THE RATE OF FORMATION OF FIBRIN FERMENT FROM PROTHROMBIN BY THE ACTION OF THROMBOKINASE AND CALCIUM CHLORIDE.

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In 1912 the rate of formation of trypsin from trypsinogen by the action of enterokinase was determined by J. Mellanby and V. J. Woolley(1). This reaction differs from that observed in such ferment actions as the inversion of cane sugar by invertase in so far that the velocity of the change is small at the outset of the reaction but proceeds with a constantly increasing acceleration. This fact has been confirmed by Vernon(2), but the hypothesis which we put forward to account for the mechanism of the change has been a matter of dispute. It became of interest therefore to determine the rate of formation of fibrin ferment from prothrombin by the action of thrombokinase and calcium chloride. The information thereby obtained shows that the processes involved in that reaction are similar to those observed in the generation of trypsin from trypsinogen and affords evidence in favour of the hypothesis which we put forward to elucidate the mechanism of the latter reaction. Incidentally the hypotheses put forward by Howell(3) on the relation of thrombokinase to antifibrin ferment and by Barratt(4) on the time factors involved in the coagulation of blood are considered in the light of the experimental facts detailed.

The preparation of fibrinogen, prothrombin and fibrin ferment. Prothrombin can be obtained from bird's blood in a state of comparative purity and in such condition, that, after the addition to it of thrombokinase and calcium chloride, a fibrin ferment solution of extraordinary potency may be obtained. Bird's blood was used in the experiments since, as was shown by Delezenne(5), such blood if carefully collected shows no tendency to coagulate, and thus constitutes an ideal medium whereby the mechanism of blood coagulation may be studied. No foreign bodies, such as potassium oxalate, sodium chloride, peptone, etc. have been added to it and the stability of the original blood affords an

adequate control for any experiments which may be made on the fibrinogen prepared from it.

Fibrinogen was prepared from fowl's plasma by dilution with ten volumes of distilled water and neutralisation of the alkalinity of the plasma by the addition to the diluted liquid of a few drops of 1 % acetic acid. The precipitated fibrinogen was obtained as a compact mass by prolonged centrifugalisation (after preliminary settling). The fibrinogen was suspended in water or dissolved in ·5 % NaCl.

Fibrin Ferment. The precipitate obtained from plasma by dilution and neutralisation does not consist of fibrinogen only, but of a complex of prothrombin and fibrinogen. On this account the precipitate when dissolved in ·5 % NaCl can be coagulated by adding to it a trace of thrombokinase, and calcium chloride to the extent of ·05 %. The mechanism involved in the change is obvious—the thrombokinase acting in conjunction with the calcium salt generates fibrin ferment from the prothrombin attached to the fibrinogen, and the fibrin ferment changes the fibrinogen, to which it is attached, into fibrin. After removal of the fibrin the clear residual liquid contains a large quantity of fibrin ferment, the coagulating activity of the expressed liquid being proportional to the strength of the original fibrinogen-prothrombin suspension.

Prothrombin. The addition of a minimal quantity of fibrin ferment to a solution of fibrinogen-prothrombin in ·5 % NaCl results in the change of the fibrinogen into fibrin. The residual fluid, after removal of the clot, possesses no active coagulating capacity but contains a quantity of prothrombin proportional to the strength of the original suspension. The absence of fibrin ferment from the expressed fluid depends upon the fact that fibrin absorbs considerable quantities of fibrin ferment (this is the basis of Gamgee's method for the preparation of fibrin ferment) and consequently the ferment added to the original fibrinogen-prothrombin solution is removed by the clot. Although the expressed fluid possesses no power of coagulating a solution of fibrinogen yet after the addition to it of a small quantity of thrombokinase and calcium chloride coagulating activity equal to that of a fibrin ferment solution develops within it after a short period of time.

Thrombokinase. The thrombokinase used in the experiments was obtained by macerating a cockerel's testis in a litre of distilled water. The clear supernatant fluid obtained after the removal of the insoluble débris contained considerable quantities of thrombokinase.

The estimation of fibrin ferment. A series of experiments were made to determine the relation between the time of coagulation and the

quantity of fibrin ferment added to a fibrinogen solution. From the figures obtained in this way the amount of ferment contained in any solution was calculated. The fibrin ferment was obtained by activating a solution of prothrombin with kinase and calcium chloride, the amount of kinase and calcium added being so small that activation of the prothrombin took twenty-four hours for completion. Small quantities of this solution possessed considerable coagulating activity, but contained negligible amounts of kinase and calcium salt. The figures given below express therefore the relation between the time of coagulation of fibrinogen by fibrin ferment and the quantity of ferment used.

·5 c.c. of a solution of fibrin ferment in ·5 % NaCl was diluted with 2 c.c. of ·5 % NaCl. Varying quantities of this solution were added to ·5 c.c. of a solution of fibrinogen in ·5 % NaCl, and the total volume was made up to 1 c.c. in every case by the addition of the necessary quantity of ·5 % NaCl. The only variable in each experiment was the amount of fibrin ferment contained in the mixture. The following results were obtained:

Fibrinogen in ·5% NaCl	NaCl ·5%	Fibrin ferment in ·5% NaCl	Coagulation time
·5 c.c.	·1 c.c.	·4 c.c.	30 secs. (*a*)
—	·2	·3	45 ,,
—	·3	·2	1 min.
—	·4	·1	2 ,,
—	·45	·05	6 ,,
—	·475	·025	20 ,,

It may be observed that with large quantities of fibrin ferment the time of coagulation is inversely proportional to the quantity of ferment added. When however the amount of ferment added falls below a minimal value the times of coagulation are indefinitely prolonged. The relation between the amount of fibrin ferment and the times of coagulation is precisely similar to that observed in the coagulation of caseinogen by pepsin or trypsin and expresses the fact that the fibrinogen complex is so large that the ferment must be adsorbed to it before coming within the effective range of the ferment's sphere of energy. The activity of the ferment solution used is worthy of note. From experiment (*a*) it may be seen that the quantity of fibrin ferment contained in the solution before dilution was such that ·08 c.c. coagulated 1 c.c. of fibrinogen in half a minute. It is impossible to prepare solutions of fibrin ferment from fibrin (Gamgee's method) or from serum (Schmidt's method) which approximate in any degree to the activity of a ferment solution obtained by the activation of a solution of prothrombin in ·5 % NaCl.

The activation of prothrombin by thrombokinase and calcium chloride.

A solution of prothrombin in ·5 % NaCl was made in the following way. 4·66 c.c. of fibrinogen suspension was dissolved by the addition of ·33 c.c. NaCl 7·5 %. This solution was coagulated by the addition to it of ·5 c.c. of fibrin ferment (coag. time 30 secs.). The fluid was expressed from the clot after complete coagulation. It contained no coagulating activity as may be observed from the following experiment:

Fibrinogen in ·5% NaCl	NaCl ·5%	Expressed fluid	Coag. time
·5 c.c.	·4	·1	No coag. in 30 mins.

The absence of coagulating power in the expressed fluid was due to the removal of the fibrin ferment added to the original fluid by the fibrin when formed.

Although the expressed fluid possessed no capacity to coagulate a solution of fibrinogen yet this fluid contained large quantities of prothrombin, a fact which was evidenced by the production of fibrin ferment in it after activation by kinase and calcium chloride.

(1) *Variable kinase.* These experimental results show the rate at which prothrombin is transformed into fibrin ferment under the influence of varying quantities of kinase and a constant quantity of calcium chloride.

A solution containing prothrombin, kinase and calcium chloride was made up thus:

Pro.	K.	$CaCl_2$(·1N)	H_2O	
·5 c.c.	·05 c.c.	·1 c.c.	·35	Solution = A

After varying intervals of time ·1 c.c. of this mixture (A) was added to 1 c.c. of a solution of fibrinogen and the time of coagulation noted. The following results were obtained:

Interval ...	2′	4′	6′	8′	10′	12′
Coag. time	30′	20′	5′	3′	1¼′	⅝′
Units of fibrin ferment	1½	2	4½	6	14	24
Increase in fibrin ferment at 2 mins. intervals		½	2½	1½	8	10

The units of fibrin ferment were calculated from the first experiment giving the time of coagulation of a fibrinogen solution by varying quantities of fibrin ferment. One unit of fibrin ferment is assumed to be that quantity of ferment which coagulates 1 c.c. of fibrinogen solution in forty minutes.

Two facts are evident from these figures (*a*) the addition of thrombokinase and calcium chloride to a prothrombin solution results in the production of a large coagulating activity, and (*b*) the rate of formation of fibrin ferment continually increases as the reaction proceeds.

To determine how far the velocity of the reaction depended upon the

relative quantities of thrombokinase and calcium chloride in the solution various other experiments were done.

In the following experiment the amount of kinase added to the prothrombin solution was increased fourfold.

Pro.	K.	$CaCl_2$ (·1N)	H_2O
·5 c.c.	·2 c.c.	·1 c.c.	·2 c.c. = B

The activity of B after varying intervals of time was determined from the capacity of ·1 c.c. of it to coagulate 1 c.c. of a solution of fibrinogen. The figures obtained are given in the following table:

Interval	0′	2′	4′
Coag. time	25′	2′	½′
Units of fibrin ferment	1¼	8	32
Increase in fibrin ferment at 2 mins. intervals		6	24

The results show the same general facts as were observed in the first experiment. The rapid production of fibrin ferment from the prothrombin is worthy of note—within four minutes the addition of kinase and calcium chloride to a prothrombin solution increased the capacity of that solution to coagulate fibrinogen twenty-fivefold.

(2) *Variable calcium chloride.* In these experiments the calcium chloride contained in the prothrombin solution was altered, the kinase being kept constant.

The following solution of prothrombin, kinase and calcium chloride was made:

Pro.	K.	H_2O	$CaCl_2$ (·1N)
·5 c.c.	·05 c.c.	·35 c.c.	·1 c.c. = X

The following figures express the capacity of ·1 c.c. of this fluid to coagulate 1 c.c. of fibrinogen solution after varying intervals of time.

Interval	2′	4′	6′	8′	10′	12′
Time taken by ·1 c.c. of X to coag. 1 c.c. of fibrinogen	30′	20′	5′	3′	1½′	⅝′
Units of fibrin ferment	1½	2	4⅓	6	12	24
Increase in fibrin ferment at 2 mins. intervals		½	2½	1⅔	6	12

In the following experiment double the amount of calcium chloride was added to the prothrombin solution.

Pro.	K.	H_2O	$CaCl_2$ (·1N)
·5 c.c.	·05 c.c.	·25 c.c.	·2 c.c. = Y

The following figures express the rate of activation:

Interval	2′	4′	6′	8′
Time taken by ·1 c.c. Y to coag. 1 c.c. of fibrinogen	20′	6′	1⅓′	⅝′
Units of fibrin ferment	2	4	13	24
Increase in fibrin ferment at 2 mins. intervals		2	9	11

The effect of increasing the calcium chloride content of the prothrombin solution is precisely the same as that produced by increasing the kinase content.

Discussion of Results.

The experimental results detailed in the previous pages show that in the formation of fibrin ferment from prothrombin by the action of thrombokinase and calcium chloride, the reaction proceeds with a constantly increasing velocity, and is, in fact, comparable in all respects to the rate of formation of trypsin from trypsinogen by enterokinase. To elucidate the mechanism of the formation of trypsin from trypsinogen by enterokinase, V. J. Woolley and the present writer put forward the following hypothesis.

Trypsinogen consists of a complex of protein and trypsin, the trypsin being adsorbed to the protein but unable to digest it. Activation by enterokinase involves two distinct processes, (*a*) the adsorption of the enterokinase by the trypsinogen, (*b*) the digestion of the protein moiety of the trypsinogen complex by the enterokinase with the consequent liberation of trypsin. As a corollary it may be stated that the experimental results demand that more than one enterokinase element must be adsorbed to any trypsinogen complex before the liberation of trypsin results. The results obtained from an analysis of the rate of formation of fibrin ferment from prothrombin by the action of thrombokinase and calcium chloride show that in this reaction a type of mechanism similar to that observed in the activation of trypsinogen by enterokinase is involved. In accordance with this observation the following hypothesis is put forward. The ultimate agent in the activation of prothrombin is the calcium ion, the potency of the calcium ion being proportional to its concentration up to about ·1 %. Pure prothrombin is not activated by calcium in any concentration, but prothrombin to which kinase has been adsorbed becomes unstable in the presence of calcium salts in proportion to the number of kinase elements contained in the complex. A slow production of fibrin ferment may be due to the presence of a minimal quantity of kinase or calcium salt; a rapid activation time to the presence of an optimal concentration of both these agents. The slow velocity of activation at the beginning of the reaction may be illustrated by the following suppositious case:

Assume that a solution contains one hundred elements of prothrombin, ten elements of kinase, and a definite concentration of calcium ions; also that before activation by the calcium ion can take place two

thrombokinase elements must be adsorbed to a prothrombin complex. It is evident that at the outset of the reaction the chance of any one prothrombin element being in active contact with two kinase elements is small, and therefore activation by the calcium ion proceeds at a slow rate. But as the change proceeds the relative proportion of thrombokinase to prothrombin becomes larger, the chance of the necessary complex of thrombokinase and prothrombin being produced continually increases, and consequently the rate of activation proceeds with an accelerated velocity.

The hypothesis put forward by Howell(3) to account for the facts observed in the coagulation of blood is similar to the above in so far as the ultimate activation of prothrombin is assigned to the calcium ion. His hypothesis differs in other fundamental details. Briefly stated, he assumes that prothrombin is transformed into fibrin ferment by the action of calcium only. In circulating blood prothrombin is combined with antithrombin and is protected thereby from the action of ionised calcium. When blood is shed thrombokinase exerts its influence by combining with antithrombin thus liberating prothrombin from its combination and allowing the calcium ions to act upon it.

This hypothesis has been considered in detail by Dale and Walpole(6), who find that their experimental results offer no support in favour of it. The facts stated in the previous pages as to the reciprocal relations between thrombokinase and calcium on the rate of activation of prothrombin contained in a solution free from antithrombin also negatives the hypothesis of Howell.

More recently Barratt concluded from his experimental observations that when a mixture of fibrinogen, prothrombin and calcium salt coagulates under the influence of kinase, the coagulation time is wholly occupied by the action of thrombin on fibrinogen. The experimental results detailed above afford no support in favour of this contention.

Summary.

1. The formation of fibrin ferment from prothrombin by the action of thrombokinase and calcium chloride proceeds in a manner similar to that observed in the activation of trypsinogen by enterokinase—the velocity of the change is small at the outset of the reaction but proceeds with a constantly increasing acceleration.

2. Thrombokinase and the calcium ion appear to hold a reciprocal relation to one another. Neither of these substances alone can activate

prothrombin, yet an increase in the rate of formation of fibrin ferment may be produced by an increase in the quantity of either of these substances in the activating mixture.

3. The following hypothesis has been put forward to account for the facts observed. The ultimate agent in the activation of prothrombin is the calcium ion. Pure prothrombin is not activated by calcium in any concentration, but prothrombin to which thrombokinase has been adsorbed becomes unstable in the presence of calcium salts in proportion to the number of kinase elements contained in the complex.

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REFERENCES.

(1) Mellanby and Woolley. This Journ. **45**. p. 370. 1912.
(2) Vernon. Ibid. **47**. p. 325. 1913.
(3) Howell. Amer. Journ. Physiol. **35**. p. 474. 1914.
(4) Barratt. Biochem. Journ. **9**. p. 511. 1915.
(5) Delezenne. Arch. de Physiol. p. 333. 1897.
(6) Dale and Walpole. Biochem. Journ. **10**. p. 331. 1916.