The Fibrinogen Molecule: Its Size, Shape, and Mode of Polymerization

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PLATE 1

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ABSTRACT

Improved electron micrographs of the shadow-cast bovine fibrinogen molecule have been obtained establishing its general morphology and dimensions in the dry state. It consists of a linear array of 3 nodules held together by a very thin thread which is estimated to have a diameter of from 8 to 15 A, though it is not clearly resolved. The two end nodules are alike but the center one is slightly smaller. Measurements of shadow lengths indicate that nodule diameters are in the range 50 to 70 A. The length of the dried molecule is 475 ± 25 A.

Adopting the molecular volume from previous physical chemical data and the general morphological features and length from electron microscopy, we calculate the diameters of the end nodules to be 65 A and the center one as 50 A. The model of the molecule so obtained is consistent with the electron microscopical observations and the data from physical chemistry.

The intermediate polymers formed when fibrinogen is activated with thrombin were also examined and found to be end-to-end aggregates of altered fibrinogen molecules which shrink in length during the process. Intermediate polymer lengths are from 1000 to 5000 A.

The nodular nature of fibrinogen, its shrinkage and end-to-end aggregation on polymerization permits us to deduce an explanation for the system of cross-bands previously observed in stained fibrin fibrils.

INTRODUCTION

The extensive literature dealing with the fibrinogen molecule and its conversion to fibrin has been reviewed at length by Scheraga and Laskowski (1) and in a briefer discussion by Waugh (2). The molecular weight of fibrinogen is well established from physical chemical data at 330,000 to 340,000 (3). That the molecule has a high asymmetry ratio is also firmly established, but the dimensions cannot be deduced without information regarding the molecular morphology which is obtainable only from electron microscopy. In the first attempts to study the fibrinogen molecule with the electron microscope, Hall (4, 5) observed nodous filaments "like a string of beads," but units were so entangled that it was not possible to establish a unique length for the molecule. The high concentration in his preparative procedure was necessary to cover the somewhat similar texture of the supporting film. Later, Porter and Hawn (6) failed to confirm Hall's observations and proposed an oblate ellipsoidal model for the fibrinogen molecule which seemed to have some advantages in explaining their observed cross-striations in fibrin (7). This model was not, however, consistent with the accepted interpretation of the physical chemical data (8), and their proposed structure for fibrin based on this model was inconsistent with later electron micrographs of fibrin made by Hall (5). Still later, Mitchel (9) also failed to confirm Hall's observations and reported finding only spherical particles. The presence of nodose filaments in fibrinogen solutions was eventually confirmed by Siegel, Mernan, and Scheraga (10) whose technique was sufficiently improved that they could discern individual "strings of beads," but there was some confusion with the substrate texture and probably some

disruption of structures due to drying. Consequently, though their micrographs were a significant improvement, they were not able to find a unit which would correspond to the molecule that existed in the presumably highly monodisperse fibrinogen solutions. Discrete fibrinogen molecules were finally observed clearly by Hall (11) using a technique that eliminates substrate structure (12–14). Since the preliminary report (11) we have gathered quantitative supporting data on the structure of the fibrinogen molecule and studied aspects of the polymerization process.

Probably the most significant result in previous work on the polymerization process has been the observation by physical chemical methods of intermediate polymers very much thinner than fully polymerized fibrin fibrils and with lengths up to about 4000 A (1). The apparent diameter of the polymer is, however, greater than the width of the hydrodynamic ellipsoidal model generally used, which has been interpreted to mean that the polymer grows by staggered side-to-side association (15). Previous electron microscope observations confirmed the existence of such a polymer and indicated that the aggregation was predominantly end-to-end, but did not rule out the possibility of some lateral overlapping (10, 16).

The most striking aspect of fully polymerized fibrin fibrils in the electron microscope is the existence in stained specimens of regular cross-striations with a period of about 230 A as first observed by Hawn and Porter (7). Later, Hall (5) showed that between the heavily stained bands there existed a lighter secondary stained band. No acceptable scheme has been proposed to account for the observed structure on the basis of the structure of fibrinogen. As a result of the studies to be described, we have devised a model which accounts for the observed bands and is consistent with other relevant data.

Materials and Methods

We have obtained excellent micrographs of bovine fibrinogen, directly from commercial Fraction I (Pentex) and from repurified fibrinogen made by D. F. Waugh according to the method of Laki (17). The latter shows a higher proportion of recognizable molecules. The repurified material was stored frozen and later thawed under tap water prior to use. In any case, the material was dialyzed for 24 to 48 hours against 0.1 per cent $(NH_4)_2CO_3$ adjusted to pH 9.5 with NH_4OH to give a stock solution of fibrinogen of 5 mg./ml.

For electron microscopy various concentrations of fibrinogen were used, but for a typical experiment a solution would contain 0.15 mg./ml. of bovine fibrinogen plus 0.06 mg./ml. of polystyrene spheres of mean diameter 880 A. The material was sprayed on to a freshly cleaved mica surface from a high pressure spray gun, shadow-cast with Pt at a shadow-to-height ratio of about 10:1, backed with SiO and collodion, and stripped on water as previously described (11–14). Micrographs were made with an R. C. A. Type EMU3-B at a magnification of about 15,000. The best populations of intact molecules were obtained when the mica surface was double-sprayed with fibrinogen and 0.2 per cent formalin, or when the formalin was added to the solution.

In the polymerization experiments we used a sample of thrombin obtained from Dr. Walter H. Seegers. The sample had an activity of 130 units per mg. (Its original activity was 640 units per mg. when first obtained some 15 years ago.) In a typical experiment, bovine fibrinogen and thrombin in 0.1 per cent $(NH_4)_2CO_3$ at pH 9.5 were mixed so that the concentration of fibrinogen was about 1.5 mg./ml. and the thrombin was 0.4 units/ml. (0.003 mg./ml.). Samples were taken at intervals varying from 30 seconds up to 2 or 3 minutes, diluted by a factor of 10 with buffer, and sprayed immediately. Best intermediate polymers were observed for clotting times between 30 and 60 seconds.

OBSERVATIONS AND RESULTS

Molecular Morphology.—A micrograph showing the typical particles observed in fibrinogen preparations is reproduced in Fig. 1. There are triads, *A*, which we identify with the fibrinogen molecule, dyads, *B*, and monads, *C*, which we believe are the result of breakage probably due to mechanical rupture during drying. Designated *D* is also a string of 4 nodules, but strings of more than 3 nodules are extremely rare except in areas where there is very obvious clumping due to high concentration.

A triad is usually straight, although the connecting thread is extremely thin. The diameter of the thread is estimated at 8 to 15 A—close to the resolution limit of the method. The two end nodules appear to be identical but the center one is a little smaller. As can be seen in the figure, doublets are more nearly the length of a triad, suggesting that in this case they are formed by a splitting off of the center nodule leaving the end two attached by a thread which, however, is not clearly seen.

In Table I are tabulated the results of particle counts from two experiments, one with a com-

TABLE I

Particle Counts From Electron Micrographs of Bovine Fibrinogen

Type of particle	o	oo	ooo	oooo	ooooo
Pentex bovine Fraction I	551	244	267	4	10
Waugh's purified bovine fraction I double-sprayed with formaldehyde	366	356	943	2	0

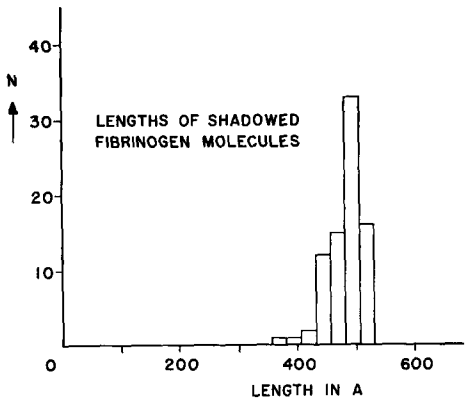
mercial bovine Fraction I, and another with highly repurified Fraction I which was stabilized with formaldehyde while sprayed. In both cases it is seen that strings of more than 3 nodules are extremely rare and can be dismissed as representing the fibrinogen molecule. In the first case, triads are a little more numerous than dyads and monads are very numerous, as would be expected in a relatively impure material. In the second instance, triads constitute about 73 per cent of the total material if we count one monad and one dyad as equal to one triad. It is remarkable that the number of monads and dyads is practically the same, strongly suggesting that they are formed by a splitting of one nodule from a triad due to disruptive forces of drying. We conclude from these data that the triad represents the fibrinogen molecule.

Although in most micrographs the nodules appear spherical, there is frequently evidence of substructure within them. Since, however, we have not clearly resolved these aspects, we shall represent them diagrammatically as spherical. It seems very likely, however, that if the resolution of the method could be improved each nodule would display a higher degree of complexity. All the micrographs shown are slightly underfocused.

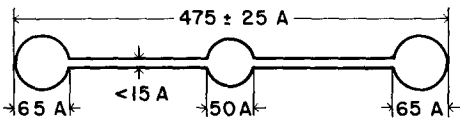
Molecular Weight.—Katz *et al.* (18) gave a molecular weight of 340,000 from light-scattering and a little later from a consideration of sedimentation, diffusion, and viscosity, Shulman (3) gave a value of 330,000 ± 10,000. As a check in the present study we ran purified bovine fibrinogen in the centrifuge according to the semi-equilibrium method of Archibald (19), which method has been described in detail elsewhere (20). The average of two runs gave a molecular weight of 324,000 which is sufficiently close to the accepted value of Shulman to establish that we are dealing with essentially the same molecule. We cannot obtain a more accurate molecular weight (or volume) from electron microscopy at the present time and therefore assume Shulman's molecular volume of 38.7×10^{-20} cm.^{3} (calculated from the anhydrous molecular weight plus the density) to be used in conjunction with electron microscopic morphology in working out a molecular model.

Dimensions.—Apparent lengths of shadowed fibrinogen molecules were measured from prints at a magnification of about 100,000. A set of results are represented graphically in Text-fig. 1. Eighty-seven per cent of the lengths are in the interval 450 to 520 A, which is a reasonably sharp peak considering the disruptive forces of drying. A number or weight average from these data is probably not significant since the molecules are in all likelihood homogeneous as to length in solution and the distribution of lengths observed is probably due to shrinkage during drying. We would think that the significant length would be either the most frequently occurring or the maximum observed, though some stretching might conceivably occur. In any case, whether we take the significant length as the most frequent or the maximum, we have a value between 500 to 520 A. This applies to the apparent length of the shadowed particle which has a cap of metal over both ends which could increase the apparent length by anything from 20 to 50 A. As a result of these considerations, we conclude that the length of the molecule is 475 ± 25 A.

In working out a model for the fibrinogen



TEXT-FIG. 1. Histogram showing distribution of measured lengths of shadowed fibrinogen molecules.



TEXT-FIG. 2. Model of fibrinogen molecule deduced from the electron microscope observations and the molecular volume from physical chemical data.

molecule we have taken the following considerations into account:

1. The over-all length is 475 ± 25 A.

2. There are 3 nodules of which the outer two are equal and the middle one is smaller.

3. We cannot measure the diameter of the nodules with sufficient accuracy for volume calculations but we estimate from shadow lengths that they are in the range 50 to 70 A.

4. From measurements of shadow lengths we estimate that the diameter of the center nodule is about 80 per cent that of the end ones.

5. The nodules are held together by a thread which is not clearly resolved. Since we have been able in general clearly to resolve rod-shaped molecules with diameters in the 15 to 20 A range, we estimate that the thread is probably about 8 to 15 A in diameter. The volume contributed by this element is small compared to the volume of the nodules.

6. We adopt Shulman's figure for the volume (anhydrous) of 38.7×10^4 A^3 and calculate the diameters of the nodules (rounded off to 5 A) from this volume. The resulting diameters are consistent with the estimates from electron microscopy given in 3 above.

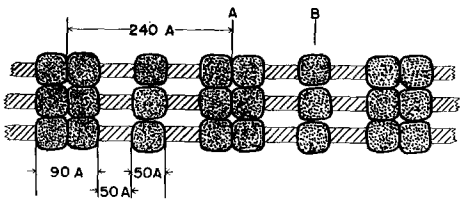
The resulting model is shown in Text-fig. 2. It is interesting to note that among the variety of models to which Shulman attempted to fit the physical chemical data a string of 4 beads bears the closest resemblance to our result, and his figure for the length is 430 A. Ellipsoidal models are much too long and much too thin. We believe that the model of Text-fig. 2 is consistent with all the significant data at present available at this level of dimensions. There are undoubtedly features of substructure which have not yet been resolved.

Polymerization of Fibrinogen.—Typical intermediate polymers prepared as described under Methods are shown in Fig. 2 and at higher magnification in Fig. 3. These strands can be seen to consist of strings of nodules, which however, are much closer together than they are in fibrinogen. Polymer lengths are from 1000 to 5000 A. The interval occupied by 3 successive nodes is usually less than 300 A. (The apparent size of the nodules in Figs. 1 and 3 are not to be compared quantitatively since apparent size depends on quantity of metal, direction of shadowing, and other factors.) It is not generally possible to pick out the precursor fibrinogen molecules in these polymers because there is some distortion of typical triads and the difference between the center and end nodules is not always evident. What appears to be two clear cut triads end-to-end is indicated in Fig. 3 *a*. The evidence is definitely for end-to-end polymerization.

DISCUSSION

At the level of dimensions given in Text-fig. 2 we have a model of the fibrinogen molecule which for the first time fits all of the pertinent data and provides a substantial basis for the interpretation of physical chemical and other data aimed at further elucidation of the structure. It is almost certain that the nodules represented in outline contain incompletely resolved substructure. No evidence has been obtained or would be expected from this electron microscope study of small peptides which might be split off in the activation by thrombin. The central nodule is certainly much too big to represent such low molecular weight products as have been observed (1). The absence of dimers and higher order polymers in fibrinogen preparations is to be noted and probably indicates a strong repulsion between the molecules. Whether the thread-nodule relationship in the triad is like a ball and chain or like a string of beads cannot be deduced from the present evidence. The occurrence of diads with the middle nodule apparently split off would suggest that the thread passes continuously through the center region.

The observations of the intermediate polymer of fibrin clarify previous concepts and suggest a plausible sequence between the fibrinogen mole-



TEXT-FIG. 3. Model devised to account for the observed cross-striations in fully polymerized fibrin as shown in Fig. 4.

cule and the observed banded structure in fibrin shown in Fig. 4. Since we cannot distinguish a unit which might be termed a fibrin molecule in Fig. 4, our model for fibrin is speculative and diagrammatic. In making such a model we must take into account the following results, however:

1. The polymerization is end-to-end which rules out the suggested side-to-side mode suggested by Ferry, Katz, and Tinoco (15).

2. There is a shrinkage of the fibrinogen in the polymer down to about one-half its original length.

3. In such a polymer, after shrinkage, the diameter is essentially that of the nodules, that is 50 to 65 A. This is in excellent agreement with hydrodynamic data (21). Lengths are also in the range expected. The staggered model was suggested partly to account for the fact that the ellipsoidal model had a diameter of 30 A which was too small to account for the diameter of the intermediate polymer.

4. The triad morphology lends itself readily to account for the system of heavy and light bands observed in stained fibrin.

With these considerations in mind and on the assumption that the fibrinogen particle maintains some degree of integrity in the process, we propose the model shown in Text-fig. 3 to account for the observed band structure in fibrin. The dimensions are taken approximately from the relative band dimensions of fibrin shown in Fig. 4.

The longitudinal shrinkage of the fibrinogen molecule in the conversion is perhaps suggestive of the syneresis effects that are observed in gross clots. We do not believe, however, that these effects are connected. Syneresis appears to result from strong interaction between large fibrils in an advanced stage of clotting where there is a large amount of side-to-side aggregation. In electron micrographs of stained fibrin clotted under close to physiological concentrations one sees bundles of fibrils lying side-by-side with the striations in register suggestive of strong lateral attractions in final stages of the clot. Under conditions designed to produce the intermediate polymer there is apparently little tendency for lateral aggregation, but lateral forces must come strongly into play as the clot develops.

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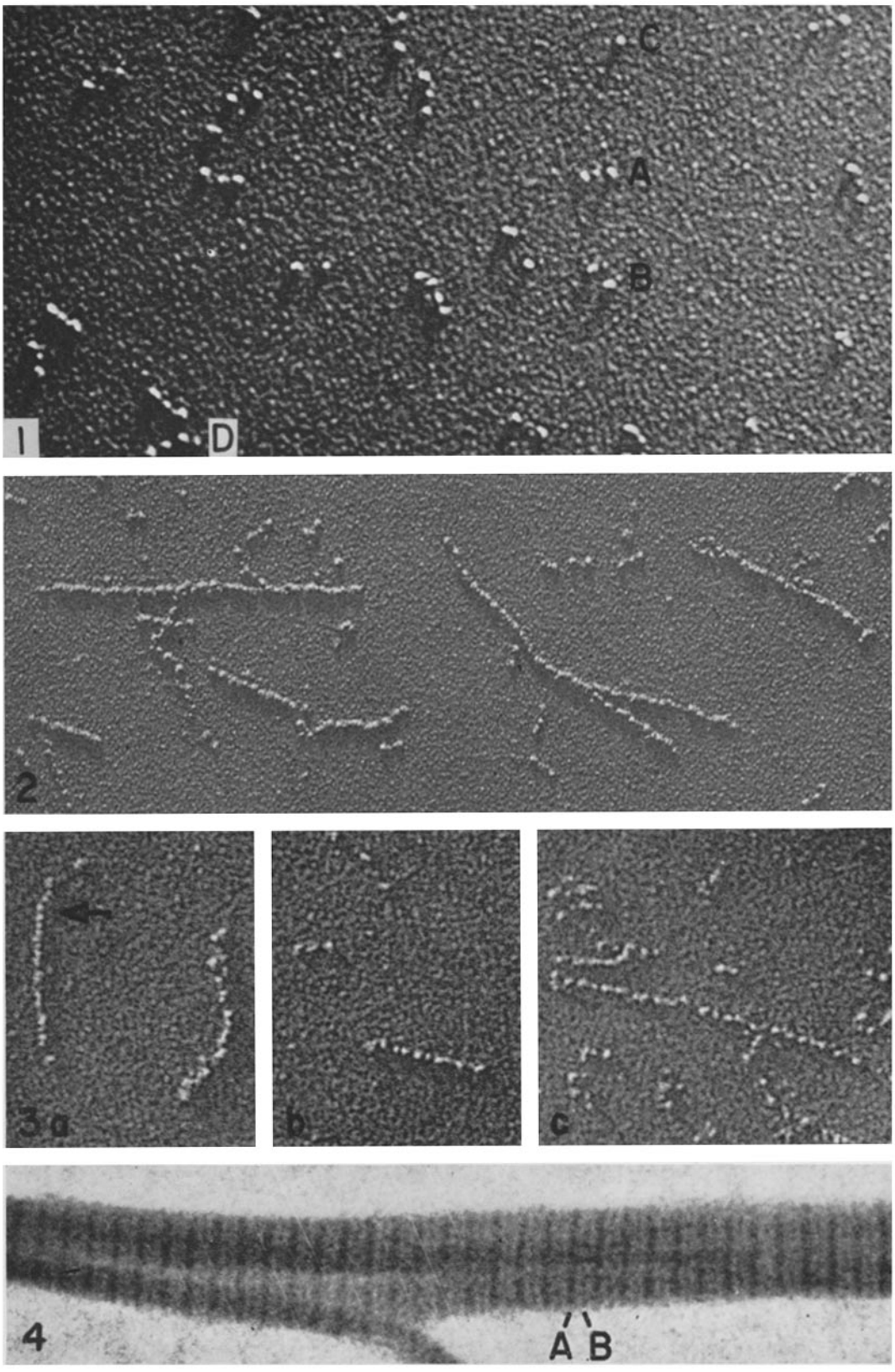
EXPLANATION OF PLATE 1

FIG. 1. Fibrinogen molecules from repurified bovine Fraction I. The intact molecules are the triads, designated *A*. Dyads, *B*, and monads, *C*, are believed to be fragments produced by the disruptive forces during drying. Strings of more than 3 nodules such as at *D* are very rare. Magnification, 156,000.

FIG. 2. Intermediate polymers consisting of end-to-end aggregations of fibrinogen molecules produced by the action of thrombin. Magnification, 100,000.

FIG. 3. Same as Fig. 2, but at higher magnification. Indicated in *a* by the arrow are two fibrinogen triads which have come together end-to-end and shrunk in length. Polymers in *b* and *c* are from other parts of the field. Magnification, 150,000.

FIG. 4. Segment of a typical fully polymerized fibrin fibril stained with phosphotungstic acid. The axial period is about 230 A and consists of alternating heavily and lightly stained bands designated *A* and *B*, respectively. Magnification, 180,000.



(Hall and Slayter: Fibrinogen molecule)