit. The microscope magnifications were calibrated with tropomyosin paracrystals, with a repeat of 39.5 nm (Caspar et al., 1969). The diameters of the fibers were measured from the electron micrographs with an optical comparator. At least several hundred fibers were measured for each set of conditions.

Electron microscope images of negatively contrasted fibrin were simulated by computer modeling programs that have been described previously (Weisel et al., 1981; Cohen et al., 1983; Weisel et al., 1985). Simulations were carried out on a Digital Equipment Corp. VAX 11/750 computer and the results displayed on an Advanced Electronics Design 767 color graphics terminal. The model for the shape of the fibrin molecule was that previously determined from analysis of the x-ray crystallographic data and electron microscope images (Weisel et al., 1985). A wide variety of molecular packing schemes was examined.

RESULTS

Electron Microscopy of Fibers Formed upon Removal of the A Fibrinopeptides Only

Negatively contrasted human fibrin, formed by clotting fibrinogen with thrombin under physiological conditions (yielding $[\alpha, \beta, \gamma]_2$), displayed a characteristic band pattern in the electron microscope (Fig. 2 *b*). Similar structural studies were carried out on fibers produced by clotting human fibrinogen with batroxobin, which removes only the A fibrinopeptides (yielding $[\alpha, B\beta, \gamma]_2$). Fig. 2 *c* shows a micrograph of such a fiber, formed at pH 7.4, 0.15 M NaCl, 37°C. Except for normal variations in staining and specimen preservation, which occur among all groups of fibers, there is no detectable difference between fibers produced with thrombin or batroxobin. It is also apparent that the diameters of both of these particular fibers are similar, but a quantitative determination of the average fiber diameters will be described later in this paper.

Electron Microscopy of Fibers Formed upon Preferential Removal of the B Fibrinopeptides

The experiment corresponding to that just described, but with venzyme (yielding $[A\alpha, \beta, \gamma]_2$), cannot be carried out because no clot is formed at 37°C (see introduction). However, the protein does polymerize at lower temperatures. Hundreds of clots produced by adding venzyme to human fibrinogen at 0°C were examined in the electron microscope. Such clots are somewhat different in gross visual appearance from those made by cleavage with thrombin or batroxobin; they have been described as "gelatinous" (Shainoff and Dardik, 1983). Electron microscopy revealed that such clots consist almost entirely of fibers similar to those prepared by using thrombin. This uniformity is important since a small percentage of A fibrinopeptides are also cleaved with venzyme (Shainoff and Dardik, 1983); if only a few normal fibers were observed, they could be dismissed as due to this artifact. Furthermore, the same results were obtained by re-precipitating pure $(A\alpha, \beta, \gamma)_2$ prepared as described by Shainoff and Dardik (1983). In this case, all of the fibers present must be made up of molecules missing the B fibrinopeptides only. A representative example of one of the fibers made by clotting human fibrinogen with venzyme is shown in Fig. 2 *a*. Most of the fibers displayed striations with a regular repeat of 22.5 nm. The band pattern of negatively stained fibers is similar to that described above. The fibers are generally not quite as well ordered as those produced by thrombin or batroxobin, but all of the same features are present. There is a broad light, stain-excluding band and three narrower light bands, with the central one being somewhat less distinct in appearance. Again, the fibers appeared to be about the same width as those produced by thrombin, but this question will be considered in more detail below.

Computer Image Simulation of the Fibrin Band Pattern

A molecular packing arrangement for fibrin fibers that accounts for the electron microscope images and is consistent with the chemical and physiological data has been described (Weisel et al., 1981, 1983, 1985*a*). To interpret the images obtained from electron microscopy of fibrinogen clotted by venzyme, it is important to determine if this molecular packing scheme is the only one that can account for the images. For this reason, the same computer programs that have been used in the past to simulate

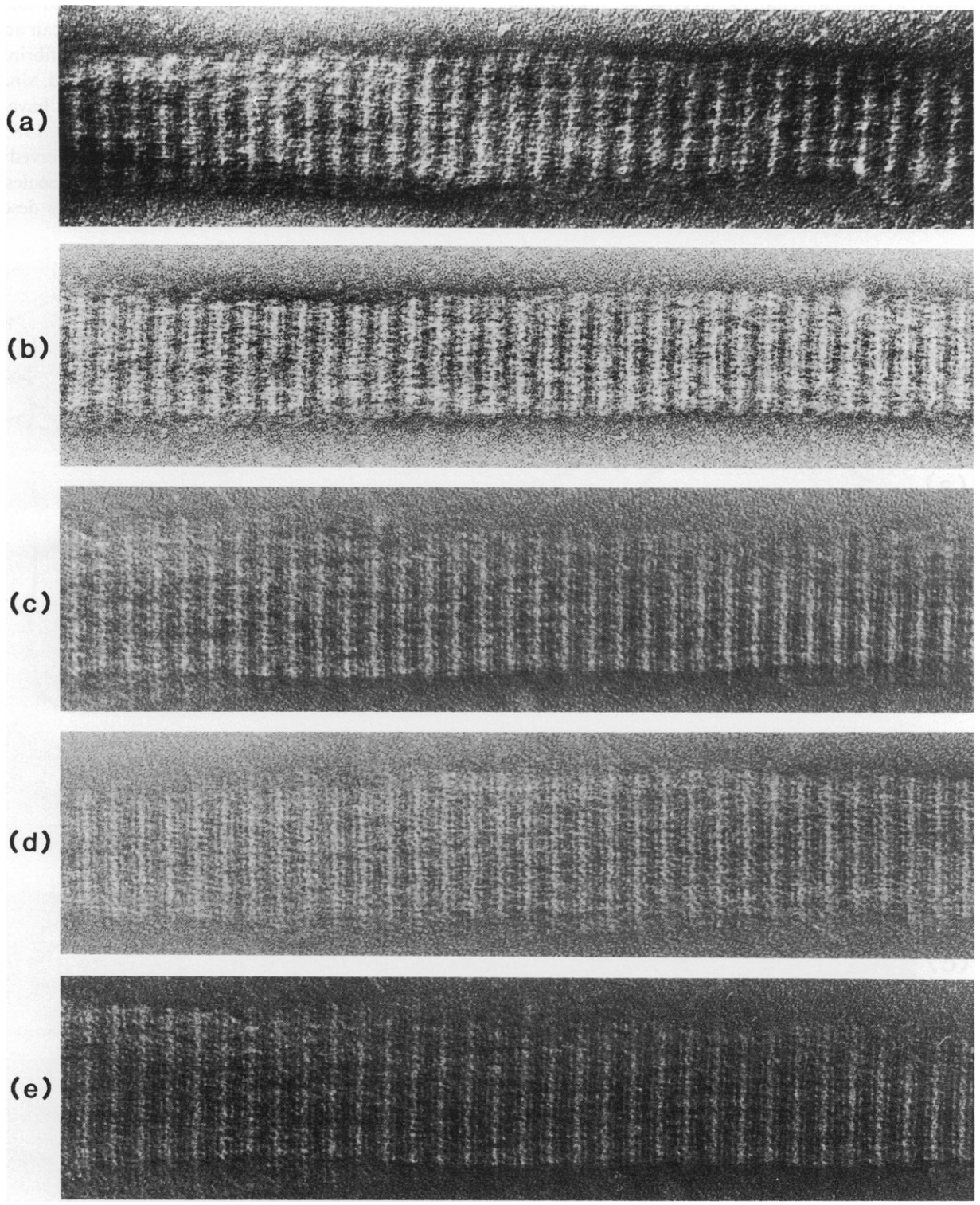

FIGURE 2 Electron micrographs of negatively contrasted fibrin fibers produced by clotting human fibrinogen with several enzymes and under various ionic conditions. Many other conditions were also examined; these are only a few representative images. All fibers were negatively contrasted with 1% uranyl acetate. The repeat is 22.5 nm, and the band patterns are similar, but there are slight differences as described in the text. The enzymes and ionic conditions for each image were as follows: (*a*) venzyme, 0.15 M NaCl, pH 7.4, 0°C; (*b*) thrombin, 0.15 M NaCl, pH 7.4, 37°C; (*c*) batroxobin, 0.15 M NaCl, pH 7.4, 37°C; (*d*) thrombin, 0.05 M NaCl, pH 7.4, 0°C; and (*e*) thrombin, 0.40 M NaCl, pH 7.4, 0°C. Bar, 0.1 μm.

electron microscope images of the various crystal forms were used to simulate the paracrystalline fibrin band pattern. The constraints on this modeling were minimal: the shape of the molecule was that determined previously and the molecules were oriented roughly along the fiber axis. As both the number of molecules and their locations in space were varied systematically, the resulting band patterns were observed on the monitor of a graphics terminal. A great number and variety of possible molecular packing schemes were attempted. This search could be exhaustive, since the pattern being simulated is only one-dimensional. By this means, it was determined that it is highly unlikely that any other packing model can account for the striations of negatively contrasted fibrin. The results of these experiments are not illustrated, since the only successful simulations were those that have been published previously (Weisel et al., 1981, 1983, 1985*a*). It appears that the only way to obtain the observed band pattern with a repeat of 22.5 nm is for the molecules to be half staggered. In other words, the previously described

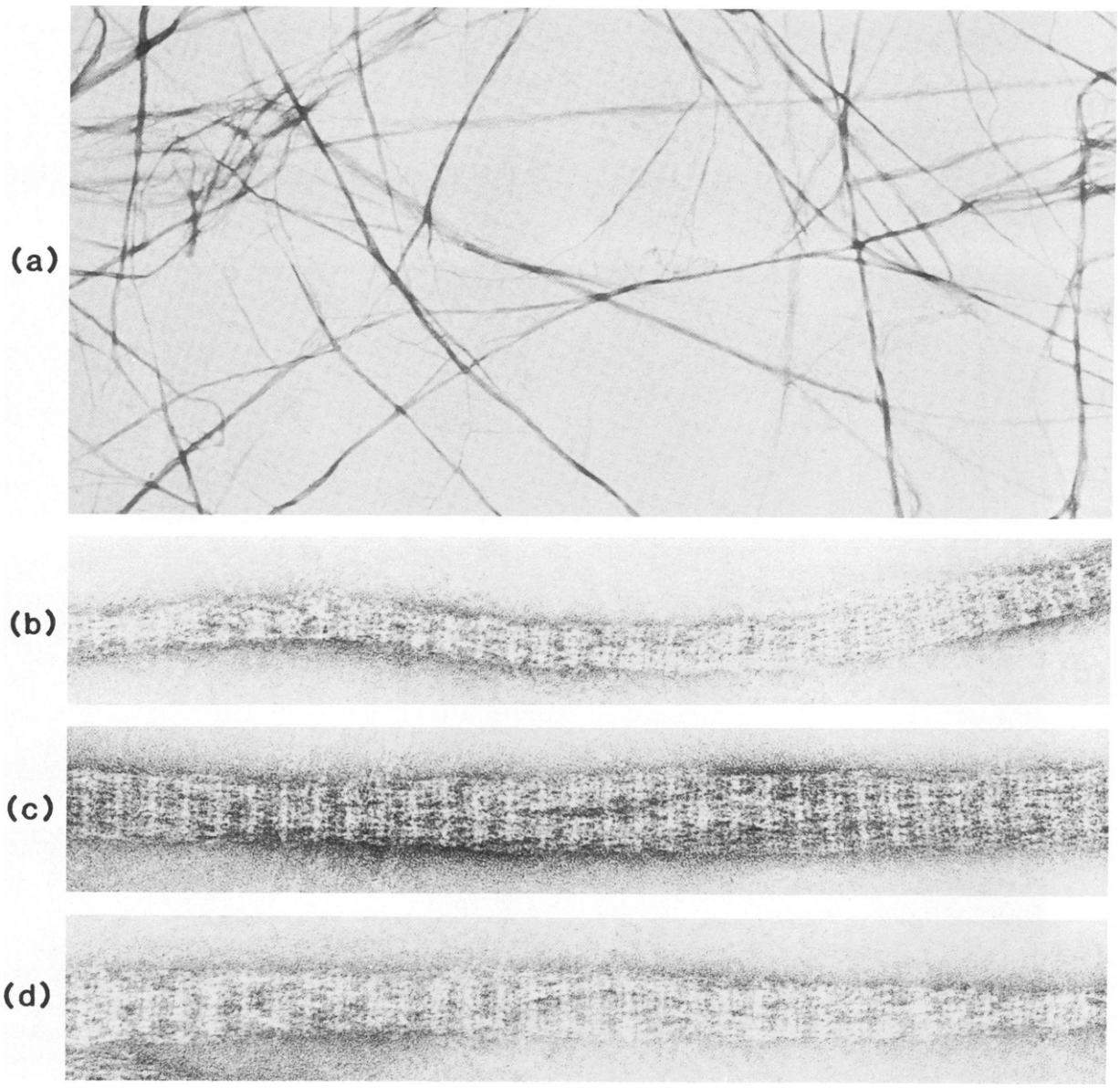

FIGURE 3 Electron micrographs of fibrin produced at high ionic strength and high pH. Fibrinogen in 0.40 M NaCl, pH 8.5 at 0°C was clotted by thrombin or batroxobin (as indicated). (*a*) Low magnification field showing many fibers produced by clotting with thrombin. The fibers are normal in appearance, except for their smaller diameter. Bar, 1.0 μm. (*b* and *c*) Fibers produced by thrombin, shown at higher magnification than in *a*. The cross-striations are similar to those of the wider fibers shown in Fig. 2. The particular fibers shown here are above the average diameter for these conditions because the band pattern is clearer; however, even the thinnest fibers show some beading, although the striations may not show up as clearly as they do in these images. (*d*) Fiber formed by cleavage of the A fibrinopeptides of fibrinogen by batroxobin. This fiber is similar in appearance to those above. The band pattern is identical to those of the fibers above and in Fig.2. (*b–d*) Bar, 0.1 μm.

molecular packing uniquely accounts for the images of fibrin fibers.

Fiber Diameter as a Function of pH, Ionic Strength, and Temperature

The initial experiments described above suggested, unexpectedly, that there are not large differences among the diameters of fibers formed after cleavage of fibrinogen with the three enzymes, thrombin, batroxobin, and venzyme. However, since there is a wide variation in fiber widths in any one experiment, any quantitative conclusions must be made on the basis of accurate measurement of a statistically significant number of fibers. To investigate further the factors influencing lateral aggregation, I also measured the diameters of fibers prepared under a variety of conditions of pH, ionic strength and temperature. Except for the variables just listed, all other conditions were kept constant. Fibers were negatively contrasted with uranyl acetate and examined in the electron microscope. At least several hundred fibers were measured for each condition. A few representative examples of electron micrographs of these fibers formed under different conditions are shown in Fig. 2; an attempt was made to illustrate the effect of variation of all of the parameters in this figure. Fibers produced by cleavage with thrombin, batroxobin, or venzyme and under ionic conditions of 0.05, 0.15, or 0.40 M NaCl and pH 6.0, 7.4, or 8.5 and 0°, 20°, or 37°C were extensively examined, although many other conditions were also tested. The band pattern is the same in all cases.

Under most of the conditions of the experiments described here, there was no readily apparent variation in fiber diameter. An exception is the fibrin formed by either batroxobin, venzyme, or thrombin at pH 8.5, 0.4 M NaCl. These are the conditions for formation of fine clots. In this buffer, nearly all of the fibers are considerably smaller in diameter, agreeing with the results of Ferry and coworkers using other techniques (most recently, see Muller et al., 1984). Fig. 3 *a* is a field of fibers prepared at high salt concentration and high pH as they appear at low magnification in the electron microscope. Fig. 3,*b–d* are micrographs of individual fibers clotted with thrombin or batroxobin and shown at the same magnification as those in Fig. 2. Although the fibers are smaller in diameter, their band pattern is identical to that of the thicker fibers.

A summary of the results of this extensive series of measurements of fiber diameters under many conditions is shown in Fig. 4. Each column represents the average of several hundred fibers; they are shaded differently to distinguish fibers formed by the three proteolytic enzymes, thrombin, batroxobin, and venzyme. It should be noted that several columns are missing because des-BB fibrin does not clot at higher temperatures. At the top of each column is a line indicating one standard deviation. The standard deviations are ~15% of the measured fiber widths, confirming the visual impression of a fairly large variation of widths. Strikingly, the average fiber widths are

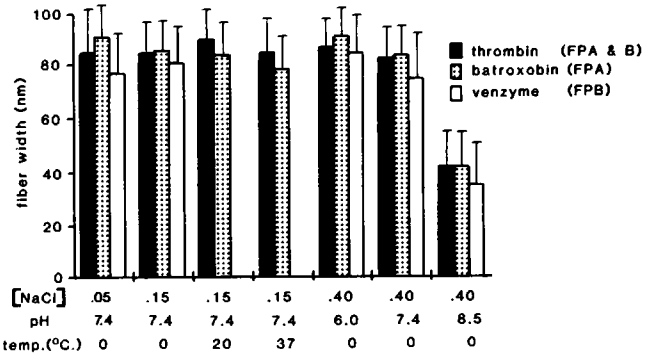

FIGURE 4 Chart of average diameters of fibers produced with different enzymes and under different environmental conditions. Each particular set of conditions is indicated at the bottom of the chart. At least several hundred fibers were measured from electron micrographs of fibers prepared under each set of conditions. Lines indicating one standard deviation from the mean are shown for each column.

the same, within the variation of one standard deviation, under all conditions and enzymes, except at pH 8.5 and 0.4 M NaCl. Under most conditions, then, the fibers are 85 ± 13 nm in diameter.

Lateral Aggregation of Fibrin Fibers

During the course of the measurements of fiber diameter, it soon became apparent that, under certain conditions, there is substantial aggregation of fibers (Fig. 5). In other words, fully formed fibers often appear to aggregate side-by-side with one another. In carrying out the experiments just described, measures were taken to minimize the bias that aggregation could introduce. Most aggregated fibers can be recognized since they usually do not associate along their entire length (Fig. 6 *a*). Thus, it is important to measure only fibers that can be observed along a substantial portion of their lengths; then, diameters can be measured in places where they are not aggregated. This one precaution eliminated nearly all aggregated fibers from consideration. Occasionally, fibers were observed that appeared to be single but showed a slight lateral discontinuity somewhere along their length (Fig. 6 *b*). A few fibers were eliminated on this basis.

Electron microscopy of fibrin at relatively low magnifications revealed a great variation in the extent of lateral aggregation of the fibers. In all of the images, fibers merged at certain points along their course and diverged again to form a three-dimensional network. Under some ionic conditions, there were only those few points of contact, and the fibers were primarily separated from one another. For example, there was little lateral aggregation of fibers in 0.15 M NaCl upon cleavage with batroxobin (Fig. 5 *a*) or in 0.40 M NaCl on cleavage with thrombin (Fig. 5 *c*). On the other hand, under other conditions, such as batroxobin cleavage in 0.05 M NaCl (Fig. 5 *b*) or thrombin in 0.15 M NaCl (Fig. 5 *d*), thick bundles of fibers associated side-by-side were observed. Depending upon the ionic conditions and the fibrinopeptides cleaved, a

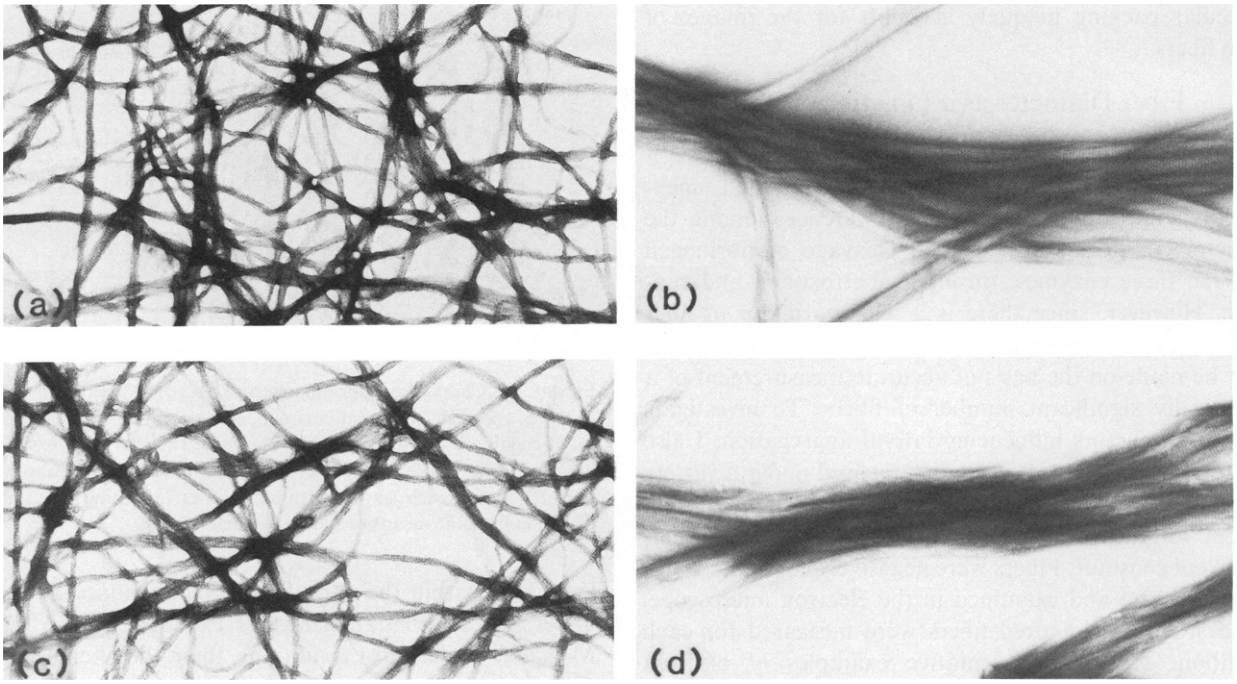

FIGURE 5 Electron micrographs of separated and aggregated fibers. Under certain clotting conditions the fibers are primarily separated, as in the micrographs on the left, while under other conditions many fibers aggregate laterally to form thicker bundles, as in the images on the right. It should be noted, however, that conclusions should not be drawn from a single image. The results shown here are typical, but a variety of images are observed for any one experiment. Also, note that the apparent diameters of individual fibers seen on first inspection of some images at low magnification can be misleading (see text and Fig. 6). Furthermore, although the results described for some conditions are clearly different from others, quantitative estimation of the extent of aggregation of the fibers has not been attempted. Clots here were produced under the following conditions: All four were clotted at pH 7.4 and at 0°C; (*a*) batroxobin, 0.15 M NaCl; (*b*) batroxobin, 0.05 M NaCl; (*c*) thrombin, 0.4 M NaCl; (*d*) thrombin, 0.15 M NaCl. Bar, 1.0 μm.

variety of states in between were also seen. In general, there is more lateral aggregation of fibers upon cleavage of both sets of fibrinopeptides and as the ionic strength or pH is decreased.

DISCUSSION

Intermolecular Binding Sites Exposed by Removal of the B Fibrinopeptides and Their Complementary Sites

In this section, I will first describe the relationship between the known biochemical data on the binding sites and structural studies on fibrin, and then show how the results described in this paper may be interpreted to provide new information on the localization and function of the B fibrinopeptides and related complementary binding sites.

Any meaningful interpretation of the structural studies presented above is dependent upon an adequate understanding of the molecular packing in the fibrin fiber. With the recent development of a more accurate model for the structure of fibrinogen (Weisel et al., 1981, 1985*a;* Cohen et al., 1983), the band pattern of negatively contrasted fibrin has now been analyzed and interpreted in some detail (Weisel et al., 1983; Weisel, J. W., manuscript submitted for publication). The results of these studies, together with biochemical data from other laboratories, indicate that the fibrin band pattern can be accounted for simply and directly from the molecular structure and packing, as follows. Fibrin monomers interact with each other via complementary binding sites in the central and distal end domains to form a protofibril made up of molecules that are half-staggered and bonded end-to-end. The broad, bright band arises from the juxtaposition of the two distal end domains (γ domains) with the central domain (disulfide knot), perhaps with some contribution from the α domain. The narrower light bands on either side come from the proximal end domains (β domain). The central light band that is somewhat variable in appearance arises from the smallest, plasmin-sensitive domain that interrupts the coiled-coil rod. As described above, the analysis of almost every conceivable variation of molecular packing has demonstrated that this interpretation is unique.

The binding sites exposed by removal of the A fibrinopeptides, called the "A" sites, are located in the central domain, while the complementary sites, called the "a" sites, are located in the γ domain (Fig. 1). Analysis of the orthogonal sheet microcrystal form, which is closely related to fibrin, has revealed aspects of the structure of the two-stranded protofibril (Weisel et al., 1983). The orthogonal sheet is made up of layers of tilted two-stranded

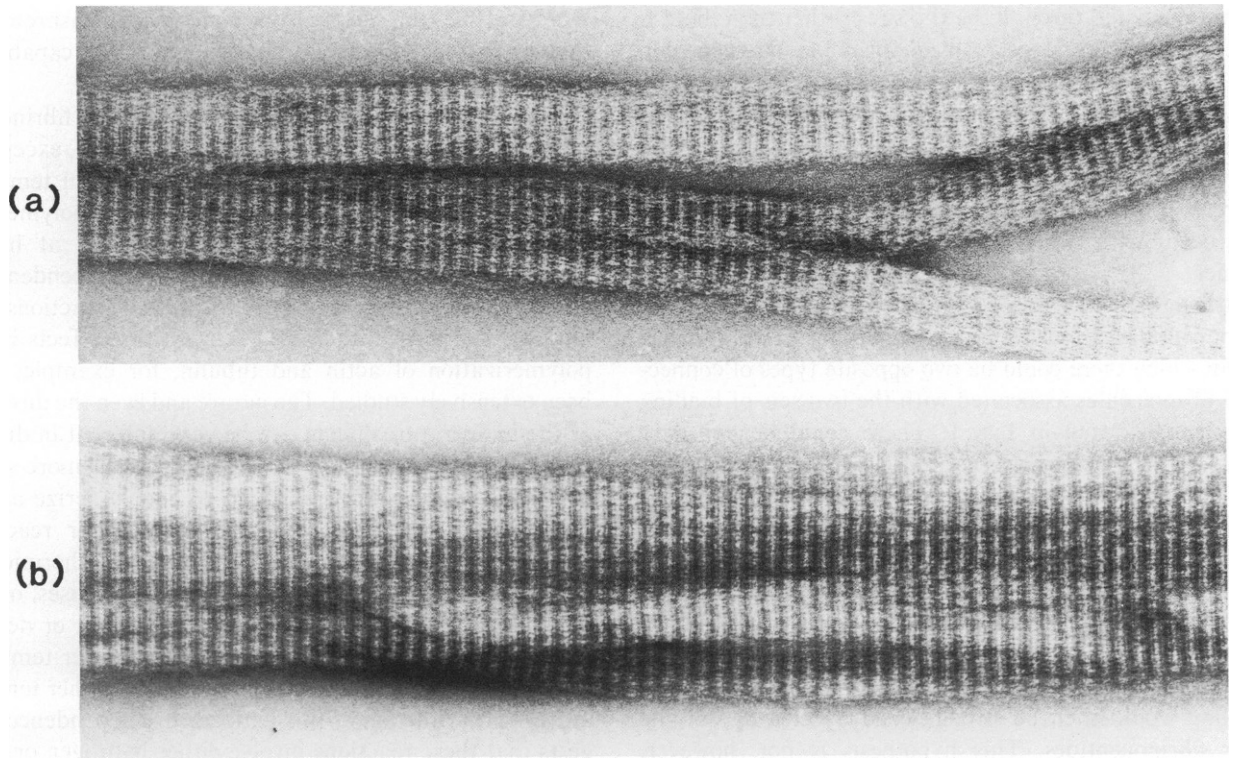

FIGURE 6 Electron micrographs of the lateral aggregation of the fibrin fibers. These micrographs are shown at higher magnification than those in Fig. 5, so that the nature of the lateral aggregation of the fibers can be seen in more detail. In many cases, the fibers line up so that the striations of adjacent fibers are in register. (*a*) In this image there is still space between the upper and middle fiber, although their striations are aligned. (*b*) Here several fibers have completely fused, but boundaries between them can occasionally be seen. Fibers that appear to be considerably thicker than normal often have this appearance (e.g., Fig. 5, *b* and *d*). If such fibers are traced, it can be seen that most of them are not be completely fused along their entire lengths. Bar. 0.1 μm.

rotofibrils. As suggested by Fowler et al. (1981), all nolecules in one filament of the protofibril face in the same irection and the two filaments making up the protofibril ace each other. The protofibril is a "closed structure" in he sense that all binding sites (two "A" sites in the central omain and one "a" in each distal end domain) interact vith molecules in the adjacent, half-staggered filament.

The "B" sites exposed by removal of the B fibrinopeptides are also located in the central domain, since they are t or near the amino terminus of the β-chain (Shainoff and Dardik, 1979). On the other hand, the "b" sites have not previously been localized. With the arrangement of protofibrils predicted from the molecular packing in the orthogonal sheet (Weisel et al., 1983) and confirmed by the simulations of the fibrin band pattern described above, the "b" sites would have to be in the distal end domain (or γ domain) to interact with the central domain of the adjacent half-staggered molecule. The β domain, or proximal end domain, is too far away to interact with the adjacent central domain (Fig. 1 and Weisel et al., 1985*a*), as it could if the molecules were staggered by one third rather than half the molecular length. This localization is strongly suggested by the structural studies in this paper, but must be confirmed by isolation of the binding peptides or other biochemical experiments.

Interactions of the two sets of binding sites cannot be identical, but they may be either very different, as depicted in Fig. 1, or similar. First, consider the simplest interpretation of the data. By this scheme, if there were separate binding sites for protofibril formation and lateral aggregation, exposed by cleavage of the A and B fibrinopeptides, respectively, then there would be no protofibril formation or lateral aggregation without exposure of the appropriate sites. It has been demonstrated experimentally, however, that normal fibers are formed upon removal of either fibrinopeptide. Upon cleavage of the B fibrinopeptides alone, at temperatures below ~15°C, fibrin fibers are formed that appear to be identical to those produced with removal of either both fibrinopeptides or the A fibrinopeptides alone. Although these various fibers result from cleavage with different enzymes, all of the details of the staining patterns are the same. From the analysis of computer simulations described above, we know that there is a unique interpretation of this staining pattern in terms of molecular structure and packing. The observation of similar images thus indicates directly that the molecular arrangement in these fibers is similar. The molecules must be half staggered and bonded end-to-end. Hence, it is likely that these fibers are also made up of two-stranded protofibrils. The intermolecular interactions would then be simi-

lar, but mediated through the B:b sites rather than the A:a sites. These results suggest a mechanism like that shown in Fig. 7, where both sets of sites are involved in the formation of protofibrils, which then aggregate with each other. According to this scheme, the A:a and B:b binding sites on one molecule interact with the same other molecule, whereas in the mechanism depicted in Fig. 1, the interactions occur with two separate molecules in a directional manner.

Although there is clearly no necessity to remove both fibrinopeptides to get lateral aggregation, there is still a way in which there could be two opposite types of connectivity of molecules associated with the two sets of binding sites, as illustrated in Fig. 1. There could be two very different kinds of protofibrils (as shown at the bottom of Fig. 1): one as in the top two rows of filaments resulting from cleavage of the A fibrinopeptides and the other represented by the middle two rows formed by removal of the B peptides. In this case, assume that once either type of protofibril has been formed by cleavage of one pair of fibrinopeptides, that protofibril could aggregate with others without removal of the second set of fibrinopeptides. Bonding would then be strengthened by cleavage of the other fibrinopeptides. This hypothesis is not, however, consistent with the data of Shainoff and Dardik (1983), in which all initial aggregates formed by removal of different fibrinopeptides have the same width but in which binding is stronger with release of both fibrinopeptides. In other words, taken as a whole, the experimental data supports the mechanism shown in Fig. 7 rather than that in Fig. 1. This overall conclusion agrees with that of Shainoff and Dardik (1983), based on physical chemical measurements, that each of the sets of binding sites are capable of functioning individually as aggregation sites.

The fibers produced after cleavage of the B fibrinopeptides are similar, then, to the other fibrin fibers, except for the fact that they do not form at physiological temperatures. In fact, even formation of insoluble polymers of des-AA fibrin (or $[\alpha, B\beta, \gamma]_2$) is eliminated at higher temperatures (41°C). Such a temperature dependence of polymerization is common in biological reactions (cf. Oosawa and Asakura, 1978). Temperature effects in the polymerization of actin and tubulin, for example, have been extensively studied. The nature and even the direction of the temperature effects can be very different in diverse systems. Both tubulin and actin polymers are more stable at higher temperatures and tend to depolymerize at low temperature; this is a common situation for reactions involving hydrophobic bonding, which is endothermic and increases in strength as the temperature increases. on the other hand, the polymerization of $(A\alpha, \beta, \gamma)_2$, or des-BB fibrin, like that of flagellin, occurs only at lower temperatures, and these structures depolymerize at higher temperatures. The direction of this temperature dependence suggests that these reactions involve either hydrogen or ionic bonding, or both, since the forces involved here increase with decreasing temperature.

Lateral Aggregation of Protofibrils to Form a Fiber

Fibrin fibers are formed by lateral aggregation of protofibrils. Since fibers result from removal of either set of

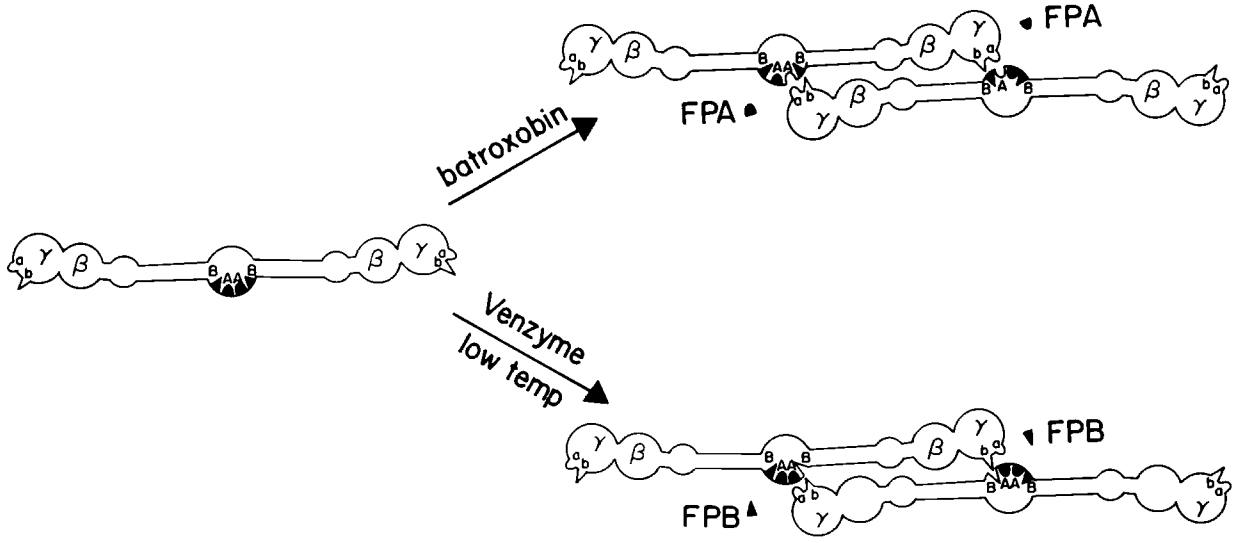

FIGURE 7 A model for fibrin assembly involving two sets of complementary binding sites: both for protofibril formation. The molecular shape and all of the symbols are the same as in Fig. 1. In this case, however, both sets of binding sites, *A*:*a* and *B*:*b*, on any one molecule interact with complementary sites on the same other molecule. In other words, the same aggregate is formed by the action of either batroxobin, which removes only the *A* fibrinopeptides, or venzyme, which cleaves only the *B* peptides. Large protofibrils and fibers are produced by venzyme only at low temperatures. The cleavage of both fibrinopeptides by thrombin results in the formation of the same, but more stable, protofibril. The evidence of both structural data presented here combined with biochemical data described elsewhere (Shainoff and Dardik, 1983; see also the discussion in the text) indicates that this scheme is more likely than that in Fig. 1.

fibrinopeptides, without the necessity of cleaving both sets, this enzymatic step must be related only to protofibril formation. The protofibrils themselves must then aggregate by a self-assembly process, i.e., all of the information necessary for the assembly of protofibrils to make a fiber is present in the protofibril. There is no switch, like the removal of the fibrinopeptides is for protofibril formation, to initiate this reaction.

There may, however, be specific binding sites involved, like those required for the formation of the protofibril itself. The high degree of order in the axial direction in fibrin could indeed suggest such specificity. If there are specific binding sites, they must be weak enough so that they are not ordinarily reactive between fibrinogen, or even individual fibrin, molecules. There is an alternative possibility to account for the lateral aggregation of protofibrils that avoids the difficulties of sites that could cause interaction of fibrinogen molecules. There could be a binding site formed by the juxtaposition of the distal end domains of two molecules bonded end-to-end that reacts with a complementary binding site that is always present in the central domain of an adjacent molecule. Both of these sites would be on the face of the molecule opposite from the A:a and B:b sites. The data of Olexa and Budzynski (1981) and Budzynski et al. (1983) could be interpreted to suggest that such binding sites may exist. These investigators found that fragment E (the central region of the molecule) binds to insolubilized, cross-linked fibrin or to D dimer (D is the end region of the molecule; D dimer is two of these, bonded end-to-end as they are in fibrin), but not to fibrinogen or fibrin monomer or fragment D alone. These experiments suggest that a new binding site is formed by the end-to-end bonding of two molecules. Such a scheme would account for the highly specific lateral aggregation of protofibrils without the involvement of the B:b sites and without having to postulate sites that are present on fibrinogen and could cause its aggregation.

To assess accurately the extent of lateral aggregation of protofibrils under various salt conditons, the diameters of several hundred fibers were measured for each set of conditions. The unexpected result of these measurements is that, except at the extremes of high salt and high pH where fine clots are produced, the diameters are remarkably constant under a wide variety of conditions. The average fiber diameter was found to be 85 ± 13 nm. With an average protofibril spacing of ~5 nm (Weisel, J. W., manuscript submitted for publication), there would be about 14 to 22 protofibrils across a typical fiber. There have been few other studies of fiber diameter by electron microscopy. An early paper by Hawn and Porter (1947) gives some measurements of fibers but does not address the issues of heterogeneity and lateral aggregation of fibers. Shah et al. (1982) measured fiber diameters under only one set of conditions, but, interestingly, they found fibers at both of the same diameters observed in the present study under different ionic conditions. Muller et al. (1984) did some measurements of clots under two conditions, but their specimens were fixed, embedded, and sectioned or critical point dried; consequently, although visualization of the three-dimensional structure is better, they could not have easily distinguished a thick fiber from several laterally aggregated fibers.

Since salt concentration, pH, and temperature would be expected to have some effect on the aggregation of protofibrils, there must be some sort of mechanism for maintaining a constant diameter. Although the mechanism is unknown at present, it is apparently not very rigorous or precise since the range of sizes has a fairly large standard deviation from the average size. At greater extremes of pH and salt concentration, such as pH 8.5 and 0.4 M NaCl, whatever forces are involved in width determination are overcome, and thinner fibers are formed. The average fiber diameter here was found to be ~30–40 nm. This value is slightly different from that measured by Muller et al. (1984) under similar ionic conditions. Considering that these authors used bovine fibrinogen rather than human and that both their methods of clot formation and preparation for electron microscopy are considerably different than those employed in the present study, I think that the agreement of data is good. It should be noted of the different methods employed that each has its own strengths and weaknesses and was chosen for its applicability to particular aspects of these two very different studies.

Lateral Aggregation of Fibers

As mentioned in the introduction, there has been a great deal of research on the physical properties of clots formed under various conditions. The striking differences between coarse and fine clots are clearly obvious, even by eye, for example. This is why the results indicating that the fibers do not vary greatly in diameter were so unexpected. Fibrin formation has also been an important focus for light-scattering studies for many years (recent work includes Burchard and Muller, 1980, 1982; Carr et al., 1977; Carr and Hermans, 1978; Cavazza et al., 1981; Hantgan and Hermans, 1979; Hantgan et al., 1980; Muller et al., 1981). Results from many of these experiments from several laboratories suggested that the fiber width varied widely.

The apparent discrepancies between results using these two different techniques have been resolved in this paper. Electron microscopy shows that, although the diameters are relatively constant, the extent of aggregation of fibers is not. This effect is graded, i.e., it appears to vary smoothly with solvent conditions. Because of this effect, the extent of aggregation of fibers is not easy to quantitate; quantitation is better left to the method of light scattering since it measures average or global properties, while electron microscopy better displays the properties of individual fibers. This is another example of the power of combining these two methods, as discussed by Hantgan et al. (1980). There are, however, dramatic differences in the appear-

ance of the clot at opposite extremes of conditions. Fig. 5 shows electron micrographs of fields of fibers at low magnification to indicate the differences in the extent of aggregation of fibers. There is relatively little aggregation of fibers produced by treatment of fibrinogen in 0.15 M NaCl with batroxobin (Fig. 5 *a*) or in 0.4 M NaCl with thrombin (Fig. 5 *c*). On the other hand, there is considerable aggregation upon cleavage with batroxobin in 0.05 M (Fig. 5 *b*) or thrombin in 0.15 M NaCl (Fig. 5 *d*); under these conditions, thick fiber bundles are often observed. The pH and temperature were the same (pH 7.4 and 0°C) for all of the experiments shown. It is important to note that at physiological ionic strength (0.15 M NaCl) there appears to be a considerable difference in the extent of aggregation of fibers depending upon whether or not the B fibrinopeptides have been released.

In contrast to previous research cited above, preliminary results from a recent light-scattering study (Carr et al., 1985) indicates that, in the presence of 5 mM $CaCl_2$, there is little difference in light-scattering parameters of clots formed with thrombin and those formed with batroxobin. Fibers made under these conditions must be analyzed by electron microscopy to interpret these new light-scattering results further. These results suggest, however, that other factors, such as calcium, also play a role in the lateral aggregation of fibrin fibers.

Little is yet known about the process of aggregation of fibers but, it is interesting to note the appearance of aggregated fibers at higher magnification in the electron microscope. Fig. 6 *a* shows several fibers that appear to be just beginning to coalesce. It may be seen that the structures quite often line up with the striations in all of the fibers in register. Fibers that appear to be very broad are occasionally seen (Fig. 6 *b*). That such structures represent not a single thick fiber but several that have fused can usually be shown by following the structures until they divide. Even when this cannot be done, slight lateral discontinuities across the width of the fiber, representing planes of fusion, can usually be seen (Fig. 6 *b*).

Why Does Fibrinogen Have Two Pairs of Fibrinopeptides?

A key question concerns the function of the B fibrinopeptides and the B:b binding sites. Part of the answer has already come from previous biochemical studies, as discussed above. It is important to note that the present structural studies are consistent with most light-scattering and other chemical and physical data. We now know, however, that all of these results must be interpreted in terms of lateral aggregation of fibers rather than simply fiber width. Removal of both sets of fibrinopeptides appears to result in more lateral aggregation of fibers than if only one is cleaved. There is a striking difference in appearance in the electron microscope that parallels the difference seen by light scattering. Like the changes in light scattering, this effect is most apparent under physiological conditions.

The cleavage of the B fibrinopeptides has been described as having an effect similar to lowering the ionic strength (Shen et al., 1977). These authors showed that, although the removal of the B fibrinopeptides does not expose sites unique for lateral aggregation, this event has profound effects on the overall form of the clot and the kinetics of clotting. There is a strong correlation between the kinetics of fiber formation and lateral aggregation. If the clotting reaction is fast, i.e., the gelation time is short, there is more lateral aggregation of fibers than if clotting is slower. Factors that cause the reaction to be faster, such as lower ionic strength or pH, or removal of the B fibrinopeptides, also tend to create more lateral aggregation. In other words, the observed lateral aggregation of fibers to form fiber bundles may be brought about by the normal process of cleavage of the B fibrinopeptides or by the nonphysiological change of ionic strength, pH, etc.

The energetics of the early stages of polymerization of fibrin with different sets of fibrinopeptides missing has been studied by Shainoff and Dardik (1983), using the techniques of analytical ultracentrifugation and column chromatography. They measured the relative strengths of binding of the B:b and A:a binding sites, as well as the effect of temperature on these reactions (see also Edgar and Prentice, 1980). The results of these studies suggest that there is strong cooperativity between the binding at the two sites. These experiments also indicate that an increased lateral aggregation of fibers may be an indirect effect of strengthening the bonding in the protofibril. Thus, a change in aggregation properties can be brought about by many factors that affect the bonding between molecules in the protofibril, such as ionic conditions, protein concentrations, or cleavage of the second set of fibrinopeptides.

Both strengthening of the bonding in the protofibril and lateral aggregation increase the stability of the clot, which has important clinical implications. For example, in vivo administration of thrombin results in intravascular coagulation, while administration of batroxobin results in defibrination. There is controversy over the mechanism of the defibrination: whether the batroxobin creates finely dispersed microclots or only soluble fibrin, either of which would be more easily lysed than a clot formed with thrombin. Furthermore, batroxobin does not activate Factor XIII, which stabilizes fibrin with covalent cross-links. In any case, a clot produced by thrombin is clearly more stable with respect to plasmin cleavage than one formed by batroxobin. Similarly, the necessity of cleavage of the B fibrinopeptides for stabilization of the clot could act as a preventative of intravascular coagulation by small amounts of thrombin that disperse from the site of tissue injury (Shen et al., 1977). Low concentrations of thrombin would result in the removal of only the A fibrinopeptides so that the clot would be readily dissolved. Alternatively, Shainoff and Dardik (1983) suggest that the function of the delayed

release of the B fibrinopeptides may be to allow the rapid dissociation of early soluble fibrin complexes so that they can equilibrate across interstitial fluids and to allow for their rapid uptake by phagocytic cells.

Molecular Mechanism of Clot Assembly

We can now identify several distinct steps as part of the molecular mechanism of formation of the fibrin clot (Fig. 8). The "switch" initiating the dramatic change in solubility of the molecule is the cleavage of the fibrinopeptides by thrombin. Under normal physiological conditions, the A peptides are cleaved first. The B fibrinopeptides are cleaved more slowly, but it is not necessary for polymerization to occur before they are removed (Nossel et al., 1983). The fibrin molecules interact via the A:a complementary binding sites discussed above to yield the two-stranded protofibril; the cleavage of the B fibrinopeptides and consequent interaction of the B:b sites strengthens the

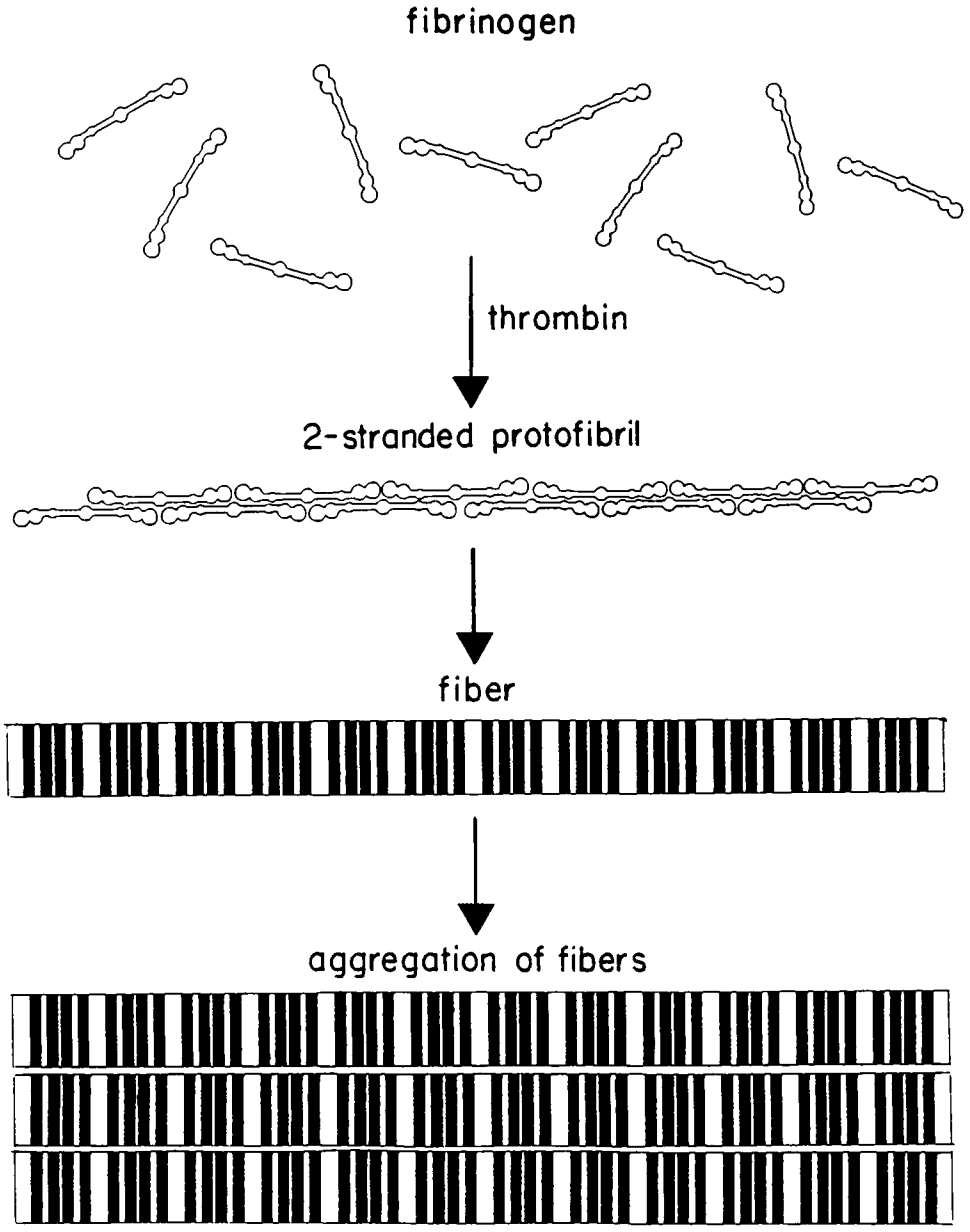

FIGURE 8 A depiction of several steps in the formation of a fibrin clot. Fibrinogen molecules are freely soluble in the blood. Upon cleavage of one or both of the fibrinopeptides by thrombin, the molecules aggregate to form a two-stranded protofibril, made up of molecules that are half staggered and bonded end-to-end. Protofibrils then aggregate laterally to form fibers. Fiber widths vary somewhat, but generally are similar under a wide variety of conditions. Finally, the fibers themselves aggregate laterally to form thicker fibers. They tend to associate in register, as shown here, but the extent of aggregation is highly dependent on the ionic and enzymatic conditions, as described in the text. Clearly, this scheme is oversimplified. It leaves out several steps studied by other investigators, including details of the formation of oligomers, branching of fibers to form a gel, and the creation of an ordered three-dimensional network. However, it is meant only to convey some of the major points of this paper.

protofibril. Protofibrils then aggregate laterally to form fibers ~85 nm in diameter. The fibers themselves also aggregate laterally, often in such a way that adjacent fibers are in register. The extent of aggregation of fibers depends upon both the solvent conditions and whether or not the B fibrinopeptides have also been cleaved. Fibers must also branch so that a three-dimensional gel is formed (Muller et al., 1984). Recently, there is evidence from gel permeation experiments and scanning electron microscopy that the fibrin gel itself is not a random network of fibers, but is somewhat ordered (Blomback et al. 1984).

The new results presented here are consistent with what has been observed for other biological assembly processes. For the most part, biological structures are assembled in a series of discrete steps. Thus, in this case, fibers of a certain size are formed from protofibrils, and larger fibers are not assembled de novo, but are made up of several smaller fibers. Such a process permits simple steps with many points of control. Furthermore, fibers with a limited width are a very efficient way to fill space with a gel or plug using only a small amount of material. The physical and mechanical properties of the clot, which influence its physiological and pathological properties, depend upon the extent of lateral aggregation of fibers, which is determined by ionic and enzymatic conditions.

I thank Drs. Carolyn Cohen, John Shainoff, and Lee Makowski for many helpful discussions and for reading the manuscript. Dr. Shainoff also generously provided venzyme necessary for some of the experiments and advice on its use.

The author is an Established Investigator of the American Heart Association and was supported by a research grant from the National Institutes of Health (HL-30954) and a grant-in-aid from the Pennsylvania Chapter of the American Heart Association. Shared instrumentation grants, for the establishment of our electron microscope and computer facilities, from the National Institutes of Health (RR-01907 to Dr. J. Murray, GM-28750 to Dr. T. Frey, and RR-01747 to Dr. Michael Summers) and the National Science Foundation (PCM82-19960 to Dr. F. Pepe) are also gratefully acknowledged.

Received for publication 13 April 1986 and in revised form 7 July 1986.

REFERENCES

Blomback, B., B. Hessel, D. Hogg, and L. Therkildsen. 1978. A two-step fibrinogen to fibrin transition in blood coagulation. *Nature (Lond.).* 275:501–505.

Blomback, B., M. Okada, B. Forslind, and U. Larsson. 1984. Fibrin gels as biological filters and interfaces. *Biorheology.* 21:93–104.

Budzinski, A. Z., S. A. Olexa, and B. V. Pandya. 1983. Fibrin polymerization sites in fibrinogen and fibrin fragments. *Ann. NY Acad. Sci.* 408:301–313.

Burchard, W., and M. Muller. 1980. Statistics of branched polymers composed of rod substructures: a model for fibrin before clot formation. *Int. J. Biol. Macromol.* 2:225–234.

Burchard, W., and M. Muller. 1982. Determination of fibrin structures by light scattering. *In* Fibrinogen—Recent Biochemical and Medical Aspects. A. Henschen, H. Graeff, and F. Lottspeich, editors. Walter de Gruyter Co., New York. 11–45.

Carr, M. E., and J. Hermans. 1978. Size and density of fibrin fibers from turbidity. *Macromolecules.* 11:46–50.

Carr, M. E., M. Kaminski, J. McDonagh, and D. A. Gabriel. 1985. Influence of ionic strength, peptide release, and calcium ion on the structure of reptilase and thrombin derived clots. *Thromb. Haemostasis.* 54:159.

Carr, M. E., L. L. Shen, and J. Hermans. 1977. Mass-length ratios of fibrin fibers from gel permeation and light scattering. *Biopolymers.* 16:1–15.

Caspar, D. L. D., C. Cohen, and W. Longley. 1969. Tropomyosin: crystal structure, polymorphism, and molecular interactions. *J. Mol. Biol.* 41:87–107.

Cavassa, C., C. Cuniberti, E. Patrone, F. Pioli, M. Rocco, and V. Terfiletti. 1981. Self-assembly of fibrin monomers: a light scattering and electron microscopic investigation. *Ital. J. Biochem.* 30:75–89.

Cohen, C., and N. M. Tooney. 1974. Crystallization of a modified fibrinogen. *Nature (Lond.).* 251:659–660.

Cohen, C., J. W. Weisel, G. N. Phillips, C. V. Stauffacher, J. P. Fillers, and E. Daub. 1983. The structure of fibrinogen and fibrin. I. Electron microscopy and x-ray crystallography of fibrinogen. *Ann. NY Acad. Sci.* 408: 194–213.

Doolittle, R. F., and A. P. Laudano. 1980. Synthetic peptide probes and the location of the fibrin polymerization sites. *Protides Biol. Fluids.* 28:311–316.

Edgar, W., and C. R. M. Prentice. 1980. The effect of temperature on formation of fibrin complexes. *Thromb. Res.* 18:758–772.

Erickson, H. P., and W. E. Fowler. 1983. Electron microscopy of fibrinogen, its plasmic fragments and small polymers. *Ann. NY Acad. Sci.* 408:146–163.

Ferry, J. D., and P. R. Morrison. 1947. Preparation and properties of serum and plasma proteins VIII. Conversion of human fibrinogen to fibrin under various conditions. *J. Am. Chem. Soc.* 69:388–400.

Fowler, W. E., R. R. Hantgan, J. Hermans, and H. P. Erickson. 1981. Structure of the fibrin protofibril. *Biochemistry.* 78:4872–4876.

Hantgan, R., W. Fowler, H. Erickson, and J. Hermans. 1980. Fibrin assembly: a comparison of electron microscopic and light scattering results. *Thromb. Haemostasis.* 44:119–124.

Hantgan, R., J. McDonagh, and J. Hermans. 1983. Fibrin assembly. *Ann. NY Acad. Sci.* 408:344–366.

Hantgan, R. R., and J. Hermans. 1979. Assembly of fibrin: a light scattering study. *J. Biol. Chem.* 254:11272–11281.

Hawn, C. V. Z., and K. R. Porter. 1947. The fine structure of clots formed from purified bovine fibrinogen and thrombin: a study with the electron microscope. *J. Exp. Med.* 86:285–297.

Horwitz, B. H., A. Varadi, and H. A. Scheraga. 1984. Localization of a fibrin gamma-chain polymerization site within segment thr-374 to glu-396 of human fibrinogen. *Proc. Natl. Acad. Sci. USA* 81:5980–5984.

Krakow, W., G. F. Endves, B. M. Siegel, and H. A. Sheraga. 1972. An electron microscopic investigation of the polymerization of bovine fibrin monomer. *J. Mol. Biol.* 71:95–103.

Laudano, A. P., B. A. Cottrell, and R. F. Doolittle. 1983. Synthetic peptides modeled on polymerization sites. *Ann. NY Acad. Sci.* 408:315–329.

Laudano, A. P., and R. F. Doolittle. 1978. Synthetic peptide derivatives that bind to fibrinogen and prevent the polymerization of fibrin monomers. *Proc. Natl. Acad. Sci. USA.* 75:3085–3089.

Laudano, A. P., and R. F. Doolittle. 1981. Influence of calcium on the binding of fibrin amino terminal peptides to fibrinogen. *Science (Wash. DC).* 212:457–459.

Laurent, T. C., and B. Blomback. 1958. On the significance of the release of two different peptides from fibrinogen during clotting. *Acta Chem. Scand.* 12:1875–1877.

Mosesson, M. W., and R. F. Doolittle. 1983. Molecular Biology of Fibrinogen and Fibrin. New York Academy of Sciences, New York.

Muller, M., H. Lasarcyk, and W. Burchard. 1981. Fibrinogen-fibrin transformation. 2. Influence of temperature, pH and of various enzymes on the intermediate structures. *Int. J. Biol. Macromol.* 3:19–24.

Muller, M. F., H. Ris, and J. D. Ferry. 1984. Electron microscopy of fine fibrin clots and fine and coarse fibrin films. *J. Mol. Biol.* 173:369–384.

Nossel, H. L., A Hurlet-Jensen, C. Y. Liu, J. A. Koehn, and R. E. Canfield. 1983. Fibrinopeptide release from fibrinogen. *Ann. NY Acad. Sci.* 408:269–279.

Olexa, S. A., and A. Z. Budzinski. 1981. Localization of a fibrin polymerization site. *J. Biol. Chem.* 258:3544–3549.

Oosawa, F., and S. Asakura. 1975. Thermodynamics of the Polymerization of Protein. Academic Press, Inc., New York.

Shah, G. A., I. A. Ferguson, T. Z. Dhall, and D. P. Dhall. 1982. Polydispersion in the diameter of fibers in fibrin networks: consequences on the measurements of mass-length ratios by permeability and turbidity. *Biopolymers.* 21:1037–1047.

Shainoff, J. R., and B. N. Dardik. 1979. Fibrinopeptide B release in fibrin formation. *Science (Wash. DC).* 204:200–202.

Shainoff, J. R., and B. N. Dardik. 1983. Fibrinopeptide B in fibrin assembly and metabolism: physiologic significance in delayed release of the peptide. *Ann. NY Acad. Sci.* 408:255–267.

Shen, L. L., J. Hermans, J. McDonagh, and R. P. McDonagh. 1977. Role of fibrinopeptide B release: comparison of fibrins produced by thrombin and ancrod. *Am. J. Physiol.* 232(6):H629–H633.

Tooney, N. M., and C. Cohen. 1972. Microcrystals of a modified fibrinogen. *Nature (Lond.).* 237:23–25.

Tooney, N. M., and C. Cohen. 1977. Crystalline states of a modified fibrinogen. *J. Mol. Biol.* 110:363–385.

Wangh, L. J., L. J. Holland, R. J. Spolski, B. S. Aprison, and J. W. Weisel. 1983. Xenopus fibrinogen: characterization of subunits and hormonal regulation of biosynthesis. *J. Biol. Chem.* 258:4599–4605.

Weisel, J., G. N. Phillips, and C. Cohen. 1983. The structure of fibrinogen and fibrin: II. Architecture of the fibrin clot. *Ann. NY Acad. Sci.* 408:367–379.

Weisel, J., S. G. Warren, and C. Cohen. 1978. Crystals of modified fibrinogen: size, shape and packing of molecules. *J. Mol. Biol.* 126:159–183.

Weisel, J. W., G. N. Phillips, and C. Cohen. 1981. A model from electron microscopy for the molecular structure of fibrinogen and fibrin. *Nature (Lond.).* 289:263–267.

Weisel, J. W., C. V. Stauffacher, E. Bullitt, and C. Cohen. 1985*a*. A model for fibrinogen: domains and sequence. *Science (Wash. DC).* 230:1388–1391.

Weisel, J. W., C. V. Stauffacher, and C. Cohen. 1985*b*. Low resolution molecular structure of fibrinogen and fibrin: Correlation of domains and sequence. *Thromb. Haemostasis.* 54:224.

Williams, R. C. 1983. Morphology of fibrinogen monomers and fibrin protofibrils. *Ann. NY Acad. Sci.* 408:180–193.