Kinetics of Fibrin Oligomer Formation Observed by Electron Microscopy†

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ABSTRACT: Fibrin oligomers, obtained by interrupting the polymerization of fibrinogen at early stages with a thrombin inhibitor, were examined by electron microscopy. The lengths of the various oligomers were determined and histograms were constructed to show their distribution. The length distribution agreed with a theory based on the assumption that thrombin releases the second A peptide more rapidly than the first, with a ratio of the release rates for the two peptides which is qualitatively in agreement with that deduced from oligomer size distributions obtained by agarose gel electrophoresis.

In the initial stages of fibrin polymerization, thrombin cleaves an A peptide from each of two Aα chains of the fibrinogen molecules (Doolittle, 1973). Loss of the A peptide exposes a binding site in the central domain of the molecule which is complementary to a site already exposed in the terminal domain of fibrinogen (Olexa & Budzynski, 1980). The thrombin-activated monomers then self-assemble in a half-staggered arrangement to form linear oligomers which have twice the cross-sectional area of a monomer (Shulman & Ferry, 1951; Ferry, 1952). A variety of evidence is consistent with the proposal (Smith, 1980) that A peptides are removed one at a time from fibrinogen to form a mixture of monofunctional and difunctional reactive units (Janmey, 1982a). Experimental studies of the kinetics of Aα chain cleavage by thrombin (Martinelli & Scheraga, 1980; Blombäck et al., 1978) and of the rate of assembly of activated fibrin monomers (Hantgan & Hermans, 1979) have shown that the activation of fibrinogen can be made slow compared to the self-assembly of the activated fibrin monomers (Bale et al., 1982). Under these conditions, at intermediate times in the initial oligomer formation, a fibrin oligomer is capped at each end by a monofunctional fibrin unit which has one central domain binding site exposed and bound and one Aα chain still available for reaction with thrombin (Smith, 1980). If a thrombin inhibitor is added to such a system during the course of oligomer formation, the growth of oligomers ceases immediately (Janmey, 1982b; Visser & Payens, 1982).

The size distribution of oligomers present during the polymerization process depends strongly on the relative concentrations of monofunctional (Aα_2) and difunctional (α_2) reactive units. Since the fibrin oligomers are held together by noncovalent interactions, oligomers of different sizes cannot easily be separated by electrophoresis or chromatography. Fibrin oligomers can, however, be covalently ligated by factor XIIIa, and these ligated oligomers, separated by using SDS[1]-agarose gel electrophoresis (Moroi et al., 1975), have proven useful in previous studies of the early events of fibrin polymerization (Bale et al., 1982; Nelb et al., 1980).

There is good evidence that ligation occurs end-to-end between the γ chains of two adjacent monomers within an oligomer (Fowler et al., 1981; Erickson et al., 1981) so that the staggered overlap pattern is not preserved by the ligations. A noncovalently bonded oligomer of degree of polymerization 20, for example, will produce two linear oligomers of 10 units each if it is ligated and then denatured prior to electrophoresis. Also, if ligation is not complete before the ligated oligomers are denatured, then staggered overlap oligomers will break into more than two strands, and the analysis will underestimate the average oligomer size present in solution before denaturation.

Despite the possibility that data obtained from electrophoresis of ligated, denatured oligomers might tend to underestimate the size of the oligomers present in the reacting mixture, analysis of such data has shown that there are larger oligomers present than can be accounted for by a reaction mechanism as outlined above where both the removal of A peptides and the association of binding sites are random events (Nelb et al., 1980; Bale et al., 1982). The shifting of the size distribution toward longer oligomers was first tentatively ascribed to nonrandom ligation by factor XIIIa (Nelb et al., 1980). Subsequent studies have proposed that the presence of larger oligomers can be explained if the peptide removal is nonrandom, with the second A peptide being more easily cleaved off than the first (Janmey, 1982a; Bale et al., 1982). Such nonrandom peptide removal had previously been proposed (Landis & Waugh, 1975).

In the present study, the staggered overlap oligomers are visualized by electron microscopy for a variety of different polymerizing solutions in which the reaction has been stopped prior to gelation. Electron microscopy has the advantage over electrophoresis in that both ligated and nonligated species can be studied in their intact states. It has an advantage, also, over light-scattering studies of the early stages of fibrin polymerization (Palmer & Fritz, 1979; Müller et al., 1981; Wiltzius et al., 1981), which can yield only an average degree of polymerization and an estimate of the breadth of the size distribution.

Electron micrographs are presented here for the solutions similar to those studied previously by gel electrophoresis (Bale et al., 1982). The size distributions obtained by electron microscopy are compared to predictions based on the theory of Janmey (1982a) based on the observations that the two A peptides are released sequentially (Smith, 1980) but at different rates (Landis & Waugh, 1975; Bale et al., 1982). Size distributions determined by electron microscopy are also compared for fibrin oligomers formed in the presence or ab-

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[1] Abbreviations: SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate.

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sence of factor XIIIa and under different conditions of pH and ionic strength.

Materials and Methods

Protein Solutions. Fibrinogen was prepared from human plasma cryoprecipitate by a slight modification of the method of Mosher & Blout (1973) and was generously provided by Dr. E. C. Williams; 99% of the protein in this preparation became incorporated into the clot under standard conditions (Roberts et al., 1974). Human fibrinogen was also obtained from Kabi (lot 64927, 96% clottable). Protein solutions were dialyzed against one of three Tris–NaCl buffers: pH 8.5, ionic strength 0.45; pH 8.5, ionic strength 0.15; pH 7.5, ionic strength 0.15. In all buffers, Tris contributed 0.05 to ionic strength. Protein concentration was determined spectrophotometrically (Blombäck, 1958). Bovine thrombin from Parke Davis was dissolved in and dialyzed against buffer at pH 8.5, ionic strength 0.45, or pH 7.5, ionic strength 0.15, and its activity was measured by clotting tests (Lundblad et al., 1976). Factor XIII (fibrin-stabilizing factor) prepared from human plasma (Lorand & Gotoh, 1970) and dissolved in Tris–NaCl buffer at pH 7.5, ionic strength 0.15, with 0.001 M EDTA, was a gift from Dr. E. C. Williams. Trasylol (proteolytic inhibitor) was obtained from FBA Pharmaceuticals. The thrombin inhibitor *p*-nitrophenyl *p*-guanidinobenzoate (NPGB) was obtained from Sigma Chemical Co. It was dissolved in redistilled dimethylformamide to make a stock solution at a concentration of 0.01 M which was diluted into 9 volumes of aqueous buffer immediately before use. Materials for agarose and polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories.

Factor XIII (1 g/L) was incubated with thrombin (23 units/L) and $CaCl_2$ (0.018 M) for 90 min at room temperature. This appeared to be the optimum time for activation to factor XIIIa under these conditions.

Ligated oligomers were prepared by mixing fibrinogen (6.8 g/L), thrombin (10 units/L), factor XIIIa (30 mg/L), $CaCl_2$ (3.2 mM), and Trasylol (2000 units/L). Aliquots of 50 μL were placed into micro test tubes, and the reaction was interrupted at successive intervals in duplicate aliquots, one by adding 200 μL of a solution containing 3% sodium dodecyl sulfate (SDS) and 9 M urea (for polyacrylamide and agarose gel electrophoresis) and one by adding 200 μL of 10^{-3} M NPGB in Tris–NaCl buffer (for electron microscopy). Polyacrylamide gel electrophoresis of samples reduced by addition of dithiothreitol and agarose gel electrophoresis of unreduced samples were performed as described previously (Bale et al., 1982). The aliquots diluted into buffer containing NPGB were often frozen in liquid nitrogen for convenience and later thawed and further diluted to give a final fibrinogen concentration of 0.34 g/L just prior to preparation of electron microscope grids. Sometimes they were diluted directly without freezing, and no qualitative differences were observed.

Oligomers without ligation were prepared in a similar way except that factor XIIIa was omitted and no gel electrophoresis was done. Also, buffers of various pH and ionic strength were used, and in some cases 1 mM EDTA was substituted for $CaCl_2$. A few partially polymerized samples were diluted into buffer not containing NPGB, placed directly onto grids, and compared with identical samples prepared as described above. One sample was also diluted to two different concentrations (0.68 and 0.14 g/L) prior to being placed on the grid.

Electron Microscopy. Samples of fibrin oligomers prepared under various conditions were fixed for examination in the electron microscope as follows. A carbon film was floated off a mica surface onto distilled water. The film was picked up on copper grids (Ernest F. Fullam, 200 mesh), and the grids were blotted on filter paper and dried under a heat lamp. The carbon film was then made hydrophilic by exposing the grids to a glow discharge in an Edwards vacuum bell jar for about 1 min.

Samples containing fibrin oligomers were diluted to 0.34 g/L as described above, a single drop was applied to each grid and allowed to rest there for several seconds. The grid was then rinsed with 3 drops of 0.01 M Tris–0.01 M $MgSO_4$, pH 8.0. Next, 2 drops of 1% uranyl acetate was applied, and the grids were blotted and dried under a heat lamp.

The samples were examined in a Phillips EM 300 electron microscope, and fibrin oligomers were photographed on 35-mm film. The oligomers were traced with a Numonics digitizer connected to a Hewlett-Packard 9825 B calculator. The dimensions, in arbitrary units, were calculated by the "Length Histogram" program of R. B. Inman (personal communication). The arbitrary units can be converted into microns by determining the length, in those units, of the gradations on standard grids (Ernest F. Fullam, 28 000 and 54 000 lines/in.).

The accuracy of this method of determining length was checked by determining the distance between the striations on those few filaments on which they could be seen: very long, somewhat thicker strands which were present after a relatively long period of incubation with thrombin. The mean distance between striations was determined to be 21 nm, in agreement with the value of 23 nm determined by electron microscopy (Weisel et al., 1978) as well as by X-ray scattering (Stryer et al., 1963; Roska et al., 1982). The error in the present length measurements is estimated to be about 10%.

Histograms were constructed from the experimental results by using the "Length Histogram" program (R. B. Inman, personal communication). The staggered overlap model predicts that for each monomer unit added to an oligomer, the length of the oligomer increases by half of the length of a monomer. Assuming a monomer length of 45 nm (Cohen & Tooney, 1974), the length of an oligomer should increase by 23 nm for each monomer added. Therefore, the oligomer lengths were sorted into bins 23 nm in size, extending from 34 to 4500 nm. Each bin is centered about an integral multiple of 23 nm and includes all oligomers with in ± 11 nm of that integral multiple. For example, bin 4 contains oligomers made up of four monomer units. The length of such an oligomer would be expected to be 5×23, or 115, nm; therefore, bin 4 contains all oligomers of length between 104 and 126 nm.

Theory

The size distribution of oligomers present when an arbitrary mixture of monofunctional and difunctional units have reacted has been derived by Flory (Flory, 1936). In terms of the number of oligomers of degree of polymerization x, the result is

$$N_x = Nr^{x-2}(1 - r) \qquad x > 1 \tag{1}$$

where N is the total number of oligomers larger than monomer and

$$r = \frac{2[\alpha_2]}{2[\alpha_2] + [A\alpha_2]} \tag{2}$$

where $[A\alpha_2]$ and $[\alpha_2]$ are the concentrations of monofunctional and difunctional units, respectively. For the case in which each of the two reactive sites on the monomer becomes activated independently of the other, r can easily be shown to be equal to y, the fraction of the total possible binding sites which has

been uncovered (Flory, 1953). For the more general case in which the two binding sites are uncovered at different rates, as in the case of fibrinogen, where the removal of one peptide seems to influence the removal of the other, the parameter r is a function of both the fraction of peptides removed, y, and q, the ratio of the maximum rates of removal of the two peptides (Janmey, 1982a). In principle, r can be calculated by integrating separately the rate expressions for the formation of monofunctional and difunctional units. The value of y can be estimated experimentally either by measuring directly the removal of A peptides or, as was done in these studies, by measuring the weight fraction of ligated γ chains for oligomers formed in the presence of XIIIa, assuming that the removal of each A peptide leads immediately to binding and ligation as confirmed by earlier studies (Bale et al., 1982). The fact that each staggered overlapped oligomer will have two more exposed binding sites than ligated γ chains makes only a small correction at all but the earliest times. The value of q cannot be measured directly but can be inferred from the values of r, determined by fits of eq 1 to the oligomer size distributions, and y, determined by polyacrylamide gel electrophoresis.

In the electron micrographs, fibrinogen monomers do not appear as clearly as fibrin oligomers, probably because of irregular background with high residual salt and some foreign matter; an accurate count of them could not be made. Since monomers are expected to be by far the most numerous species present at the early stage of reaction considered here, it was not possible to determine the number fractions of oligomers in terms of the total number of all species, but only in terms of the total number of oligomers of degree of polymerization $x \geq 2$. A comparison of the number of oligomers of size x calculated from eq 1 and the experimental results given in the histograms is made by assigning the theoretical value of dimer to be equal to the experimental result (expressed in arbitrary units with 1.0 for the most frequent) and calculating the remaining values relative to that at $x = 2$.

Results

General Appearance of Oligomers. The molecules visible in electron micrographs of partially polymerized fibrin are almost all rodlike, though they appear to be somewhat flexible. The length of these linear molecules varies over a wide range, but their thickness appears to be nearly uniform and approximately 21 ± 4 nm. A few of the molecules, usually the longest ones, appear to be twice the thickness of the others. A representative field of molecules is shown in Figure 1. Neither the qualitative appearance nor the size distribution was noticeably affected by the method by which polymerization was stopped. The presence of EDTA (1 mM) or $CaCl_2$ (3.2 mM) also had no appreciable effect, except that molecules prepared in the presence of EDTA did not seem to stain as well, as has been reported previously (Williams, 1981). Branched structures such as those thought to be formed early in the course of polymerization on the basis of light-scattering measurements (Burchard & Müller, 1980) or seen in electron micrographs of films made from fine clots (M. Müller, H. Ris, and J. D. Ferry, unpublished results) were very rarely seen and then only in more concentrated preparations in which they could not be distinguished from partially overlapping linear oligomers.

A quantitative comparison of the size distribution of oligomers obtained from one partially polymerized solution which was diluted to different extents prior to being applied to the grids showed no differences in either the lengths or thicknesses of the oligomers. This is taken as evidence that the structures observed in the electron microscope are not due to several chains of oligomers aggregating side-by-side in a nonspecific manner during preparation of the sample.

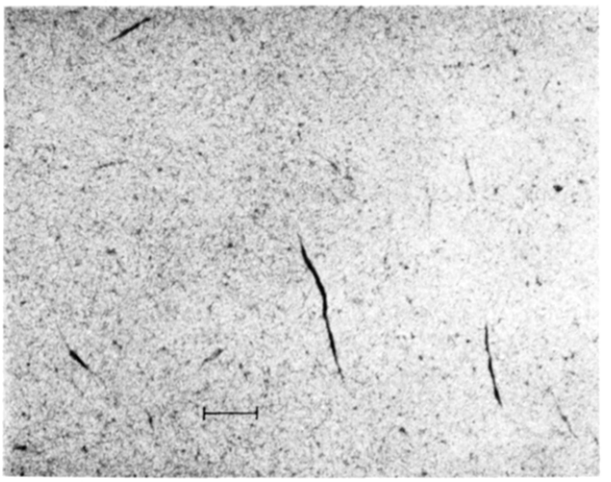

FIGURE 1: Representative field of fibrin oligomers formed by the action of thrombin (10 units/L) on fibrinogen (6.8 g/L) in the presence of factor XIIIa (30 mg/L) for 16 min (representative field corresponding to Figure 2b). Bar corresponds to 500 nm.

The structures seen in these micrographs appear qualitatively the same as those discussed by Hantgan et al. (1980), consistent with the conclusion that these were in fact made of half-staggered units; however, their method gave better resolution of the details of the oligomer structure. It may be noted that they prepared oligomers by incubating a very low concentration of fibrinogen with an excess of thrombin. In that case, fully difunctional units, which have perhaps lost their B peptides as well, assemble at random in a process in which the rate-limiting step is aggregation of binding sites instead of peptide removal. In contrast, the oligomers in the present study are presumably composed of a mixture of monofunctional and difunctional units which probably retain a large fraction of their B peptides. The size distribution of the oligomers seen by Fowler et al., which was not reported, should be that expected for the random polycondensation of difunctional units at an incomplete extent of polymerization, namely, the most probable distribution given by Flory (1953). Although the size distribution of oligomers formed under conditions in which reaction with thrombin is the rate-limiting step should be dramatically different from the most probable, the oligomers themselves appear to be the same. The presence of B peptides appears to play no role in the initial linear oligomer formation (Müller et al., 1981).

Length Distributions of Oligomers. The length distributions of linear oligomers seen in electron micrographs of solutions taken from a polymerizing mixture of fibrinogen at two different times are seen in Figure 2. The best fits of the data by eq 1 were obtained with $r = 0.87$ for the 12-min sample (Figure 2a) and $r = 0.95$ for the 16-min sample (Figure 2b). However, as estimated by polyacrylamide gel electrophoresis of the same samples after reduction, the values for y were 0.17 and 0.20 for the 12- and 16-min samples, respectively. The large differences between r and y are good evidence for the nonrandom nature of peptide release, with the second peptide being more easily removed than the first. Although the substantial uncertainties in the measurements of both r and y can lead to only a rough estimate of q, the results are $q = 30$ from the data of Figure 2a and $q = 50$ from Figure 2b. Earlier experiments have yielded values of q ranging from 16 (Bale et al., 1982) to 40 (Landis & Waugh, 1975). This nonrandom peptide release means that, even at a point early in the assembly process, when only a few monomers have reacted with

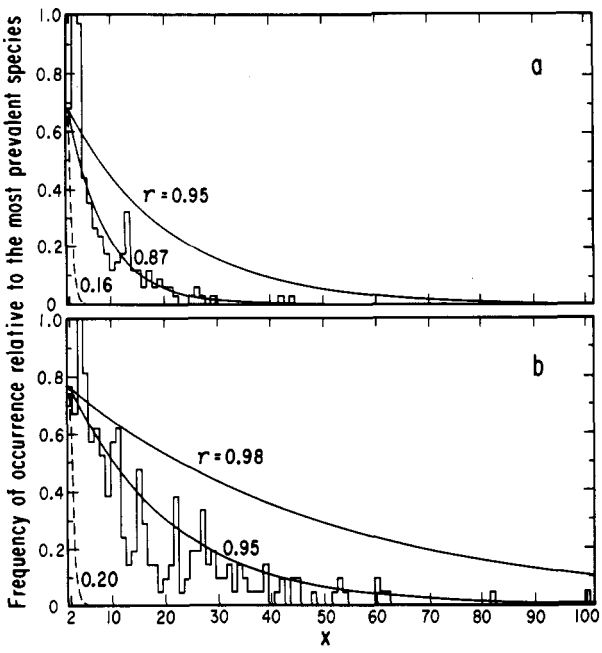

FIGURE 2: Size distribution of fibrin oligomers after polymerization of fibrinogen (6.8 g/L) by thrombin (10 units/L) in the presence of factor XIIIa (30 mg/L). (a) 12-min reaction time, 197 oligomers counted; (b) 16-min reaction time, 247 oligomers counted. x is the degree of polymerization as represented by bin number described in the text. Theoretical curves are shown for various values of r; dashed curves correspond to $q = 1$.

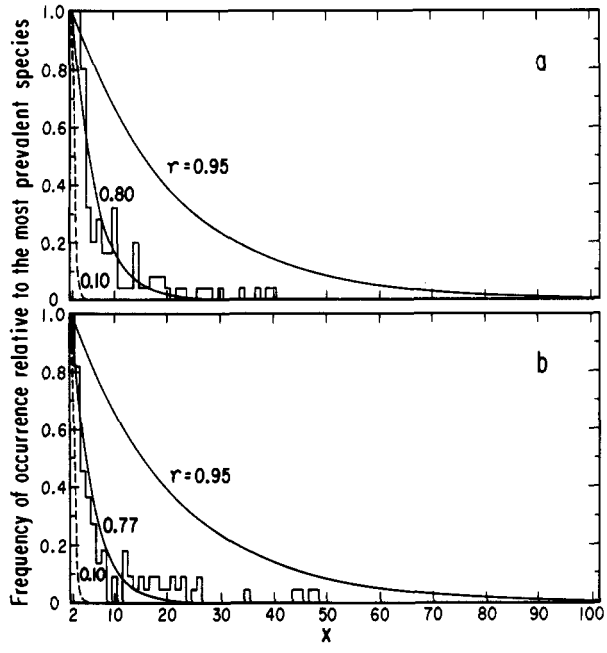

FIGURE 3: Size distribution of fibrin oligomers after 10 min of polymerization of fibrinogen (6.8 g/L) by thrombin (10 units/L) without factor XIIIa. (a) pH 7.5, ionic strength 0.15, 133 oligomers counted; (b) pH 8.5, ionic strength 0.45, 102 oligomers counted. Theoretical values are shown for various values of r; dashed curves correspond to $q = 1$.

thrombin, the vast majority of activated monomers will be difunctional. For example, with $y = 0.17$ and $q = 30$, $r \simeq 0.87$; the ratio $[\alpha_2]/[A\alpha_2]$ is 3.3.

Similar size distributions of ligated species obtained from agarose gel electrophoresis (data not shown) give results which are qualitatively consistent with those derived from electron micrographs. However, the ligated species are slightly smaller than would be predicted from molecules seen in electron micrographs if ligation were complete; assuming the same values of y as were used in the estimates of q from electron micrographs, the best theoretical fits to the size distribution of ligated oligomers for both the 12- and 16-min samples were obtained by assuming $q = 16$.

The effect of pH and ionic strength on the initial oligomer formation is seen in parts a and b of Figure 3. Polymerizing mixtures of fibrinogen and thrombin differing only in the pH and ionic strength (pH 8.5, $\mu = 0.45$, and pH 7.5, $\mu = 0.15$) were allowed to react for the same amount of time. The size distributions are strikingly similar as was the general appearance of the oligomers. The distribution formed at low salt and pH is fit approximately by an r of 0.80 and that at high pH and salt by an r of 0.77. Assuming a value for y of 0.10, consistent with experimental results obtained from similar samples made in the presence of factor XIIIa, the value of q obtained is about 30–50. The similarity of the two size distributions is consistent with recent evidence that thrombin activity is not appreciably altered over this range of pH and ionic strength (Hermans & McDonagh, 1982) and that the differences in clots formed under these conditions do not reflect differences in initial oligomer formation. The distribution of oligomers formed at pH 8.5, ionic strength 0.15 (data not shown), was similar to that of the other two samples. The differences in pH and ionic strength, although having little effect on initial oligomer formation, did change the final clot structure and the clotting times of the polymerizing solutions substantially. The clotting times were 12 and 50 min for the low and high pH and ionic strength samples, respectively.

Discussion

The major physiological role of fibrin formation is to create a three-dimensional network structure quickly, and yet in a way that is limited and controlled. Enzymatic regulation of the polymerization process is established if removal of A peptides is the rate-limiting step in protofibril formation. The rapidity of protofibril growth depends on the mechanism whereby monomers and small oligomers become attached to growing protofibrils. If thrombin removes A peptides at random, then protofibrils long enough to aggregate laterally or to form an interpenetrating array sufficient to establish a network structure will not be present until a rather large fraction of the peptides has been removed. If, on the other hand, thrombin removes both A peptides simultaneously, then large oligomers will form quickly, but their growth will not be limited by the action of thrombin. A third mechanism, in which thrombin unmasks sequentially the binding sites covered by A peptides but does so in a manner which favors the creation of a disproportionate number of difunctional units, allows for the presence of long oligomers early in the reaction but ensures that these oligomers cannot grow further unless acted on by thrombin.

Experimental evidence for this third mechanism, obtained from an analysis of the size distribution of ligated oligomers which remain after protofibrils are denatured in SDS–urea prior to electrophoresis (Bale et al., 1982), is here confirmed by visualization of the linear oligomers formed both with and without ligation and under a variety of conditions of pH and ionic strength. The electron microscopic studies suggest that the size distribution is even more shifted toward long oligomers, compared to a most probable distribution, than earlier experiments concluded.

A fourth possibility, that thrombin produces only difunctional units but that these units bind to fibrinogen (which has the terminal domain binding sites already exposed), accounts for the limited growth of protofibrils but does not explain the existence of long oligomers early in the reaction. When only

relatively few monomers are activated, the fibrinogen, present in excess, would act as a buffer to keep oligomers from growing until a certain fraction of monomers had been activated (Brass et al., 1976). Instead, the long oligomers form very soon and grow steadily. Therefore, although the binding of fibrinogen to the ends of oligomers, or to fibrin monomers, may play some role in their formation and has been shown to inhibit the repolymerization of dispersed fibrin monomers (Belitser et al., 1982), it does not seem to control the rate of oligomer growth early in their formation.

One possible explanation for the quantitative difference between the size distributions determined from electron micrographs and gel electrophoresis, leading to a higher value of q deduced from the former, is that electron microscopy might tend toward overcounting of larger oligomers, which are easier to see. Also the fact that some of the oligomers appear to be a bit thicker than the average suggests that some of the structures are not single oligomers. It has been assumed that, in the sample preparation, the rinsing step does not selectively remove smaller adsorbed oligomers prior to staining. Any such tendency would be interpreted as a higher value of q and might be partly responsible for the difference observed. However, it could not invalidate the general conclusion that $q > 1$, since the very existence of a perceptible number of large oligomers at an early reaction stage would be incompatible with $q = 1$, as shown by the dashed curves in Figures 2 and 3. The really critical portion of the size distribution for determining q is in the range $x = 5$–15. The fact that the general features of the size distribution are unaffected by dilution and also by differences in reaction time (Figure 2) is further evidence that selective adsorption does not play an important role in the interpretation of these results.

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Registry No. Thrombin, 9002-04-4; blood coagulation factor XIIIa, 9067-75-8.

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