THE FINE STRUCTURE OF CLOTS FORMED FROM PURIFIED
BOVINE FIBRINOGEN AND THROMBIN: A STUDY
WITH THE ELECTRON MICROSCOPE*

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PLATES 29 TO 31
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The electron microscope can provide information on the fine structure of
blood clots which may be useful in the consideration of the over-all physical-
chemical description of the clotting mechanism. Wolpers and Ruska (1) (1939)
presented the results of their studies of fibrin produced from whole blood
plasma. Subsequently (2) they studied the pellicles from the spinal fluids of
cases of tuberculous meningitis and noted the same general structure as had
been encountered in the clots of blood plasma. These they described as being
made up of micellar bundles, often arranged in parallel to form thicker strands
which were held in a network by the intercommunicating branches. They also
described cross-striations in the micellar bundles and in the composite fibers;
these striations showed periodicities ranging from 200 to 350 Å. No similar
striations were found in clots from blood plasma and it was concluded that stria-
tions were a characteristic of the fibrin of the spinal fluid pellicles. Schmitt
and Hobson (3) investigated similar spinal fluid pellicles and found striations in
preparations from one case of seven investigated.

The methods for isolating and purifying proteins of the blood plasma, both
human and bovine, developed in the Department of Physical Chemistry of
Harvard Medical School by Cohn and his associates (4-7) have made available
certain of the proteins in states of greater purity and in greater quantity than
had been obtained by previous methods. Along with others of these, fibrinogen
and thrombin have been quite extensively studied and it is known that the
mechanical properties of the clots formed from them can be varied within wide
limits by control of such factors as pH, the temperature at which the interaction
of fibrinogen and thrombin takes place, the concentrations of the reacting sub-
stances and the presence of various chemical substances (8). In the course
of investigations on the culture and behavior of tissue cells in clots from purified
bovine fibrinogen and thrombin (9), it seemed desirable to make some parallel
studies on the influence of the above factors on the fine structure of the clot.

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The observations in this initial report are primarily concerned with the effects of alterations of pH.

**Materials and Methods**

The fibrinogen and thrombin preparations used in these experiments were generously provided by the Chemical Research and Development Department of Armour and Company, Chicago. The fibrinogen (lot C-185A) contained 79 per cent clotable nitrogen and was furnished in the form of a dried powder of which approximately 40 per cent by weight was sodium citrate. The thrombin (lot P-38) had a clotting activity of 27 units per mg. of total weight. These substances were dissolved in buffered physiological saline or in Tyrode's solution as indicated in the figure legends. Determinations of the pH were made with the glass electrode. The solutions were allowed to react at room temperature (22.1–23.0°C.).

For convenient study by means of the electron microscope it was desirable to obtain a clot sufficiently thin to permit a clear resolution of individual fibers and yet thick enough to provide a picture of the tridimensional orientation of the fibrin strands. The following technique was devised. Its results have been entirely reproducible, and satisfactory for the observations thus far undertaken:

Conventional glass microscopic slides (1 inch × 3 inch) were coated with a thin film of formvar by immersing them in a 0.15 per cent solution of this material in ethylene dichloride, and allowing the solvent to evaporate. The resulting film was approximately 200 Å in thickness.

A clotting mixture was prepared by the addition of the thrombin in buffered saline solution at a concentration of 10 units per cc. to the fibrinogen in similarly buffered solution at a concentration of 0.06 per cent. To 5 cc. of the fibrinogen solution in a short test tube, 0.5 cc. of the solution of thrombin was added and mixed rapidly by pipetting. The coated slide was immersed in the clotting mixture for 15 seconds, then removed and placed horizontally over water in a covered Petri dish. The clotting process was allowed to proceed undisturbed for 2½ minutes. At the end of this time the clot in the test tube was well formed, as was the film of clot on the coated slide. The latter was then flooded with a solution of phosphotungstic acid (11), 0.2 per cent, for approximately 15 minutes. At the end of this time, the phosphotungstic acid was removed by flooding the slide with distilled water. While under water, small portions of the clot-covered film of formvar were peeled away from the glass and mounted on the conventional 3 mm. discs of wire cloth used to support objects for electron microscopy. The preparations were then quickly drained and dried and examined.

The clot from the test tube was removed and compressed to obtain free fluid for pH determinations. It was assumed that the measurement of the pH of the clot in the test tube, which was the source of the clot on the microscope slide, gave at least a first approximation to the pH value of the latter. The measurements of pH of the various solutions were recorded throughout, but those of the fluid obtained from the final clots were considered to be the most significant.

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1. The unit is defined as the amount required to clot 1 cc. of a standard fibrinogen solution in 15 seconds (6) or the amount which clots a 1 per cent solution of fibrinogen in a test tube 1 cm. in diameter at 25°C., pH 6.3, in approximately 45 seconds (10).

2. This is a polyvinyl formal resin, sold by the Shawinigan Products Corporation. It seems to be particularly valuable for making preparations by these methods, presumably because of its tensile strength. It is insoluble in H₂O and probably does not interact with the fibrinogen or thrombin or influence the character of the formed clot.
The buffers used were freshly prepared Sørensen's phosphate mixtures and in two experiments the fibrinogen was dissolved in Tyrode's solution (Earle's modification). The alteration to acidity was obtained in the latter by maintaining a partial tension of CO₂ over the solutions and clotting mixture. In some experiments the vapors of osmium tetroxide were used in place of the phosphotungstic acid, with no marked variation in the results.

An RCA electron microscope, type E.M.U., was used for all microscopy. Most of the micrographs were taken at magnifications between 5000 and 7000 and enlarged photographically. Particular care was taken to use an electron beam of minimal intensity so as to avoid, as far as possible, any alterations in the material.

**Observations**

In an attempt at an orderly presentation of data we have arbitrarily subdivided the description of certain of the characteristics of the clots formed at three different values of pH. The measurements represent mean values derived from the study of many micrographic fields in the several preparations made at each of the several pH intervals studied. A more nearly statistical analysis is in preparation, but the approximations here recorded seem adequate for the purpose of an initial presentation.

**Coarse Structure of the Fibrin Network.**—The general architecture of the clots is illustrated in Figs. 1 to 4. It appears that under the conditions of these several experiments the unit of structure is an elongated fiber (to be called a unit fiber), and that such fibers are joined laterally in various ways to form compound fibers (Fig. 5). This results in a tridimensional network of branching strands. Studies of the micrographs also show evidence of the tapering character of the unit fibers. Many of these appear to have their tapered tips incorporated in incompletely polymerized material on the plastic film.

In the clots formed at pH 8.5 (Fig. 1), most of the fibers are single and show relatively infrequent fusion with other single or unit fibers so that but few compound fibers are formed (Fig. 1). The fibers are twisted and curved. The interstices are, on the average, very small.

At pH 7.6 the formation of more numerous compound fibers is apparent (Fig. 2). These are much less curved, particularly over the intervals where two or more unit fibers lie bundled in parallel longitudinal association. The interstices are generally larger.

The micrographs of clots formed at pH 6.3 show the more generalized formation of compound fibers, and only a few independent unit fibers (Figs. 3 and 4). The broad compound fibers are often straight and the interstices vary widely in size and shape but are of larger average size. The number of fibers in the broad strands varies from place to place as component fibers branch from the main bundles to join other single or compound fibers. As many as eight unit strands can be demonstrated in some of the broad compound fibers. Often the point

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The authors are grateful to Dr. R. M. Taylor for permission to use the instrument belonging to the laboratories of the International Health Division of The Rockefeller Foundation.
of juxtaposition of several fibers appears as a nidus from which multiple branchings appear to be derived (Fig. 6).

The average values of the diameters of representative unit fibers formed at these different pH values are indicated in Table I. These were derived from measurement of separate, single fibers and also units within compound fibers where the numbers and limits of the components could be made out with certainty.

It is evident that the greater the hydrogen ion concentration in the clotting mixture the larger the average diameter of the unit fiber, single or in bundles. The larger unit fibers at the lower pH, and particularly the frequent close lateral association of two or more other unit fibers produce the characteristic coarse architectural features described.

It is also of interest to note that in the background of the micrographs there was a significantly greater amount of amorphous unpolymerized protein precipitate in the preparations clotted at pH 8.5 than in those clotted at pH 6.3.

<table>
<thead>
<tr>
<th>pH</th>
<th>6.5</th>
<th>7.6</th>
<th>8.5</th>
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<tr>
<td>mµ</td>
<td>60</td>
<td>49</td>
<td>36</td>
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**Fine Structure of the Individual Fibers.**—Examination of the individual fibers in the electron micrographs (Figs. 4 to 7) shows a striking cross-striated appearance essentially similar to that noted only in human spinal fluid pellicle fibrin by Ruska and Wolpers (2). This is due to alternate bands of relatively high and low density to electrons. These striae are more readily seen in the fibers formed at the acid pH than in those formed at the alkaline pH. While most of our preparations were treated with phosphotungstic acid, it is to be noted that these periodic striae were clearly visible as well in preparations fixed in vapors of osmium tetroxide. Also, in preparations covered with gold by the so-called shadow-cast technique (12), some fibers show marginal prominences which correspond to the cross-striations (Fig. 7).

One of the most striking features of the fine structure is the coinciding striations of laterally associated unit fibers. Often this coincidence can be seen to extend across the several fibers of a large bundle. The longitudinal outlines of the individual fibers in such bundles are not lost (Figs. 5 and 6).

The tapered ends of the unit fibers show the characteristic striations, and signs of the same periodicity are to be seen in the incompletely polymerized material, particularly in the preparations clotted at pH 6.3. The fine fibrillar material visible in the background of the gold-shadowed preparation shows a comparable periodic beading (Fig. 7). Average lengths of the periods at three
different pH values are presented in Table II. In a few fields some of the fibers looked as if stretched. Measurements of the lengths of the periods of the cross-striations in these stretched fibers show them to be increased significantly.

There are certain technical difficulties encountered in making and analyzing the required measurements. Among these may be mentioned the lengthening of the distance from one dark band to the next as the apparent result of stretching. Also, there is a tendency for the periodicity to be more regular in compound fibers, and in these the distances between dark bands appear slightly shorter than in individual fibers. In addition, the striations are less well defined in fibers of clots formed at pH 8.5 than in those formed at pH 7.6 or pH 6.3. When these factors are taken into account, it seems improbable that the slight variations of the recorded average values of Table II have significance. Indeed the average periodicity throughout the clots formed at the three values of pH is strikingly constant.

### TABLE II

<table>
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<tr>
<th>pH</th>
<th>6.3</th>
<th>7.6</th>
<th>8.5</th>
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<tr>
<td>Å</td>
<td>245</td>
<td>243</td>
<td>253</td>
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**DISCUSSION**

Any attempt to describe the details of the mechanism of the clotting of whole blood from the observations recorded in this report would be premature and speculative. The method of study we have outlined has obvious limitations, particularly since the preparations examined in the electron microscope have been dehydrated in the vacuum of that instrument and certain distortions and contractions must be assumed to have taken place. Furthermore, the system studied has been simplified by the use of the two protein substances assumed to interact in the final stage of the clotting mechanism, and only the effect of alterations of pH has been considered in this initial report. Nevertheless, the general appearance of the clots is essentially similar to that of those studied by Wolpers and Ruska (1, 2) who used as objects clots formed from whole blood, plasma, and spinal fluid under more nearly physiological conditions.

Ferry and Morrison (10) studied the clotting of the solutions of human fibrinogen by the addition of human thrombin under various conditions of concentration of the reacting proteins, pH, ionic strength, temperature, and the addition of certain polyhydroxyl compounds, and they concluded that clotting is to be interpreted as a three-dimensional polymerization. They proposed as the structure of the fine clot a network of chains, consisting of
fibrinogen molecules joined end to end, cross-linked partly at least by primary chemical bonds. They suggested that the coarse clot is a network of bundles of such chains, cross-linked largely by secondary bonds and as result of lateral association. Our own observations tend to bear out in large part these hypotheses. However, at the concentrations of fibrinogen, thrombin, and hydrogen ion we have studied, and under the other described conditions of our experiments, it would seem that the fine, grossly transparent clot formed at pH 8.5 is composed of unit fibers of multiple, rather than single chains of fibrinogen molecules. The marked increase in the diameter of these unit fibers in clots formed at pH 6.3, and especially the tendency of the unit fibers to form compound fibers by lateral association could well account for the opacity of the gross clot at this pH. The tendency to form compound fibers may also explain in part the syneresis of coarse clots that is observable in the gross.

The striking regularity of the cross-striations of the fibers at the different values of pH studied is difficult of interpretation. Measurements of double refraction of flow, viscosity, osmotic pressure, and sedimentation in the ultracentrifuge of solutions of human fibrinogen have led to a tentative description of the model of the molecule as an elongated ellipsoid of revolution about 35 × 700 Å, with a molecular weight of about 500,000 (13). Bovine fibrinogen recently has been subjected to osmometric and viscometric studies with essentially the same results (14). If it is assumed that the primary interaction of fibrinogen and thrombin results in an end-to-end linkage of such long molecules, with subsequent lateral association, it is not easy to reconcile the measured periodicity of the unit strands with the dimensions of the proposed molecular model. Possibly, in polymerization, the shape of the fibrinogen molecule is altered by extensive folding and consequent shortening. The region of greatest folding could be, as suggested for collagen (15), the region of greatest density; i.e., the dark striation.

Study of the incompletely polymerized material to be seen in the backgrounds of the clots (Fig. 7) has yielded some evidence for the end-to-end linkage of elongated structures of the orders of magnitude of the proposed fibrinogen molecules. The limit of resolution in the better micrographs seems to lie between 5 mμ and 10 mμ. The finest chains visible show longitudinally arranged densities and irregularities and may represent single strands or "protofibrils" (15) of elongated molecules, linked end to end. Also, it would appear that the unit fibers are built up by the lateral association of several such chains (Fig. 7). In this association the regions of greatest density coincide in a remarkable way to give the characteristic banded or striated appearance. Even when the unit fibers come to lie in close association, some force operates to line up the striae so that they coincide directly across the compound strand (Figs. 5 and 6).

Another model which could fit many of the data would be a disc-shaped ellipsoid of revolution 10 Å in thickness with a diameter of 350 Å (13).
A discussion of the nature of the forces involved in the precise lateral association of molecular chains and bundles of such chains (unit fibers) so that the striations correspond in fibers lying side by side is not within the scope of this report. However, as Ferry and Morrison (10) have pointed out, the conditions which favor decreasing coarseness of the fibers are those which would be expected to diminish attractive forces and the tendency to aggregation. Since the isoelectric point of human fibrinogen is at about pH 5.5, an increase of pH from 6.3 to 8.5 would increase the net charge of the molecule and hence the mutual electrostatic energy which might be expected to result in diminution of electrostatic attractive forces between the large molecules.

SUMMARY

1. A technique has been described for the preparation of clots from purified fibrinogen and thrombin of bovine origin which are suitable for study with the electron microscope. Experiments have been carried out to compare the fine structure of clots prepared at various values of pH.

2. The clots are composed of meshworks of single and compound fibers. At pH 8.5 the unit fibers have a smaller average diameter than those formed at pH 7.6 or pH 6.3. The tendency for the lateral association of unit fibers into compound fibers is markedly increased as the pH is decreased.

3. A striking feature of all the clots studied is cross-striation of the unit fibers. The periodicity of these striae is quite constant throughout (approximately 250 Å). There is a precise coincidence of the striations of the individual unit fibers where these are associated side by side to form compound fibers.

BIBLIOGRAPHY

EXPLANATION OF PLATES

PLATE 29

Fig. 1. Micrograph of clot formed at pH 8.5: in Tyrode's solution, bicarbonate buffer. Treated with 0.2 per cent phosphotungstic acid solution.

The unit fibers (A) are thin and often curved. Relatively few compound fibers (B) are formed by lateral association of the units. The interstices are fairly uniform and small. Much of the background material appears non-fibrous. × 15,200.

Fig. 2. Micrograph of clot formed at pH 7.6: in 0.85 per cent saline, Sörensen's phosphate buffer. Treated with 0.2 per cent phosphotungstic acid solution.

The unit fibers are broader and many compound fibers are to be seen. These are less curved. The interstices vary widely in size and shape but many are larger than in Fig. 1. The background material is for the most part fibrillar. × 15,200.

Fig. 3. Micrograph of clot formed at pH 6.3: in 0.85 per cent saline, Sörensen's phosphate buffer. Treated with 0.2 per cent phosphotungstic acid solution.

Many coarse strands formed of associated unit fibers characterize the clot. The unit fibers are broader than in Figs. 1 and 2. The interstices show marked variation in size and shape. Relatively little background material is visible and that which can be seen seems polymerized into long fibrils. × 15,200.
PLATE 30

Fig. 4. Micrograph of clot formed at pH 6.3: in 0.85 per cent NaCl, Sörensen's phosphate buffer. Treated with 0.4 per cent phosphotungstic acid solution. This illustrates the branching of the compound fibers and the other general architectural features of the coarse clot. Characteristic cross-striations are visible in most of the compound fibers, as well as in the single fibers, including their tapering ends. × 30,500.
(Hawn and Porter: Electron microscope study of clots)
Fig. 5. Micrograph of fibrin formed at pH 6.8: in Tyrode's solution, bicarbonate buffer. Treated with 0.2 per cent phosphotungstic acid solution.

The several compound fibers show the striking coincidence of the striations where the unit fibers lie in lateral association. × 40,000.

Fig. 6. Micrograph of fibrin formed at pH 6.3: in 0.85 per cent NaCl, Sörensen's phosphate buffer. Treated with 0.4 per cent phosphotungstic acid solution.

Detail of striations showing striking coincidence across a flattened bundle formed by the lateral association of approximately eight unit fibers. × 44,000.

Fig. 7. Micrograph of fibrin formed at pH 6.8: in Tyrode's solution, bicarbonate buffer. Treated with 0.2 per cent phosphotungstic acid solution. Gold-shadowed.

This preparation shows details of fiber structure. Fine fibrils lie in parallel with coinciding densities. Some of the resulting fibers appear flattened. It is evident also that the finely fibrillar background material shows a periodicity similar to that in the larger fibers. × 50,000.