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MUSCULAR CONTRACTION, BLOOD COAGULATION.

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Abbreviations used in this volume.

ATP = Adenylpyrophosphate or adenosinetriphosphate.
ADP = Adenylphosphate or adenosinediphosphate.
DRF = Double refraction of flow.

In this volume two expressions are used for the characterisation of actomyosin:

1. *% Actomyosin.* By % actomyosin is meant the quantity of actin in 100 parts of actomyosin. So for instance, 16,7% actomyosin contains 16,7 g actin in every 100 g of actomyosin.

2. *% Activity.* By % activity of actomyosin is meant the fall of viscosity of actomyosin, (dissolved in neutral 0,6 M KCl) on addition of ATP — relative to the fall of viscosity of a 16,7% actomyosin. If we say, for instance, that an actomyosin is 100% active, this means, it shows the same fall of viscosity as a 16,7% actomyosin. (16,7% is the activity of our myosin B, see vol. I. of these studies.)

The actin content of an actomyosin can be calculated from its % activity by means of the curves given by F. B. STRAUB in this volume.

The autocatalytic formation of thrombin and the clotting defect of hemophilic blood.*

by

K. LAKI

The coagulation of blood is the conversion of a soluble protein, the fibrinogen, into the insoluble fibrin. This conversion of fibrinogen is brought about by the action of thrombin, which is formed from prothrombin during the process of blood clotting. The formatin of thrombin increases during the process of clotting and follows the type of an autocatalytic reaction.

This autocatalytic type of the formation of thrombin has aroused considerable interest among students of blood coagulation and many theories have been put forward to

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explain it. As knowledge about blood coagulation has increased and more and more components taking part in it have been isolated and investigated, it has become evident that the classical theory of blood coagulation is inadequate as an explanation of this autocatalytic type of the formation of thrombin. The processes known at present to be taking part in coagulation are not autocatalytic. Purified thrombin is not able to catalyse the thrombin formation from purified prothrombin. The conversion of fibrinogen into fibrin[1] is not autocatalytic nor is the formation of thrombin from prothrombin by the action of tissue kinase.[2]

The possibility that some hitherto unknown substance or mechanism is responsible for the autocatalytic formation of thrombin was indicated by the experiments of FISCHER[3] and especially of ASTRUP.[2] ASTRUP demonstrated that in the so called Mellanby-fibrinogen solutions the autocatalytic formation of thrombin was broken down and that even a slight acidification of the normal plasma was enough to destroy the autocatalytic formation of thrombin.

In a series of investigations performed in recent years on the hemophilic patients of the Clinic of Internal Medicin of Szeged, in collaboration with L. SZÉCSÉNYI NAGY, we compared the blood coagulation of normal persons with that of hemophilic patients.* Following quantitatively the formation of thrombin during the whole process of coagulation we came to the conclusion that hemophilic blood lacked the autocatalytic formation of thrombin and that the solution of the problem involved the necessity of elucidating the mechanism responsible for the autocatalytic formation of thrombin.

According to ASTRUP's and our findings Mellanby-fibrinogen and hemophilic blood resemble each other, both lacking the autocatalytic formation of thrombin.

It was found in the course of the investigation on the clotting of hemophilic blood that small amounts of fibrinogen prepared according to the method of the author in vitro restored not only the time of coagulation to normal but also

* A brief account of this work was read before the meeting of the Hungarian Physiological Society in 1942, and the detailed account will appear elsewhere.

the formation of thrombin. 0,01 cc. of fibrinogen solution containing 17 γ of protein was enough to restore the clotting of 1 cc. of hemophilic blood to normal.

Experiments soon revealed that some contaminating substance present in fibrinogen solutions is responsible for the effect. I call this substance *„plasmakinin."*

The resemblance between hemophilic blood and Mellanby-fibrinogen became closer when it was found that the same plasmakinin solution which restored the clotting of hemophilic blood to normal also accelerated the clotting of Mellanby-fibrinogen solution. The Mellanby-fibrinogen thus seemed to be a suitable material for the investigation of the mode of action of plasmakinin.

In the present communication the preparation of a crude solution containing plasmakinin will be described together with experiments showing the action of plasmakinin on Mellanby-fibrinogen. It will be seen that the addition of plasmakinin to Mellanby-fibrinogen leads to the formation of kinase, and that this formation of kinase increases in time. With the increase of kinase also increases the quantity of thrombin. Thus in view of these findings plasmakinin shows itself as an important factor in the formation of thrombin and the lack of it explains the hemophilic clotting defect.

Experimental.

Preparation of plasmakinin.

Oxalated cattle blood (containing 0,2% Na-oxalate) collected in the slaughter house was centrifuged and the clear plasma was sucked off. To the clear plasma saturated ammoniumsulfate was added in small portions until 0,25 saturation was reached. The precipitate which consisted mainly of fibrinogen was centrifuged. After discarding the supernatant plasma, the precipitate was brought into a solution of 0,7% NaCl (containing 0,2% Na-oxalate). Starting with 300 cc. of plasma, the precipitate was brought into 80 cc. of the NaCl solution. The precipitate dissolved slowly and gave a turbid solution. To remove the ammoniumsulfate this solution was dialysed in a cellophane tube against ten volumes of NaCl

solution for about 6 hours in the cold. The solution was then thoroughly centrifuged and used as PK_1 (first plasmakinin solution) in the experiments. 0,01 cc. of this solution given to 1 cc. of hemophilic blood is usually enough to restore the clotting time to normal.

Further purification was achieved by coagulating the fibrinogen. For this purpose 4 cc. of purified thrombin* solution were added to 80 cc. of PK_1 solution. Within an hour a firm clot was formed. The fibrin clot, after standing for a few hours in the ice chest was broken into pieces by means of a spatel then put on a cloth and the liquid, enclosed in the clot, pressed out. The fibrin-free liquid was left to stand in the ice chest; some further formation of a small amount of fibrin occured. Removing the clot by centrifugation a clear solution was obtained, which was almost free of fibrinogen and contained practically no thrombin. (The thrombin was probably adsorbed by the fibrin and was removed with it.) This solution (PK_2) often has the same activity as PK_1 though usually the activity is somewhat less. The preliminary experiments about the nature of this substance show that boiling to 100° destroys the activity of plasmakinin solutions. Warming to 50° for 5 minutes in neutral or slightly alkaline solution does not influence the activity but warming to this temperature in acid solution (N/40 acetic acid) or precipitation with alcohol or with acetone completely destroys the activity.

Preparation of Mellanby-fibrinogen (MF).

Cattle oxalate plasma obtained as above was gradually acidified by adding *N* acetic acid. During the course of acidification the pH was controlled colorimetrically. In order to test the pH 1 cc. of plasma was pipetted into test tube and 1 drop of bromocresolpurple** was added to it. Acetic acid was added till the colour of the dye became greenish-yellow. The acidified plasma was then poured into ten volumes of distilled water: a voluminous precipitate appeared which was allowed to settle

* 0,1 cc. of the thrombin solution gave a clotting time of 1' 30" when added to 1 cc. of oxalated plasma.

** 0,1 g. of bromocresolpurple dissolved in 18,5 cc. of *N*/100 NaOH was diluted with water to 250 cc.

overnight in the cold. The fluid was then sucked off and 15 cc. of Ca-free Ringer* solution were added to the precipitate for every 100 cc. of plasma which was used for the preparation. This turbid solution, (— made up to 40 cc. with water —) was allowed to stand at room temperature for about 12 hours and then centrifuged. The precipitate was discarded and the solution placed into the ice chest overnight during which time some formation of fibrin clot occured. This clot can easily be removed and after centrifugation a clear slightly yellow solution is obtained. This solution contains 3—6 mg. of fibrinogen and yields, on the addition of tissue kinase and calcium, as much thrombin per cc. as 1 cc. of the original plasma. 0,03 cc. of this solution added to 1 cc. of hemophilic blood reduces the clotting time only slightly, which shows that the active substance responsible for the normal clotting is greatly reduced in MF solutions.

The Mellanby-fibrinogen can be kept for weeks in frozen state and gives a clear solution when thawed. Storing in an ice chest is not very convenient owing to the formation of fibrin clot which must be removed before using the solution. In order to serve as a test solution for the action of kinase or plasmakinin the MF was diluted five times with *M*/20 borate buffer. This clear solution clots only after the addition of Ca and it takes usually 25 minutes before the first fibrin clot is detectable. If the clotting time is much shorter, then the original undiluted MF solution should be left overnight in an ice chest. After removing the fibrin clot which has been formed the solution can be treated as above and a suitable test solution will be obtained.

Kinase and plasmakinin have a similar effect on MF: both reduce the clotting time.

The action of tissue kinase on MF.

The clotting time of MF depends on the quantity of kinase used as demonstrated by fig. 1. The curve was obtained by plotting the clotting time against the quantities of kinase. The experiment was performed in the following way: Into small test tubes different quantities of very diluted brain

* The Ringer solution contained 0,23% $NaHCO_3$.

kinase* were pipetted. Then 1 cc. of recalcified MF solution was added after being diluted with borate buffer of pH 7,8. (The concentration of $CaCl_2$ was 10^- *M.*) One tube without kinase served as control. By shaking the tubes gently from time to time the appearance of the first fibrin clot can be observed sharply and the time of its formation measured. It is necessery to use test tubes made of the same glass because the glass wall

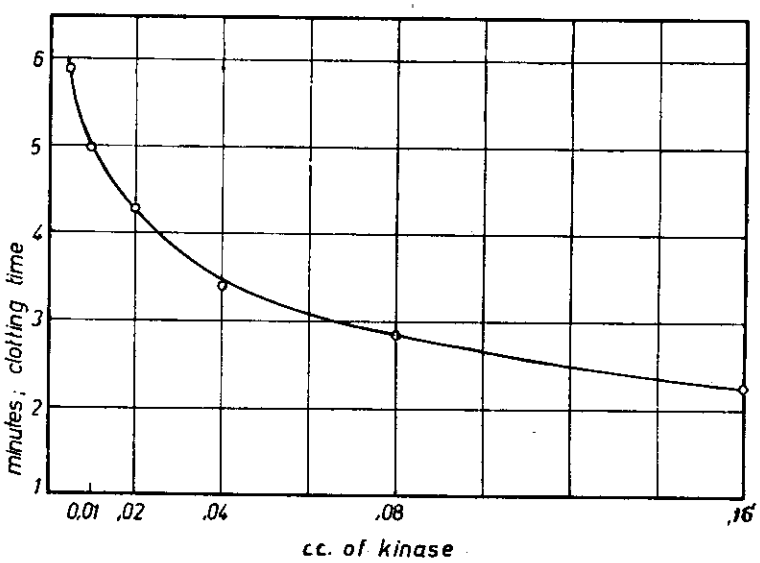

Fig. 1.

somewhat influences the clotting time of MF solutions. In all the experiments presented in this paper glass of Jena was used.

The concentration of Ca influences the clotting time to a great extent, the optimum is at 2×10^{-3} *M* $CaCl_2$. Fig. 2. gives the data of an experiment in which the concentration of Ca was varied and the kinase quantity kept constant. The experimental technic was the same as described above. The $CaCl_2$ solution and the kinase was pipetted into the tubes first, then 1 cc. of MF solution, which was diluted with borate buffer of pH 8,0 was added and the clotting time observed. In fig. 2. the clotting time is plotted against the $CaCl_2$ concentrations.

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* Rabbit's brain was extracted with an equal volume of 0,9% NaCl solution.

The action of plasmakinin on MF.

Like kinase, plasmakinin also catalyses the clotting of MF. Data of a similar experiment which was performed as above, using various quantities of PK_1 and PK_2 instead of kinase, are presented in table 1. It can be seen how the clotting time depends on the quantity of plasmakinin solutions. In the first column the dilution of plasmakinin is given and in the second the clotting time. PK_2 has somewhat longer clotting times indicating some loss in activity.

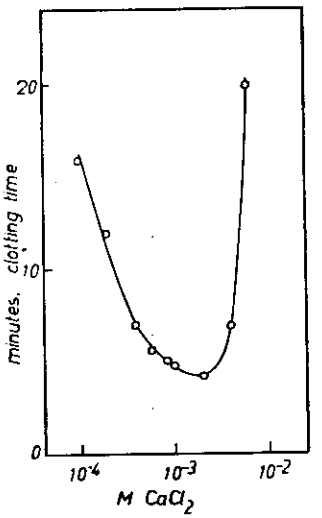

Fig. 2.

Table 1.

The clotting time of Mellanby-fibrinogen on the addition of various quantities of PK_1 and PK_2.

Quantity of PK_1 or PK_2 added to 1 cc. of MF.	Clotting time in the presence of PK_1 or PK_2	
0,05 cc. of . . . . . PK_1 or PK_2	10 min.	10 min.
„ 5 times diluted . . . . „	15 „	16 „
„ 25 „ . . . . „	25 „	29 „

Although the simplest explanation of the action of plasmakinin is that it acts as a kinase, it may be of interest to discuss briefly the possibility that other factor present in plasmakinin solutions might be responsible for the observed effect. The possibility that thrombin is responsible can easily be ruled out because PK_1 does not contain thrombin at all. The accelerating effect can hardly be attributed to prothrombin. MF contains much more prothrombin and the slight increase in prothrombin

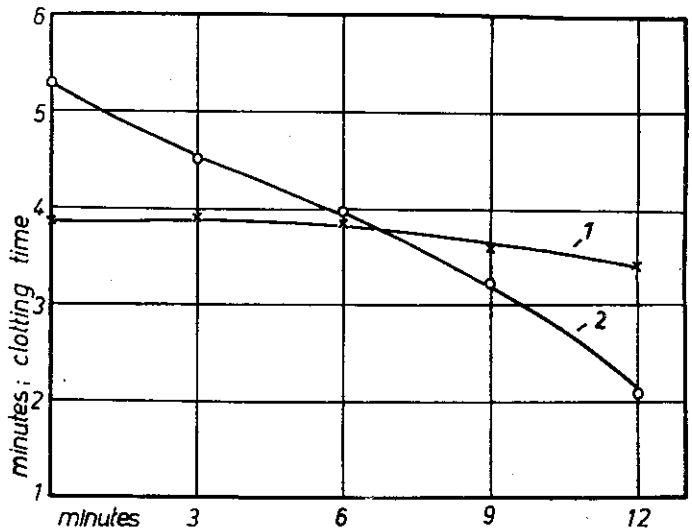

Fig. 3.

due to the addition of plasmakinin cannot be responsible for the effect. There was found no correlation between the fibrinogen quantities of plasmakinin solutions and their action on MF. The most plausible explanation remains that plasmakinin acts as kinase.

There is now increasing evidence that blood plasma contains some kind of soluble kinase. HOWELL,[4] using hemophilic plasma as a test, isolated a substance which, according to him, represents a kinase very similar in its action to the kinase in platelets or in tissues. According to FEISSLY[5] the kinase activity is connected with a viscous protein fraction of the serum. LENGGENHAGER[6] studying the clotting defect in hemophilic blood, postulated the existence of an inactive kinase present in normal plasma but lacking in hemophilic plasma. APITZ[7]

calls this plasma substance which the hemophilic plasma lacks, X-factor. WIDENBAUER and REICHEL[8] also came to the conclusion that blood plasma contained a kinaselike substance in an inactive prestadium, and whinh becomes active only when the clotting of blood starts.

It was found in our recent experiments that cattle plasma does not contain kinase whereas after clotting the serum does contain. Further experiments revealed that the kinase present in serum appears when the clotting starts and that this formation of kinase depends on the presence of Ca ions.

In view of these findings it seemed surprising that plasmakinin obtained by a simple ammoniumsulfate fractionation of plasma should act as kinase when tested with MF. The experiment in which the action of kinase and plasmakinin was compared showed that plasmakinin is not an active kinase but becomes kinase after it has been added to MF.

Comparison of the action of kinase with plasmakinin.

In the following experiment recalcified MF was allowed to stand at room temperature and at various times samples were taken out to test the activity of tissue kinase and of plasmakinin. The experimental procedure was the following: At various times samples of 1 cc. were taken out from the recalcinated MF and brought into small test tubes, one part of which contained kinase while the otthers plasmakinin solution. There were thus two series of test tubes. The tubes in one series contained 0,04 cc. of suitably diluted brain kinase and the tubes in the other 0,04 cc. of PK_1. Into the first tube of each series the MF solution was pipetted in the moment of recalcination. After 3, 6, 9, 12 minutes had passed MF was pipetted into the 2nd, 3rd, 4th and 5th tubes of each series. Thus there were tubes in which MF was added after it had stood for 0, 3, 6, 9, 12 minutes at room temperature. The appearance of the first fibrin clot in the tubes was observed and recorded as clotting time. The clotting time of MF without PK_1 or kinase was 25 minutes. The clotting times in the presence of PK_1 and kinase were much shorter as can be seen on fig. 3 where the clotting time is plotted against the time which elapsed after recalcination. Curve 1 represents the results obtained with kinase and curve 2 the results with PK_1.

In can be seen that it is almost immaterial for the action of tissue kinase how old the MF was. The clotting time in the first tube is nearly the same as in the last where MF stood for 12 minutes after recalcination. There was no substance formed to activate tissue kinase. In the case of PK_1 however the age of MF had marked effect. The older the MF solution is the more pronounced the effect. The first clotting time is much longer than the last. This shortening of the clotting time shows that in MF a substance was formed which acted on PK_1 in such

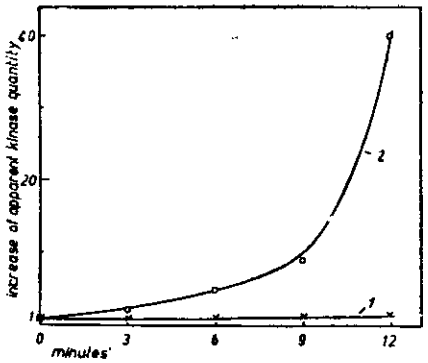

Fig. 4.

a way as if its kinase content had been increased. This apparent increase of kinase quantity is more pronounced when instead of the clotting time the apparent kinase quantity is represented. If the action of PK_1 on MF is equivalent to a certain quantity of kinase then every clotting time obtained with PK_1 corresponds to a certain quantity of kinase. This quantity of kinase can be read from the curve of fig. 1. Taking the kinase quantity corresponding to the first clotting time as a unit it can readily be calculated how many times the apparent kinase quintity has increased. If the increase of kinase quantity, calculated this way, is plotted against the time which passed after recalcination curve 2 of fig. 4 results. The data for curve 1 were calculated in a similar way: the first clotting time obtained with tissue kinase being taken as a unit, the increase in kinase quantity was calculated. As fig. 4 shows a certain quantity of kinase always represents the same quantity, independent of how old the recalcinated MF is. But the apparent kinase quantity of plasmakinin increases

according to the time which has passed after recalcination. In the light of this experiment plasmakinin seems to be an inactive substance which only gives rise to the formation of kinase upon the action of an other factor. This factor formed in MF is probably the thrombin itself, if not, it must be an as yet unknown substance, the formation of which strictly follows the formation of thrombin.

Summing up the results of the above experiment two facts emerge: a) During coagulation kinase is formed. b) For this formation of kinase the plasmakinin is responsible plus an other substance which is the thrombin or an unknown substance.

Discussion.

In view of the findings described in this paper the clotting of blood can be pictured in the following way: The reaction starts with the conversion of a small amount of prothrombin into thrombin by the action of platelets or tissue kinase. The thrombin converts fibrinogen into fibrin, parallel with this process kinase is formed from plasmakinin. This new kinase converts more prothrombin into thrombin, which is followed again by the formation of an other amount of kinase. Thus during the clotting process there is an increased formation of kinase and consequently an increased formation of thrombin too. This simultaneous formation of thrombin and kinase explains the autocatalytic type of the formation of thrombin.

The clotting system in hemophilic blood lacks this mechanism due to the lack of the plasmakinin. The addition of this substance restores the autocatalytic mechanism and with it the normal clotting.

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