XXII. THE ACTION OF THROMBIN UPON FIBRINOGEN.

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METHOD.

THE solutions of fibrinogen, thrombin and sodium chloride employed in these experiments contained about 0.5 % of tri-sodium citrate.

As source of fibrinogen human plasma was ordinarily used, being prepared by mixing 12 cc. of blood, immediately after withdrawal from a vein of the forearm, with 1 cc. of 0.85 % sodium chloride solution to which 0.13 g. sodium citrate had been added. After centrifugalisation the plasma was pipetted off and stored at 0°. In a few experiments fibrinogen was precipitated by pouring human plasma into twenty volumes of distilled water through which a stream of carbon dioxide had been passed, the precipitate being separated by centrifugalisation and then redissolved in citrated 0.85 % sodium chloride solution.

As source of thrombin freshly prepared whipped blood, to which 1 % of sodium citrate had been added as soon as separation of fibrin was completed, was used in a few experiments. As, however, the range of concentration of thrombin obtainable in this way was small, in most experiments the thrombin employed consisted of the venom of *Echis carinatus*¹ dissolved in 0.85 % sodium chloride solution, to which 0.5 % of sodium citrate had been added [Barratt, 1915]; the limit of utility of this form of thrombin was about 1 in 30,000,000 and the highest concentration used rarely exceeded 1 in 3000; it was completely destroyed by heating to 75° for ten minutes.

Dark background illumination was obtained by means of a paraboloid condenser with central stop, an arc lamp being used as source of light.

Coagulation was usually studied at room temperature, the mixture of fibrinogen and thrombin, which formed the subject of observation, being placed upon a glass slide, within an area ringed with vaseline, and closed by a coverslip: the thickness of the layer of fluid examined was usually about 50 μ . Occasionally coagulation was carried out in small glass tubes.

¹ For a supply of this I am indebted to the kindness of Dr C. J. Martin, Director of the Lister Institute.

FIBRIL FORMATION.

When a liquid containing fibrinogen is mixed with a solution of thrombin in the presence of sodium citrate or oxalate coagulation occurs, due to the formation of fibrin fibrils. If the production of such fibrils occurs with sufficient slowness the course of the process can readily be studied¹.

Fig. 1 represents the appearance, under a dark background illumination, of a mixture of (1) citrated human blood (containing fibrinogen, but no thrombin), and (2) whipped human blood² (containing thrombin, but no fibrinogen): at the end of three hours a fine fibril of fibrin, about 0.17μ thick, previously invisible or on the threshold of visibility, was observed

Fig. 1. Early stage of coagulation of a mixture of citrated human blood and whipped human blood (subsequently citrated). Film preparation three hours old. A single fibril is seen. In addition to red cells highly refractile dancing particles and a polymorphonuclear leucocyte are seen. $\times 2300$.

lying across the field. At the end of a further period of twenty hours, this fibril was found to have increased considerably in thickness, while a second delicate fibril, about 0.2μ thick and 25μ long, had appeared (Fig. 2): the length of the large fibril was found on measurement to be 0.342 mm. Fibrils vary in length from 20μ or less to 500μ or more; their apparent diameter ranges from about 0.17μ to 1.1μ ; the smallest fibrils do not present any

¹ Fibril formation has been investigated ultramicroscopically by several observers, two views having been advanced as to the nature of the process occurring. According to Mayer [1907] and Cesana [1908] coagulation consists in the appearance of very fine granules, which increasing in size arrange themselves in rows, forming threads, subsequently becoming numerous and thus giving rise to a network. Stübel [1914] compares coagulation to a crystallisation process and Howell [1914, 1916] states that fibrin is deposited not as a network, but as separate needles or crystals, which are massed to form a close meshwork.

² Citrated after separation of fibrin.

obvious structure, but the largest exhibit an irregular imperfectly reticular aspect (Fig. 3 c), due to refractile material, which is in more or less marked contrast to the rest of the substance of the fibril, the pattern thus produced sometimes extending along the whole of the fibril, sometimes being observed in short lengths of fibril, the intervening portion being apparently homogeneous. No further increase in size or number of fibrils could be recognised subsequently in the microscopic field shown in Fig. 2.

Two processes are therefore concerned in the formation of fibrin fibrils: (1) production of primitive fibrils; and (2) subsequent growth of these in presence of fibrinogen. The number of the finest fibrils actually seen depends

Fig. 2. Same as Fig. 1, twenty hours later. The fibril seen in the former figure has increased considerably in size and a second fine fibril has appeared. The leucocyte has become swollen and its granules, which were fixed in the preceding figure, are now in oscillatory motion. $\times 2300$.

upon the intensity of the illumination employed: with a poor arc light only a few fine fibrils are seen; the more intense the arc, the larger the number of exceedingly fine fibrils which can be rendered visible. The possibility must therefore be borne in mind that in mixtures of fibrinogen and thrombin excessively small fibrils may be already in existence, which cannot be rendered visible until their thickness has been increased by further addition of fibrin. It will be shown later that the exact moment at which fibrils are first formed can be studied only by indirect methods (p. 201). On the other hand the time of first appearance of visible fibrils can be determined by direct observation: to this method of investigation most of the results obtained in the present work are due.

In order to determine the relative amounts of fibrinogen and thrombin taking part in coagulation the following experiment was made.

Exp. A. Equal volumes of citrated human blood plasma (fibrinogen solution) and citrated 1 in 8,000,000 venom (thrombin) solution were mixed together. Coagulation occurred at room temperature (18°). At the end of sixteen hours a portion of serum expressed from the clot was mixed with an equal volume of venom solution (1 in 100,000) and allowed to evaporate at room temperature to about one-tenth of its bulk: no further coagulation occurred.

The amount of fibrinogen contained in the plasma employed was determined by precipitation with twenty volumes of distilled water through which a stream of carbon dioxide had been passed, the precipitate being then dried to constant weight at 120°. The plasma contained 0.78 % of fibrinogen¹.

In the above experiment the weight of fibrinogen employed was more than 60,000 times as great as that of the venom added. It follows therefore that fibrin arises by transformation of fibrinogen: thrombin merely acts as a determinant, that is to say catalytically².

Fibrils of fibrin are frequently attached to each other and may coalesce over part of their length, but no branching occurs (*cp.* Figs. 1 to 3). When present in sufficiently scanty numbers they are observed to be quite separate and to be unconnected with any of the suspended particles present, so that their characters can be readily studied, especially if the conditions of experiment are such as to lead to the production of fibrils of relatively large size: they are then seen to be attached to the surfaces of glass bounding the layer of liquid in which they have been formed.

Whatever may be the ultimate mode of production of these fibrils it is obvious that they form separate entities and that they are therefore particulate in origin, their appearance being determined by the addition of thrombin and the material of which they are constructed being provided by fibrinogen. Such particles of origin appear to be amicroscopic, for none of the particles seen in plasma or solution of fibrinogen can be observed to disappear or diminish in number after fibril formation is completed, and again the number of particles of origin, estimated from the number of fibrils developing, is very considerably in excess of the number of particles ordinarily observable in fibrinogen solution by ultramicroscopic methods. Presumably these particles of origin of the fibrils may be contained either (1) in the venom solution or (2) in the fibrinogen solution, employed in experiments on fibril formation. As a preliminary step in the investigation of this point the appearance presented at the junction of a solution of fibrinogen with a liquid containing thrombin was next studied.

¹ *Cp.* note, p. 197.

² Howell [1910] concludes that thrombin probably does not act upon fibrinogen after the manner of an enzyme. The solution of thrombin employed by this observer could, however, be heated to the boiling-point without completely losing its coagulative action on fibrinogen.

Fig. 3. Fibril formation at junction of droplets of (1) citrated human blood plasma and (2) citrated solution of venom of *Echis carinatus* (1 in 8,000,000). Film preparation six days old. *a* is taken at the plasma side. In *a* the fibrils are fine and numerous; in *b* most of the fibrils are larger, though less numerous; in *c* the fibrils are still further reduced in number, a few of relatively considerable size exhibiting a reticular pattern. Highly refractile dancing particles are seen among the fibrils; in *c* a fixed collection of particles attached to fibrils is observed. $\times 2300$.

Exp. B. A film preparation was made by placing droplets of human blood plasma and of a 1 in 8,000,000 solution of venom of *Echis carinatus* (both liquids being citrated) respectively near each other on a glass slide, within an area ringed with vaseline. A coverslip was then applied and lightly pressed down, so as to allow the droplets to come in contact at their opposed edges with little or no mixing of the liquids, evaporation being prevented by the ring of vaseline. The appearance of the junction of liquids at the end of six

days is shown in Fig. 3. At the side of the junction near the venom solution (*a*) very fine, exceedingly numerous fibrils are seen; in the middle (*b*) larger, less numerous fibrils; at the side next the fibrinogen solution (*c*) only relatively few fibrils, mostly long, of large diameter, and exhibiting an irregular pattern are observed.

Fibrin fibrils it may be observed are usually found to be unequal not only in length but also in thickness. After the lapse of a fortnight or three weeks, when they appear to have reached their maximum thickness, it may be observed that, mixed with the largest fibrils, medium-sized and fine fibrils are also seen (Fig. 3 *c*); when the fibrils do not exceed a medium value fine fibrils may be seen as well (Fig. 3 *b*); but when liminal visibility of fibrils has been reached differences of thickness can no longer be recognised.

It will be noted that those fibrils which are situated near the venom solution are contained in a fluid which is relatively rich in venom and correspondingly poor in fibrinogen, while at the opposite aspect of the junction of liquids the fibrils are present in a liquid in which the concentration of fibrinogen reaches its maximum, the venom being exceedingly dilute.

In order to observe more fully the effect of dilution of fibrinogen and venom respectively the series of experiments in Tables I and II were carried out. In these experiments an attempt has been made to give the actual number of fibrils observed in the field of the microscope: the higher counts are only approximate, owing to the large number and fineness of the fibrils present; the lower counts are more reliable owing to the smaller number and larger size of the fibrils. The enumeration of fibrils present in the field of the microscope is attended with difficulty if the fibrils are very numerous or are exceedingly fine. In the former case (*cp.* Fig. 3 *a*) delimitation of fibrils cannot be effected: it is a matter of uncertainty whether any given length of fibril should be regarded as a separate fibril or is merely the continuation of an adjacent fibril. Again, when fine fibrils are being examined, it is difficult to avoid overlooking some of the finest, which are indeed barely visible even with the maximum intensity of illumination available. It is obvious, therefore, that, if the fibrils under observation are very numerous or are exceedingly fine, enumeration is merely approximate. On the other hand if the conditions of experiment are such that the fibrils are few in number and if, in addition, they are relatively thick, counting ceases to be difficult and accuracy of enumeration is attainable. In Table I the effect of an 81-fold dilution of venom is exhibited. It will be seen that, within the range of concentration employed, the number of fibrils visible appears to be roughly proportional to the amount of venom present. This relation, it may be observed, does not continue to hold if the range of concentration of venom is made considerably more extensive: under these circumstances a point is reached at which no further increase occurs, but an actual diminution in the number of fibrils recognisable may be noted (*cp.* p. 209). With the highest venom concentration employed

in Table I, fibril formation is not only abundant but also rapid, occurring within a few minutes, while with the lowest concentration its course is very slow, fibrils not appearing until after the lapse of many hours: in the former case only a limited increase in thickness of the fibrils subsequently occurs; in the latter the amount of fibrinogen available is, owing to the smaller number of fibrils, relatively considerable and in consequence marked though slow growth takes place, which may not reach its maximum for several days.

Table I.

Equal parts of citrated human plasma (undiluted) and of citrated venom solution, the latter in varying dilutions, were placed on a glass slide and, immediately after mixing, a coverslip applied, the thickness of fluid examined being 0.05 mm.

Venom	Fibrils per field	Appearance of fibrils
in 300,000	1730	Very fine, retracted
„ 900,000	640	Fine
„ 2,700,000	120	Medium
„ 8,100,000	50	Moderately coarse
„ 24,300,000	20	Coarse

In Table II the effect of dilution of plasma is exhibited: it is seen that, with diluted plasma, the number of fibrils diminishes, but the diminution is no longer accompanied by an increase in thickness, as in the preceding experiments, the fibrils remaining fine. With the higher dilutions no fibrils can be recognised. Fibrils make their appearance in diluted plasma more slowly than in undiluted plasma.

Table II.

Equal parts of citrated human plasma, in varying dilutions, and of citrated venom, diluted 1 in 8,100,000, were mixed together as in the preceding Table.

Plasma	Fibrils per field	Appearance of fibrils
100 %	43	Moderately coarse
33	7	Fine
11	—	No fibrils visible
3.7	—	„ „
1.2	—	„ „

Returning once more to the appearances presented by fibrin fibrils at the junction of two liquids containing respectively plasma and venom (*Exp. B*, p. 193, and Fig. 3 *a, b, c*), we are now, by the aid of the data obtained from the above dilution experiments, in a position to state that at the venom side of the junction of liquids (where venom is relatively abundant and fibrinogen dilute) the numerous fine short fibrils observed have been produced rapidly, while at the fibrinogen side of the junction (where plasma is almost undiluted and venom is highly dilute) the long, relatively coarse fibrils, which are seen in comparatively small numbers, have been produced with considerable slowness.

This mode of formation of fibrin fibrils in the presence of varying concentrations of venom indicates the source of the particles of origin of the fibrils.

These particles of origin, which, it may be surmised, are contained either in the thrombin solution or in the fibrinogen solution, employed for fibril production, may conceivably consist of either thrombin or fibrinogen. If the particles of origin consist of thrombin the effect of dilution of the thrombin solution, the concentration of fibrinogen remaining unchanged, would be to diminish the number of fibrils without affecting their time of formation. Such, however, is not the case: when the concentration of thrombin is lowered fibrils make their appearance (in diminished numbers) after a lengthened interval, the duration of which is dependent upon the degree of dilution of thrombin. The hypothesis that the particles of origin of fibrin fibrils consist of thrombin is therefore untenable. If, on the other hand, the particles of origin consist of fibrinogen the effect of dilution of thrombin, acting catalytically, would be exhibited by delay of formation of fibrils, and if the particles of origin were unequal (for example, in consequence of variability in size, as is not inherently improbable) in respect of their response to thrombin, the fibrils appearing would be reduced in number: since both of these effects are actually observed it is seen that the latter hypothesis, namely that the particles of origin of fibrin fibrils consist of fibrinogen, is tenable in so far as it is consistent not only with the delay in appearance of the fibrils but also with the diminution of their number¹. Further experimental proof of the validity of this hypothesis will now be given.

FIBRINOGEN SOLUTION.

Since growth of fibrils can be observed in a fibrinogen solution the further assumption must be made that, while part of the fibrinogen is in suspension, another part is in solution: in other words the liquid containing fibrinogen must be diphasic, forming a hydrosol, the disperse phase furnishing the particles of origin of the primitive fibrils, while the continuous phase, by affording a further supply of fibrinogen to the primitive fibrils, enables an increase of their diameter to occur, both particulate and added fibrinogen alike changing under the influence of thrombin into fibrin.

A point has now been reached at which it is possible to put hypothesis to the test of experiment. If fibrinogen solution forms a two-phase system the volume of the concentrated phase which, it has just been inferred, provides the particles of origin of fibrils will diminish on dilution, that of the dilute phase undergoing a corresponding increase, until ultimately a dilution is reached, below which the concentration of the dilute phase, previously maintained unchanged, will now begin to diminish with further addition of diluting fluid.

In order to test this conclusion an attempt was made to determine the concentration of the continuous phase present in varying dilutions of plasma.

¹ The effect of dilution of fibrinogen solution upon the thickness of fibrils is readily explicable. The latter in low concentrations of fibrinogen can receive only a small supply of this substance and in consequence remain exceedingly small.

To this end the experiments on fibril formation, shown in Table III, in which the time of first appearance of fibrils in a series of mixtures of fibrinogen and venom is observed, were made. This method of enquiry is based upon the assumption that, since one of the two substances interacting in the production of fibrils, namely venom, is employed in the same concentration in all experiments, fibril formation will therefore proceed at a monomolecular reaction rate according to the equation

$$-lC = kt + a,$$

where C = concentration of continuous phase, t = time of first appearance of fibrils, and k and a are constants. If this assumption is verified it would become possible to determine the degree of dilution of plasma below which diminution of concentration of the continuous phase commences, and to ascertain the relative concentration of continuous phase corresponding to any given dilution of plasma. The value of the constant k is independent of that of a : if the temperature of experiment is maintained invariable its value is dependent upon the concentration of thrombin employed. If the curve is made to commence at the axis of ordinates so that the vertical part corresponds with this axis, then $-a$ represents the natural logarithm of the lowest concentration of plasma at which the continuous phase is saturated.

Table III.

Citrated human plasma, in varying dilutions, was mixed with citrated venom, 1 in 30,000, in the proportion of eight parts of the former to one part of the latter, and the time at which fibrin fibrils were first observed noted.

Exp.	Plasma	Fibrils first seen at end of:
1	100 %	5 minutes
2	90 "	6 "
3	80 "	5 "
4	70 "	7 "
5	60 "	12 "
6	50 "	50 "
7	40 "	113 "
8	30 "	175 "
9	20 "	262 "
10	10 "	No fibrils observed

The observations when plotted with dilution of plasma as ordinates and time of first appearance of fibrils as abscissae were found to describe a curve (Fig. 4 *a*) so long as the concentration of plasma employed did not exceed 61 % (undiluted plasma¹ being represented by 100): above this point the curve was replaced by a vertical line. The curve corresponds to the concentration of the continuous phase, which reaches its maximum at a dilution of

¹ The precipitate (dried to constant weight at 120°) obtained from plasma by adding twenty volumes of distilled water, through which a stream of carbon dioxide had been passed, amounted to 0.8 % of the plasma employed. This precipitate is known to contain, in addition to fibrinogen, prothrombin and thrombokinase. It is not at present possible to obtain fibrinogen in even an approximately pure condition.

plasma represented by 61 %: at this point the conversion of the disperse phase into the continuous phase is apparently complete. Below a dilution of 61 % the concentration of the continuous phase becomes lowered when more fluid

Fig. 4 a and b. Graphic representation of experiments in Table III. Ordinates in Fig. 4 a indicate the total concentration of fibrinogen contained in the plasma employed, that of undiluted plasma being represented by 100; ordinates in Fig. 4 b give the concentration of fibrinogen in the continuous phase, that corresponding to 61 % in Fig. 4 a being here represented by 100. Abscissae represent time, in hours, at which fibrin fibrils were first observed. The curves are constructed from values of $k=0.0042$ and $a=-4.623$. Exps. 1 to 4 are not indicated in Fig. 4 b.

is added; above this dilution no further change in the concentration of the continuous phase occurs, saturation having been attained. In Fig. 4 b, the same curve is again given the ordinates now indicating the concentration of the continuous phase, saturation of which (corresponding to 61 % of plasma

in Fig. 4 a) is represented by 100. If the values of the constants, determined from *Exps.* 5 to 10 (Table III), are taken at

$$a = -4.623, \quad k = 0.0042,$$

the calculated reaction times, given in Table IV, exhibit little divergence from those actually observed. From these experiments it may therefore be concluded that fibrinogen solution is diphasic. That the individual particles of the disperse phase have not, however, completely disappeared when the transition point of the curve is reached, is shown by the circumstance that fibril formation does not cease at this point. Nevertheless the sharpness of the transition point and the fair correspondence of calculated and observed times appear to indicate that the amount of fibrinogen still remaining, with increasing dilution of plasma, in the particles of the disperse phase after the maximum concentration of the continuous phase has been passed, is relatively insignificant, and thus the assumption, made in Table IV, col. 2, that the concentration of the latter phase now becomes proportional to the further dilution appears to be at any rate approximately correct. The significance of the absence of complete disappearance of the particles of the disperse phase, at the transition point, will be referred to later (p. 208).

Table IV.

Calculated time of appearance of fibrin fibrils in the experiments recorded in Table III.

Exp.	Plasma	Fibrinogen in continuous phase ¹	Reaction calculated	Time observed
1	100 %	100 %	5.5 min.	5 min.
2	90 "	100 "	5.5 "	6 "
3	80 "	100 "	5.5 "	5 "
4	70 "	100 "	5.5 "	7 "
5	60 "	98.5 "	10 "	12 "
6	50 "	82.0 "	53 "	50 "
7	40 "	65.6 "	107 "	113 "
8	30 "	49.2 "	178 "	175 "
9	20 "	32.8 "	271 "	262 "
10	10 "	16.4 "	437 "	No fibrils observed

If the dilution of plasma is considerable no visible fibrils are obtainable on adding thrombin: in Table II, for example, this was found to be the case with a dilution of 11 % of plasma. When, however, a portion of this highly diluted plasma was placed on a microscope slide and allowed to evaporate at room temperature to about one-sixth of its bulk, and then a small amount of venom (1 in 30,000) added, a striking result was obtained: at the end of fifteen minutes abundant fibril formation was found to have occurred; in a control experiment, performed at the same time, in which care was taken to prevent evaporation, no fibrils appeared.

It is thus evident that the simple addition of thrombin as a test for fibrinogen under all circumstances is not admissible. If the continuous phase is sufficiently dilute (*cp.* p. 203), no coagulation is recognisable, no fibrils

¹ The maximum concentration, which is present in 61 % dilution of plasma, being represented by 100.

are observable: by evaporation, however, the original concentration of fibrinogen may be regained and the former coagulability reproduced.

Incidentally the ease with which a highly diluted continuous phase can be concentrated, with reappearance of visible fibrils, in experiments made with small quantities of diluted plasma, placed on a glass slide, emphasises the necessity of very carefully avoiding evaporation in such experiments when determinations of the time of first appearance of fibrils are being made.

MECHANISM OF FORMATION OF FIBRIN FIBRILS.

With a view of throwing further light upon the processes concerned in coagulation, the following hypothesis as to the mechanism of production of fibrin fibrils may now be advanced.

In attempting to explain the mode of occurrence of this phenomenon in a liquid containing fibrinogen and thrombin I picture to myself fibrils originating from spherical particles, exhibiting Brownian movement like the particles ordinarily seen in plasma, but presumably of considerably smaller dimensions, which under the influence of thrombin have become semi-solid, viscid and sticky. One of these oscillating particles comes, let us suppose, into contact with the surface of the glass slide, upon which the mixture of fibrinogen and venom solutions, used for experiment, has been placed. Recoil then occurs, the particle changing its direction, continuing its oscillatory movement, and ultimately, after an exceedingly zig-zag course, colliding a second time with, let us imagine, the glass slip covering the fluid in which it is contained. But inasmuch as the particle is viscid and sticky it became adherent to the glass slide at the first impact and then, upon recoil, became drawn out, so that when the second collision with the cover-slip occurred a primitive fibril had thus been formed, which now became adherent by its previously free end (containing the remainder of the particle of origin) to the cover-slip, the fibril being thus attached at each extremity to a glass surface. By the continued action of thrombin the further change of the primitive fibril, thus formed, into fibrin is completed: the fibril becomes solid and, at the same time undergoing contraction, becomes also tense. Further addition of fibrinogen, from the continuous phase, to the primitive fibril next occurs, the new supply becoming in its turn, under the influence of thrombin, converted into solid fibrin. In this way a relatively thick fibril, visible under ultramicroscopic illumination, is ultimately produced. Adhesion of the primitive fibrils is not limited to glass surfaces. Attachment to other fibrils is common, especially when the latter are abundant, and adhesion to the particles seen singly or in groups in fibrinogen solution is also frequently observed¹.

¹ The latter in many cases seems, however, to be a late event, accompanying and partly dependent upon gradual subsidence of the particles under the force of gravity. Characteristic rosette figures, consisting of fibrils radiating from a group of granules, may thus be formed.

Both particles of origin and primitive fibrils appear to be amicroscopic (*cp.* p. 205).

FURTHER CHARACTERS OF FIBRINOGEN.

In relation to fibril formation the processes occurring prior to the first appearance of visible fibrils appear to be, as already indicated, three in number: (1) change, under the influence of thrombin, of the particle of origin, consisting of fibrinogen in a concentrated condition, presumably forming a liquid droplet, into a viscid, sticky, semi-solid mass; (2) conversion of the latter, by virtue of its Brownian movement, leading to contact with neighbouring surfaces, into a primitive fibril stretching between two surfaces to which its ends adhere, and changing under the further action of thrombin into fibrin; (3) growth of the fibril by deposition of fibrinogen from the continuous phase, the fibrinogen so deposited being changed in its turn, by the thrombin present, into fibrin. In the series of experiments, made to determine the time of first appearance of visible fibrils, given in Table III and Fig. 4, the period occupied by the first two processes must be alike in all experiments, whatever the dilution of fibrinogen may be, since the initial concentration of thrombin is the same throughout the series. This period was estimated for the dilution of plasma corresponding to *Exp.* 5, Table III, in the following way. As soon as the mixture of fibrinogen and thrombin had been prepared it was stirred continuously for five minutes, portions being removed for examination at the end of one, two, three, four and five minutes respectively, and the approximate number of visible fibrils appearing in a fixed volume of the sample determined. Such primitive fibrils as were formed before taking the sample would have been already removed by stirring; the visible fibrils observed in the samples must, therefore, indicate primitive fibrils formed after removal and would correspond to particles of origin which had not been converted into primitive fibrils at the time of removal of the sample. The observations made are given below:

Time	Fibrils		%
	observed	calculated	
0 min.	—	732	100
0.07 "	—	659	90
1 "	135	147	20
2 "	33	30	4
3 "	5.5	5.9	0.8
4 "	in exceedingly scanty numbers	1	0.2
5 "	"	0.2	0.03

These experiments, which are represented graphically in Fig. 5, indicate that the change of particles of origin into primitive fibrils proceeds at a monomolecular reaction rate, the constants of the reaction being $k = 1.6$ (time being reckoned in minutes) and $a = -6.58$: the calculated number of fibrils, given in the third column (and in percentage form in the fourth), agree with the observed values within the range of experimental error. The time occupied in the formation of primitive fibrils appears to be small: according to the above

calculation four-fifths of the maximum number of fibrils obtainable are formed within one minute of mixing fibrinogen and thrombin, and one-tenth within the first four seconds (0.07 min.)¹. On the other hand it will be noted that in the experiments recorded in Table III, in which the same concentration of thrombin was used as in the observations given above, the minimum time of first appearance of visible fibrils was five minutes. Since the fibrils first appearing form less than one-tenth of those ultimately seen it is clear that the reaction actually measured in the experiments recorded in Table III is essentially the process represented above by (3), namely growth of primitive fibrils, the processes (1) and (2) together occupying a negligible fraction of the period of first appearance of visible fibrils.

Fig. 5. Removal of primitive fibrils from mixture of fibrinogen and thrombin by stirring with a glass rod. Ordinates represent the number of fibrils appearing in samples removed after varying periods of stirring; abscissae represent time in minutes. Details of experiments are given in text. The curve is constructed from values of $k=1.6$ and $a=-6.58$.

It will be seen from Tables III and IV that fibrils may be formed, not only in undiluted plasma (*Exp. 1*), but also when only 20 % of plasma is present (*Exp. 9*). In the former experiment the maximum concentration of both disperse and continuous phases obtains; in the latter the disperse phase has nearly completely disappeared and the continuous phase has only 33 % of its maximum value (transition point of curve at 61 % of plasma). If an

¹ In mixing fibrinogen and thrombin solutions stirring should be as far as possible avoided in experiments in which fibril counting is attempted.

endeavour is made to count the fibrils present¹, as in the experiments recorded in Table II, it is found that the approximate number present does not at first exhibit any marked decrease, but the fibrils become very fine and when dilution of the continuous phase is considerable cease to be visible owing to their extreme tenuity. Below a series of experiments performed under the same conditions as in Table III (the concentration of thrombin being constant, while fibrinogen is employed in varying degrees of dilution) is given, in which an attempt is made to enumerate the fibrils present in a given small volume of each mixture of fibrinogen and thrombin. These results are plotted in Fig. 6. The higher

Fig. 6. Approximate number of fibrils obtained on coagulation of varying dilutions of citrated human plasma. Ordinates represent fibrils; abscissae represent percentage of plasma. Details of experiments are given in text. The fibril counts indicate a curve of the type shown.

¹ The enumeration is approximate: the error is considerable when fibrils are abundant.

counts are only approximate, owing to difficulties already referred to, but the type of curve formed by the fibrils is indicated in the figure: the dilution of plasma at which visible fibrils cease to be obtainable is determined graphically to correspond to 28 % of plasma. Since the maximum concentration of the continuous phase of fibrinogen in these experiments was found to be present in 75 % of plasma and in all concentrations of plasma above this point, it follows that, in this series of experiments, fibrils cease to be visible when the continuous phase is reduced to 37 % of its saturation concentration. This result and those of three other similar determinations are given below:

Series	Continuous phase of fibrinogen
<i>A</i>	26 %
<i>B</i>	30 „
<i>C</i>	40 „
<i>E</i>	37 „

The mean of the series corresponds to 33 % of the maximum concentration of the continuous phase.

The action of thrombin in converting fibrinogen in the concentrated phase into fibrin is not exerted solely upon the dispersed particles of this phase, as is shown by the following experiment.

Exp. C. Precipitated fibrinogen, obtained by adding to plasma twenty volumes of distilled water through which a stream of carbon dioxide had been passed, was suspended in distilled water so as to form a thick emulsion, composed of closely packed masses of fine motionless granules, 0.4μ or less in diameter. This emulsion was divided into two parts, one of which (*B*) was used for control experiments; to the other venom, contained in distilled water, was added, the concentration of venom in the mixture being about 1 in 1,000,000. At the end of forty-eight hours the mixture had formed a soft, semi-solid, friable mass, consisting of densely packed fine granules free from fibrils: this was broken up as completely as possible and suspended in distilled water (*A*). Portions of *A* and *B* were used for experiment as follows:

		+4 parts citrated 0.85 % NaCl 1 part containing	Microscopic appearance with dark ground illumination
1.	<i>A</i>	—	No dispersion. No fibrils.
2.	<i>A</i>	venom 1 in 30,000	Marked dispersion. No fibrils.
3.	<i>A</i>	„ 2.3 %	Nearly complete dispersion. No fibrils.
4.	<i>B</i>	—	Complete dispersion. No fibrils.
5.	<i>B</i>	venom 1 in 30,000	Complete dispersion. Fibrils fairly numerous.

The last observation was repeated in a small test-tube: a loose coagulum formed, which had already retracted at the end of three hours.

In the above experiment it is seen that under the action of thrombin precipitated particles of fibrinogen are converted into fibrin without any

change of form, fibril formation being impossible owing to the immobility of the particles. The particles of fibrinogen, however, adhere to each other and to the numerous adventitious particles which accompany the precipitated fibrinogen, so that the latter cannot be dispersed on adding 0.85 % sodium chloride solution. If, however, the sodium chloride solution contains venom in sufficient concentration to dissolve the altered fibrinogen particles, then dispersion occurs. That the fibrinogen is converted into fibrin is indicated by the effect of relatively concentrated venom in producing lysis (see p. 209). It does not appear that the presence of sodium chloride is necessary for the transformation of fibrinogen into fibrin. Direct conversion of some of the dispersed particles of fibrinogen in the concentrated phase does not seem inherently improbable, but investigation of this point is attended with difficulty owing to the presence of numerous adventitious dancing particles in liquids containing fibrinogen.

That the suspended particles of the concentrated phase of fibrinogen are unequal, presumably, in size is shown by the circumstance that (1) primitive fibrils are formed at different periods of time (p. 201), while visible fibrils (2) are of different size (Figs. 1-3), (3) do not all appear at the same time (p. 190), and (4) are reduced in number when highly dilute thrombin is employed (Table II; also p. 202).

The size of the particles which give rise to primitive fibrils does not appear to be capable of measurement, nor can the diameter of primitive fibrils be at present determined, but it is nevertheless possible to give, for both of these, upper limits of size, which cannot be exceeded. Thus the finest fibril which could be recognised with the illumination employed in these experiments had an apparent diameter of about 0.15μ . Assuming the length of such a fibril to be 50μ , its volume would be represented by a sphere of 0.46μ radius. Such a relatively large particle, however, though possessing oscillatory movement, would be much too defective in translatory motion to give rise to a primitive fibril 50μ long. On the other hand the smallest recognisable particle, having an apparent diameter of 0.15μ , though still possessing insufficient translatory movement, would have the same volume as a fibril 50μ long and 0.0067μ in diameter. These two values, therefore, may be taken to represent maximum limits for the diameter of a particle of origin and for the thickness of a primitive fibril respectively.

The very irregular translatory movement of an oscillating particle having an apparent diameter of 0.15μ did not, as a rule, exceed 10μ in the course of a minute. Since fibrin fibrils may reach 400μ or more in length and more than four-fifths of the primitive fibrils may be already formed within a minute of the addition of thrombin (p. 201), it follows that the average size of the particles of origin of the fibrils must be much less than that of the smallest visible particles observable in these experiments. Several observers have attempted to measure the mean amplitude of oscillation and degree of translation of particles of known diameter [*cp.* R. Zsigmondy, 1905, pp. 104-111],

but sufficient data do not appear to be yet available to enable the limits of size of such colloidal particles of fibrinogen as possess enough translatory motion to be capable of giving rise to primitive fibrils to be determined.

The maximum or saturation concentration of the continuous phase of fibrinogen in plasma obtained from the same source must be assumed to be constant under the same conditions of experiment (concentration of thrombin and temperature) since the time of first appearance of visible fibrils remains unchanged¹. It is therefore possible, when the transition point of the reaction rate curve has been determined (*cp.* Table IV, Fig. 4 *b*), to express the concentration of the disperse phase in terms of the saturation concentration of the continuous phase. If this is done in respect of the same sample of plasma at different periods after collection, it is found that the disperse phase of fibrinogen gradually diminishes in amount on keeping. This disappearance of the disperse phase is exhibited in the following series of experiments each series being carried out as indicated in Table III:

Series	Age of plasma	Concentration of disperse phase of fibrinogen		
		observed	calculated	in percentage form
—	0 days	—	219	100
A	6 "	163	162	74
B	12 "	120	120	55
C	18 "	92	89	41
D	25 "	64	63	29
E	39 "	33	31	14

In the second column the period after bleeding, at which the experiments were made, is shown; in the third column the concentration of the disperse phase is given in terms of the continuous phase, represented by 100²; in the fourth column these concentrations (and also the initial concentration) are calculated on the assumption that the change of the disperse phase proceeds at a logarithmic rate, the constants of the reaction being estimated at $k = 0.05$ (time being measured in days) and $a = -5.38$; in the fifth column the concentrations given in the fourth are presented in percentage form. The close correspondence between observed and calculated values, exhibited graphically in Fig. 7, establishes the truth of the assumption that the disappearance of the disperse phase proceeds at a monomolecular reaction rate. This occurs equally when the colloidal particles of this phase are suspended in undiluted or in diluted plasma, thus being independent, within the limits of experiment, of the dilution. The continuous phase of fibrinogen does not appear to be affected by the addition of thrombin: if any disappearance of this phase occurred it

¹ In Series B, C, D, the same stock solution of thrombin (kept at 0°) was employed: the time of first appearance of visible fibrils in undiluted plasma or in plasma dilutions lying above the transition point of the corresponding curve was 8.5 minutes. In Series A and E in which freshly prepared thrombin solutions were used this period was slightly reduced.

² In the series of experiments given in Table III, for example, the concentration of the disperse phase would be represented by $\frac{39 \times 100}{61} = 63.9$.

would presumably proceed at a constant rate in all concentrations of plasma above the transition point, in other words so long as the maximum concentration of the continuous phase is maintained, but of this there is no evidence in experiments of the type of those given in Table III.

Fig. 7. Disappearance of the disperse phase of fibrinogen, in citrated plasma, on keeping. Ordinates represent concentration of disperse phase in terms of maximum concentration of continuous phase, represented by 100; abscissae represent time in days. Details of experiments are given in text. The curve is constructed from values of $k=0.05$ and $a=-5.38$.

The disappearance of the disperse phase on keeping is in part, if not wholly, due to fibril formation, for when this disappearance is considerable, flocculent masses, which are found on microscopical examination to consist of radiating collections of coarse fibrils mixed with aggregations of granules,

are observed in the plasma, which has become very turbid though still liquid. Whether in addition any of the particles of the disperse phase, in consequence, it may be, of their relatively large size or of an accidental failure to collide with a surface before conversion into fibrin was complete, become directly converted into fibrin is uncertain, the presence of numerous dancing particles, as already pointed out, making it difficult to determine this point.

The particles of the disperse phase, so long as the change of fibrinogen into fibrin is incomplete, consist of a mixture of fibrin and fibrinogen. If neither of these substances is soluble in or miscible with the other no explanation is forthcoming of the failure of the disperse particles to disappear completely at the transition point of the curve, nor is the growth of primitive fibrils accounted for. If, however, it is assumed that fibrin, or any product intermediate between fibrin and fibrinogen, is soluble in fibrinogen in the disperse phase, then the concentration of fibrinogen in disperse particles in which partial change into fibrin had occurred would be lowered and would no longer be in equilibrium with the continuous phase when the latter was saturated. In consequence of this disturbance of equilibrium fibrinogen would pass from the continuous phase into the particles of the disperse phase (or into the primitive fibrils, if the particles had already given rise to fibrils). In this way a new equilibrium between the disperse phase (or primitive fibrils) and the continuous phase would be reached with increasing dilution of the latter. As more of the disperse phase (or primitive fibrils) became converted, under the further action of thrombin, into fibrin this new equilibrium would, in its turn, be disturbed and a further transfer of fibrinogen from the continuous phase into the disperse particles (or fibrils) would occur, these processes continuing until finally all the fibrinogen had been converted into fibrin.

The above appears to be the simplest hypothesis which will account both for the absence of complete disappearance of disperse particles of fibrinogen at the transition point of the reaction rate curve (Fig. 4) and also for the growth of primitive fibrils: it may therefore on this account be, provisionally at any rate, adopted. Two corollaries, it may be pointed out, follow from this hypothesis. The first is that the concentration of the continuous phase below the transition point is not strictly proportional to the further dilution: since, however, the amount of fibrinogen present in the disperse phase, when determined by experiments of the type of those shown in Table III, appears to be very small, it may be surmised that the difference in question is also inconsiderable. The second corollary is that if any of the disperse particles are too large to possess the degree of translatory movement necessary for their conversion into primitive fibrils, undergoing in consequence direct conversion into fibrin, such particles must also subsequently increase in size at the expense of fibrinogen present in the continuous phase, in the same manner as primitive fibrils.

When relatively concentrated venom of *Echis carinatus* is employed as source of thrombin a further action occurs: the fibrils which make their

appearance subsequently disappear, undergoing lysis¹. Thus a 1 in 3000 solution of venom, added to an equal volume of undiluted plasma, leads to abundant fibril formation in about three minutes. At the end of two hours at room temperature (16°) most of the fibrils have disappeared, those remaining being thinned and for the most part broken, forming débris which falls to the bottom of the layer of fluid examined and is later completely dissolved. The occurrence of this lysis in particles of precipitated fibrinogen, which have undergone direct conversion into fibrin, has been already described (p. 204). On the hypothesis that lysis of fibrin is not due to an adventitious proteolytic ferment, but is a true thrombin effect, the change of fibrinogen in the concentrated phase into fibrin, under the action of thrombin, would appear to be merely a preliminary stage of the lytic action of thrombin.

BEARING ON GEL FORMATION IN GENERAL.

The behaviour of fibrinogen under the action of thrombin suggests that in any colloidal solution the disperse phase, if it consists of liquid particles, may, when passing into the solid state under the influence of a fall of temperature or of ferment action, give rise to fibrils, if the particles are sufficiently small, or form a suspension of solid particles, if of relatively large size. In this way may be explained the formation of the characteristic structure, consisting of a mixture of network and granules, sometimes the one sometimes the other predominating or being present exclusively, met with in cell plasm, subjected to the action of fixatives [see Wilson, 1904], and also in gels after similar fixation [Hardy, 1899].

SUMMARY.

1. Fibrinogen in solution is diphasic, consisting of a concentrated phase, which is dispersed, and a dilute phase, which is continuous.
2. Under the action of thrombin fibrin is formed out of fibrinogen in the concentrated phase: if this phase is in the form of a precipitate its particles become directly converted into fibrin; if this phase is dispersed its suspended particles give rise to fibrin fibrils, forming a coagulum.
3. The action of thrombin upon fibrinogen in the concentrated phase is purely catalytic. No effect appears to be exerted by thrombin upon fibrinogen in the continuous phase.

¹ Cp. Houssay and Negrete [1918]. These authors observe that solid proteins with the exception of fibrin are not dissolved: the action of venom thus appears to be specifically directed to fibrin.

4. The following hypothesis of the mode of formation of fibrin fibrils is advanced:

Fibrin fibrils are particulate in origin.

The particles of origin, consisting of fibrinogen in relatively high concentration, contained in a solution of fibrinogen of low concentration, and possessing oscillatory and translatory movement, (1) become, under the action of thrombin, semi-solid, viscid and sticky, and (2) form, as the result of collisions, adhesions to solid surfaces, with the result that they (3) become drawn out into primitive fibrils.

Increase in thickness of the primitive fibrils takes place by addition of fibrinogen, derived from the solution of fibrinogen of low concentration.

5. When fibrinogen in the concentrated phase has become partially changed into fibrin, the concentration of fibrinogen in this phase is lowered, disturbance of equilibrium of the two phases resulting, and fibrinogen passing from the dilute into the concentrated phase. In this way may be explained the "growth" of fibrin fibrils. This growth is observed to occur at a monomolecular rate.

6. If, as source of thrombin, venom in sufficient concentration is used, the fibrin formed, whether consisting of fibrils or of particles of fibrinogen in the concentrated phase which have been directly transformed into fibrin, later undergoes lysis.

7. The action of thrombin upon fibrinogen affords an explanation of the mechanism of production of the reticular structure observed in gels and cell plasm.

REFERENCES.

- Barratt (1915). *Proc. Roy. Soc. B.* **87**, 179.
 Cesana (1908). *Arch. Fisiol.* **5**, 180.
 Hardy (1899). *J. Physiol.* **24**, 158.
 Houssay and Negrete (1918). *Revista del Instituto Bacteriologico del Departamento Nacional de Higiene*, Buenos Aires, **1**, 341.
 Howell (1910). *Amer. J. Physiol.* **26**, 253.
 — (1914). *Amer. J. Physiol.* **35**, 143.
 — (1916). *Amer. J. Physiol.* **40**, 526.
 Mayer (1907). *Compt. rend. Soc. Biol.* **63**, 658.
 Stübel (1914). *Pflüger's Arch.* **156**, 361.
 Wilson (1904). *The Cell* (London, Macmillan).
 Zsigmondy (1905). *Zur Erkenntnis der Kolloide* (Jena).