ELECTRON MICROSCOPY OF FIBRINOGEN AND FIBRIN

BY C. E. HALL

(From the Department of Biology, Massachusetts Institute of Technology, Cambridge)

PLATE 3

(Received for publication, February 4, 1949)

It has been demonstrated through electron microscopy and x-ray diffraction that there are present, in certain protein fibrils, structural periodicities having dimensions up to several hundred angstrom units. Examples are collagen (1-3), paramyosin (4, 5), the trichocysts of Paramecium (6, 7), and fibrin (8). The application of phosphotungstic acid (4) and other heavy metal compounds greatly enhances the visibility of the structures in electron micrographs and often makes structural variations apparent which are not perceptible in the unstained fibrils. It has not been established definitely whether staining involves a selective chemical reaction. However, since metal shadowing reveals in most cases that the fibril surface is corrugated with the high points corresponding to the highly staining region, it seems probable that stain deposits according to the local concentration of protein. The regions which stain more lightly, appearing as slight depressions on shadow-casting, probably represent portions which have shrunk during drying. Little, if anything, is known concerning the genesis of these macro periods or the reason for their occurrence in protein fibers. The investigation to be described was aimed at a clarification of these phenomena and is confined to fibrin and the precursor, fibrinogen.

Microscope Techniques

All micrographs were made with an RCA type B microscope which has a self-biased gun, compensated lens (9), no physical objective aperture, and is operated at 65,000 volts accelerating potential. Although space does not permit a discussion of the physical aspects of electron microscopy, it is pertinent to note that concurrent studies were made of the structure of specimen supports, the structure of metals used for shadow-casting (10), afocal imaging phenomena, and the effects of lens asymmetries on the image. If, for example, the objective lens is astigmatic, images may exhibit spurious asymmetries, giving the false impression of fibrosity in the object. As an aid to interpretation and the recognition of possible image defects, through focus series were recorded. Uranium, platinum, and nickel have a suitably fine structure for shadow-casting when deposited in thin enough layers. Uranium gives excellent results in estimated thicknesses of 3 to 6 Å in the plane of the film. Nickel has a remarkably fine
structure (11, 12) and is useful in thicknesses of less than 10 Å. Shadow angles were 3:1 or 4:1.

Observations on Fibrin

Specimens of bovine fibrin for electron microscopy were prepared in the following manner: Bovine Fraction I (Armour) and thrombin (Seegers, 640 units per mg.) were dissolved separately in 0.9 per cent NaCl buffered to about pH 6.5 with KH$_2$PO$_4$ buffer solution. Concentrations of Fraction I (including citrate) in solution varied from 0.5 to 2 mg. per cc., while concentrations of thrombin were of the order of 10 units per cc. Portions of the two solutions, frequently of the order of 1:1, were thoroughly mixed in a test-tube and a drop was placed immediately on a standard specimen screen with collodion or SiO supporting film. The development of the clot in the test-tube can be followed by eye. After varying times (30 seconds and up) according to the experiment, the excess solution was washed from the specimen screen with salt solution, stained with phospho-12-tungstic acid, washed with twice distilled water, and dried. The concentration of phospho-12-tungstic acid (Anachemia, Ltd., Montreal) was usually 0.1 per cent and sometimes 1 per cent. Usually it was unbuffered, but in some experiments the pH was raised to about 5.4 with potassium acid phthalate buffer. In one experiment, sodium citrate buffer was used (pH 6.5) and, in others, the clot was formed in unbuffered 1 per cent NaCl, pH 5.8.

The electron microscope observations of stained fibrils from bovine fibrin clots confirm the observations of Hawn and Porter (8) on the existence and approximate dimensions of the axial macro period. Measurements of over 200 fibrils from several preparations yield an average of 227 Å. All measurements in the group were between 190 and 270 Å with over 80 per cent in the range 210 to 240 Å. The spacings along individual fibrils are remarkably constant (to the order of 2 or 3 per cent) compared to variations between separate fibrils. Variations as high as 20 per cent of the mean were observed between fibrils in a single specimen. No explanation can be given for the variation from one fibril to another or for the sometimes significant variations in average spacing from one preparation to another. Since the fibrils were formed on the specimen screen, tensions due to manipulation were kept to a minimum. Tensions caused by drying might be a factor, but since the fibrils were well supported and the supporting films did not break, it does not seem likely that random variations of the order of 20 per cent could be accounted for in this way. It is clear, however, that the structure is not rigid.

The author is indebted to Dr. Walter H. Seegers, Wayne University, Michigan, for the sample of thrombin used in this work.
As shown in Fig. 1, the axial macro period consists of a series of highly staining and sharply delimited bands, designated A, which are about 70 A wide and are separated by a region of lower scattering power about 160 A wide. In addition to the structure previously reported, there is a finer stained band (designated as B in Fig. 1), about 30 A wide, mid-way between the prominent bands.

Fibrin fibrils clotted from a sample of highly purified human fibrinogen* (Fig. 2) show a stained structure which is indistinguishable from that observed in fibrin from bovine Fraction I. The average axial spacing from a number of plates was 214 A, which is in the range of values found for the bovine material.

When the image quality is favorable, substructure is visible within the stained bands, giving the fibrils, ultimately, a particulate appearance which may be discernible in the micrograph in Fig. 2. The particles are of the order of 50 A and less in diameter and are not well resolved. In such preparations there is always a background of finer fibrin fibrils of indistinct structure approaching in dimensions the resolution limit (or to be more precise, the visibility limit) of the microscope. These finer fibrils are about 200 A and less in diameter and do not possess the characteristic striated appearance. Similarly, the striation gradually dissolves into a more or less randomly particulate structure toward tapered ends where the width is about 200 A and less. It is concluded that the absence of well defined striations under these circumstances is not due to a lack of resolution, but represents an actual disorder in the structure. The effect may be seen in Fig. 2 where the fibril parted from the bundle toward the lower right is not clearly striated. Local regions of stain concentration are visible which tend to form a vague striation in a few places, but the structure is disordered compared to that in the thick bundle. The sharpness of the stained bands increases noticeably with fibril width. Also apparent in Fig. 2 is the tendency of striations to align between adjacent fibrils in a bundle, as was noted by Hawn and Porter (8). Throughout the present investigation, this coincidence was observed with such high frequency and with such high precision that it is deemed significant. The effect seems to indicate the presence, at some stage during fiber formation, of lateral attractive forces which are at an optimum when there is lateral coincidence of like bands.

Some specimens containing well formed fibrils such as those shown in Figs. 1 and 2 were shadow-cast. The observations show that the stained regions, both A and B bands, represent slight elevations.

*The sample, kindly provided by Dr. J. T. Edsall of the Harvard Medical School, was from Run 183, Fraction I-2A and was 94 per cent clottable when prepared in 1945.
Observations on Fibrinogen

Specimens from bovine Fraction I and human fibrinogen were prepared by placing a drop of the material in 1 per cent NaCl on a collodion or SiO film and drawing off the excess by touching a piece of filter paper to the edge of the screen. In some experiments, a fixative such as formalin or phosphotungstic acid was applied before drying and, in others, the specimen was allowed to dry without fixation. In any case, the final treatment was a wash in twice distilled water to remove salt and citrate, after which the specimens were shadow-cast along with control films. The variations in these procedures seemed not to produce any marked differences in the observed structure of the protein layer adhering to the film. The concentration of the sample was varied over a wide range, from about 2 mg. per cc. down to about 0.001 mg. per cc. The original intention was to secure a distribution of isolated particles over the surface of the film, but difficulty was encountered in securing this condition, because the protein tends to deposit in clumps. Also, the necessary dilution factor is so great that the effect of impurities in reagents and water becomes a consideration. Furthermore, although the film structure is relatively fine, it does complicate the interpretation. Consequently, specimens made with very high dilution factors were not such as to invite confidence. On the other hand, specimens made from concentrations which resulted in a covered supporting film leave something to be desired, because the fibrinogen particles are intermingled. With the latter condition, however, it can be concluded with some confidence that the observed structure is mainly due to the protein deposited from the original sample and the problem is to resolve the elements from the mass.

In the shadowing procedure, control films were placed beside the fibrinogen preparations. Fig. 3 shows a small, typical portion of an electron micrograph made from bovine Fraction I. There is a rather confusing aggregation of particles among which numerous filamentous elements may be distinguished. In Fig. 4 are shown micrographs of a collodion control film (a) which had been washed with twice distilled water and a micrograph of a specimen made from the sample of human fibrinogen (b). The control film exhibits a structure which is about the coarsest of any recorded; yet there is no mistaking the difference between it and the fibrinogen sample. The fibrinogen completely covers the film and although it consists of a somewhat confusing aggregation, it may be seen to contain filamentous elements similar to those from the bovine Fraction I shown in Fig. 3.

In Fig. 5, b is shown a thick layer from bovine Fraction I shadowed very lightly with nickel in order to present the fine detail within the filaments to better advantage than is the case in either Figs. 3 or 4. Filamentous elements may be distinguished, but they are seen to be non-uniform in
thickness, appearing somewhat like a string of beads. This type of structure is characteristic of high resolution micrographs of fibrinogen preparations when they have been shadowed with a suitably thin layer of metal. It is concluded that the nodose structure of fibrinogen filaments as indicated in the electron micrographs is significant.

Measurements were made of filaments which could be discerned in micrographs similar to those shown. The lengths varied from 200 to 1100 Å with 85 per cent between 300 and 800 Å. The average was about 600 Å. Widths, which cannot be determined with high accuracy, are estimated to be in the range 30 to 40 Å across the wider portions. The observations demonstrate the presence in fibrinogen preparations of asymmetric elements in accordance with the conclusions reached from physicochemical methods, and the estimated widths are not significantly different from what would be expected from experiments on double refraction of flow (13). The correlation is unsatisfactory, however, to the extent that the electron microscope observations fail to indicate a unique length, even though there are distinguishable many filaments with lengths very close to the 700 Å predicted. (The filaments which are marked in Figs. 3, 4, and 5 are close to 700 Å in length.) In view of the difficulty of identifying continuous filaments and locating their ends in electron micrographs, the fault lies quite possibly, but not necessarily, with the electron microscope observations. It would be desirable to improve the electron microscope technique so that a fair sample of isolated, identifiable elements could be measured with certainty before concluding that the two methods are inconsistent. In any event, there is no unequivocal relation between the lengths of fibrinogen filaments as determined by either method and the dimensions of the macro period as seen with the electron microscope.

**Formation of Fibrin**

The absence of correlation between lengths of the precursor particles and the magnitude of the striations in fibrin fibrils indicates that fiber formation is not simply a matter of assembling semirigid units in the manner of crystal growth as would be suggested by the extreme regularity of the final structure. Some attention was therefore given to a study of fibers during formation through the use of short clotting times and decreasing amounts of thrombin, with a view to obtaining evidence concerning the manner in which fibrinogen filaments associate. It has been noted that with the ultimate resolution stained fibrin appears particulate and the stained bands appear to represent regions where there is a relatively high concentration of stained particles. It is not likely that the particles represent elementary units. They are probably aggregates of smaller, unresolved elements. This appearance is consistent with the particulate
structure observed in fibrinogen filaments as shown in Fig. 5. When the thrombin concentration was decreased so that the clot formed very slowly, most of the fibrils showed a randomly particulate structure and little if any evidence of striations. Often, a fibril would be striated in some portions and unstriated in others. In the transition regions, the impression is gained that the occurrence of striations results from local concentrations of particles which are visible, but randomly disposed, in the unstriated portions. Although there is no marked longitudinal structure in such stained fibrils, shadow-casting reveals longitudinal filaments, indicating that formation of fibrin involves mostly lateral association of fibrinogen filaments. The observations suggest that in the clotting process the fibrinogen filaments associate laterally, after which there is a readjustment of the constituents producing variations of protein concentration along the fiber axis. The regions of higher concentration appear relatively dense after staining or as elevations when the dried material is shadow-cast.

It is to be noted that fibrils sometimes formed in solutions as they stood without any thrombin being added (some thrombin may be present as a trace in the fibrinogen material), but such fibrils have not been observed to be striated. Similarly, fibrils formed with very low thrombin concentration were mostly unstriated.

**DISCUSSION**

No satisfactory theory has yet been developed to account for macro periods of the sort described. It has been proposed by Astbury (14, 15) that the large periods are directly related to the much smaller dimensions of polypeptide chains as determined by x-ray analysis. According to this proposal, the macro periods represent the extent along the fiber axis of a single chain molecule in specific configuration. The long spacings should therefore be an integral multiple of certain of the short spacings. Some of the defects of the theory from the x-ray standpoint have been discussed by Bear (2). A serious difficulty arises from the fact that experimentally the short spacings are singularly unaffected by changes in dimensions of the long spacings. The electron microscope results indicate that the macro periods are associated with the distribution of particles having dimensions between those of the short and the long spacings. Structural distinctions visible in electron micrographs may possibly represent significant structural differences on the polypeptide level of dimensions, but before such a correlation could be made, it would be desirable to have an estimate of the size of the diffracting regions which produce the wide angle pattern. Wide angle patterns, remarkably similar to those for certain other fibrous proteins, have been shown for fibrinogen and fibrin (16), but no long spacings have been reported for either. The inhomogeneities which are apparent
in electron micrographs of both of these substances suggest that the diffracting regions producing the wide angle pattern are probably quite small, possibly in the range of 50 Å and less.

It must be acknowledged that the 700 Å length of fibrinogen filaments as determined from flow birefringence data (13) is quite close to 3 times the average fibrin spacing as reported here. The possibility therefore suggests itself that the fibrinogen filament contains three preformed macro periods and that the periodicity in fibrin results from the orderly aggregation of preexistent structures in the manner of crystal growth. This concept of the mechanism is attractive in its simplicity, but cannot be supported by the existing evidence. It is not permissible to attribute this degree of significance to the average spacing without accounting specifically for the rather wide variation in spacing between separate fibrils. If the periodicity were constructed of such an orderly array, it should appear at all stages of aggregation, for example, in the finest fibrin fibrils. The nodose character of fibrinogen filaments is suggestive of a preexistent stria tion, but it lacks the extreme regularity of that in fibrin. Also, the electron microscope evidence indicates that there is a random distribution of filament lengths in fibrinogen.

It is noteworthy that, although there is considerable variation in spacing between separate fibrils, there is a high constancy in the spacing within individual fibrils or fibril bundles. It appears that there is an interdependence of spacing in contiguous structures and that, once initiated, the periodicity is propagated with considerable precision throughout the fibril. In future efforts to account for this anomalous distribution of matter, due attention should be given to the possible effects of colloidal forces in this essentially colloidal system. From the chemical standpoint the "molecular" units may very well be those particulate elements which border on the electron microscope limit of resolution, in the neighborhood of 50 Å and less.

SUMMARY

1. The macro period in fibrin fibrils from bovine Fraction I is shown to consist of a narrow stain-receptive band mid-way between two denser and wider stain-receptive bands whose average distance center to center along the fibril axis is about 230 Å.

2. Fibrin fibrils formed from a highly purified sample of human fibrinogen were shown to have a structure indistinguishable from that observed in the bovine preparations. The average dimension of the macro period was 215 Å for the human material, which value is not considered to be significantly different from that of bovine fibrin, since there is a considerable variation in the value of the macro period between individual fibrils for both materials.
3. Within the range of resolution available for the investigation, stained fibrin fibrils appear to be constituted of particles having dimensions in the range of 30 to 50 Å.

4. Bovine Fraction I and the sample of human fibrinogen were shown to consist in large part of filamentous elements with an average length of about 600 Å and an estimated width of about 30 or 40 Å. With the ultimate resolution available, fibrinogen filaments appear nodose, not unlike a string of beads.

5. It is concluded that fibrin is produced through a predominantly lateral association of fibrinogen filaments. The characteristic axial periodic structure is interpreted as consisting of periodic variations of protein concentration resulting from local axial displacements of material to preferred positions at an advanced stage of fiber formation. No significant relation was established between the lengths of fibrinogen filaments and the dimensions of the macro period in fibrin.

BIBLIOGRAPHY


EXPLANATION OF PLATE 3

Fig. 1. Fibrin fibril from bovine Fraction I, clotted in 0.9 per cent NaCl at pH 6.5, stained with 0.1 per cent phospho-12-tungstic acid. X 217,000.

Fig. 2. Fibrin fibrils from purified human fibrinogen, clotted in 1 per cent NaCl, pH about 6, stained with 0.1 per cent phospho-12-tungstic acid. X 186,000.

Fig. 3. Bovine Fraction I, 0.01 mg. per cc. from 1 per cent NaCl, shadowed with uranium. X 164,000.

Fig. 4. (a) Collodion control film treated with distilled water. (b) Human fibrinogen, 0.05 mg. per cc. from 0.8 per cent NaCl. Shadow-cast with uranium. X 181,000.

Fig. 5. (a) Collodion control film. (b) Bovine Fraction I, shadowed with nickel. X 200,000.
ELECTRON MICROSCOPY OF FIBRINOGEN AND FIBRIN
C. E. Hall


Access the most updated version of this article at http://www.jbc.org/content/179/2/857.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/179/2/857.citation.full.html#ref-list-1