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THE FORMATION OF THROMBOPLASTIN IN HUMAN BLOOD

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When normal blood is taken into a glass tube it clots in from 5 to 10 min. This clotting is presumably due to a thromboplastin contained in the blood, since significant admixture with tissue thromboplastin can be avoided. From the fact that the blood does not normally clot within the vessels it may be inferred that intrinsic blood thromboplastin is either very feeble or exists in an inactive form.

The existence of an inactive precursor of blood thromboplastin was postulated by Collingwood & MacMahon (1912), who called it 'prothrombokinase' and believed that it was derived from the platelets. Macfarlane (1942) suggested that a plasma factor and a lipoid reacted together on contact with a foreign surface to produce active thromboplastin. Quick (1947) and Brinkhous (1947) both carried out experiments suggesting that thromboplastin was produced by the interaction of the platelets with 'antihaemophilic globulin', the plasma factor that is lacking in haemophilia. Quick (1947) has called this plasma factor 'thromboplastinogen' believing it to be the precursor of thromboplastin, and that it is activated by platelets.

A number of attempts have been made to verify these suppositions by the actual isolation of thromboplastin from blood; the results have suggested that blood thromboplastin is of feeble activity. The platelets were found by Ware, Fahey & Seegers (1948) to contain 'only a small amount of thromboplastin, if any'. Antihaemophilic globulin alone has no thromboplastic action. By mixing platelets with antihaemophilic globulin Ferguson (1949) and Shinowara (1951) were able to produce thromboplastic activity, but it was considerably less than that of tissue extracts. This finding is apparently in keeping with the long normal clotting time of 5–10 min, as compared with the clotting time of 12–15 sec that occurs when an optimum amount of tissue thromboplastin is added to the blood. From the dilution curve of such tissue thromboplastin it

can be deduced that the untreated blood clotting time of 5–10 min is the result of a thromboplastic activity of not more than a 1/100,000 dilution of the optimum tissue extract. Most workers have therefore assumed that the activity of blood thromboplastin is weak. This assumption, which has many implications in the interpretation of clotting defects in disease, will be seen to be fallacious.

When blood is placed in a glass tube and the concentration of thrombin therein is determined at intervals it is seen that during the first few minutes no thrombin appears; then there is a sudden generation of thrombin which causes rapid clotting and a steeply rising thrombin concentration followed by a decline in concentration as inactivation by antithrombin overtakes the rate of thrombin formation (Biggs & Macfarlane, 1952 and unpublished data).

These observations do not support the usual concept of the presence of a feeble thromboplastin which causes slow thrombin formation over a period of several minutes; they suggest rather that thromboplastin does not appear until just before clotting. Since the rate of thrombin generation is as rapid in untreated blood as it is when the optimum amount of tissue extract is added, it seems likely that the intrinsic blood thromboplastin, once formed, is as potent as the most active tissue preparation available. The relatively long clotting time of normal blood is probably an index of the delay in thromboplastin generation, rather than of thromboplastin activity, and the short clotting time produced by added tissue extracts containing pre-formed thromboplastin is due to the avoidance of this delay.

These observations have stimulated an attempt to demonstrate this potent thromboplastic activity of the blood by a new technique. To obtain conclusive results the components essential for thromboplastin formation must be separated from blood, shown to be free from prothrombin, and, after their interaction, the activity of the product must be tested by its ability to clot normal plasma.

From previous work several probable components can be named. Platelets are likely to be essential because citrated plasma entirely free from platelets will not clot on recalcification and, in plasma poor in platelets, prothrombin is converted to thrombin very slowly. The so-called antihaemophilic globulin, associated with the fibrinogen fraction of normal plasma is probably also necessary because haemophilic blood, which lacks this factor, shows a great delay in prothrombin conversion. A third factor, called 'factor VII' (Koller, Loeliger & Duckert, 1951) may also be required. This substance which has been called 'convertin' (Owren, 1950), 'co-thromboplastin' (Mann & Hurn, 1951), 'serum prothrombin conversion accelerator' (de Vries, Alexander & Goldstein, 1949) is apparently needed for the reaction of brain extracts with prothrombin (Owren, 1950; Koller *et al.* 1951) and might reasonably be expected to be necessary for the action of the blood thromboplastin system.

Factor VII is a substance in the globulin fraction of normal serum; it can be adsorbed by $Al(OH)_3$; it is probably formed during clotting from a precursor in the plasma. It is the purpose of this communication to demonstrate that blood thromboplastin is formed from the three components, platelets, antihaemophilic globulin and a serum fraction, the activity of which is assumed to be due to its factor VII content.

METHODS

Collection of blood. 9 ml. human venous blood is mixed with 1 ml. of 3·8 % (w/v) sodium citrate solution. The mixture is centrifuged at 1,500 rev/min for 5 min to obtain 'platelet rich' plasma. To obtain 'platelet-poor' plasma the blood is centrifuged either at 3000 rev/min for 30 min or at 15,000 rev/min for 5 min.

Phosphate buffer pH 8 is prepared by dissolving 11·875 g $Na_2HPO_4.2H_2O$ in 1000 ml. of distilled water.

Platelet suspension. Platelet rich plasma is centrifuged at 15,000 rev/min for 5 min and the clear plasma decanted. This platelet-poor plasma, if prepared from normal blood, may be used as substrate for the demonstration of thromboplastin (see 'The generation of blood thromboplastin', p. 92). The deposit of platelets is washed twice with 0·85 % (w/v) NaCl and the final deposit resuspended in a volume of 0·85 % NaCl equal to one third of the volume of plasma from which the platelets were derived. If the blood and plasma used for preparation of platelets are collected into silicone-coated tubes the yield of platelets is higher and the platelets are more easily resuspended.

Antihaemophilic globulin. In tests it is necessary that the antihaemophilic globulin should be separated from prothrombin. A crude preparation made by adsorbing plasma with $Al(OH)_3$ may be used or, if desired, a more purified material can be made.

To prepare the crude $Al(OH)_3$-treated plasma, 0·1–0·4 ml. of aluminium hydroxide Cα prepared by the method of Bertho & Grassman (1938) is added to 2 ml. of citrated plasma. The mixture is incubated at 37° C for 5–15 min. After this time the $Al(OH)_3$, which has adsorbed factor VII and prothrombin, is removed by centrifuging. The one-stage prothrombin test, as described by Biggs (1951), is carried out on the supernatant plasma. The clotting time by this method should lie between 1 and 5 min. If too much $Al(OH)_3$ is used some of the antihaemophilic activity may be lost. This crude preparation of antihaemophilic globulin may be used at a dilution of one in five with 0·85 % NaCl.

A more purified material may be made by taking a measured volume of the plasma treated with $Al(OH)_3$ and adding half the volume of saturated $(NH_4)_2SO_4$ to make one-third saturation. The precipitate is collected by centrifuging and dissolved in a volume of 0·85 % NaCl equal to that of the original plasma. The solution is dialysed at 5° C for 12 hr against 0·85 % NaCl. The solution is then heated to 56° C for 5–25 min, depending on the size of the container, and the precipitate of denatured fibrinogen is removed. The supernatant liquid retains much of its ability to shorten the clotting time of haemophilic blood and can be dried by the lyophilic method. In tests the dried material is dissolved in distilled water at a concentration of 100 mg/4 ml.

Factor VII. Factor VII occurs in normal serum where it is naturally separated from prothrombin. The untreated serum at a dilution of one in ten with 0·85 % NaCl may be used as a source of factor VII or, if desired, a more purified material can be made.

To make the purified material normal serum is treated with $Al(OH)_3$ using 0·5 ml. $Al(OH)_3$ to 10 ml. of serum. The mixture is allowed to stand at 37° C for 15 min. The $Al(OH)_3$ is then separated by centrifuging and the supernatant is discarded. The $Al(OH)_3$ deposit is washed twice with cold distilled water and the factor VII is eluted from it by mixing with phosphate buffer at pH 8 and allowing the mixture to stand for 1 hr at 37° C. The volume of buffer used is one-quarter of the original serum volume. The $Al(OH)_3$ is then removed by centrifuging and discarded. The supernatant

containing factor VII is dialysed overnight against 0·85% NaCl and may then be dried lyophilically. For use the dried material is dissolved in distilled water at a concentration of 1 mg/ml.

The generation of blood thromboplastin. For the generation of thromboplastin there are four requirements; platelet suspension, antihaemophilic globulin, factor VII and M/40 $CaCl_2$. 0·3 ml. of platelet suspension, 0·3 ml. of antihaemophilic globulin, 0·3 ml. of factor VII are pipetted into a tube and placed in a water bath at 37° C, then 0·3 ml. of $CaCl_2$ are added and a stopwatch is started. Ready in the water bath are a number of small tubes each containing 0·1 ml. of platelet-poor citrated plasma, the substrate for this test which should be pipetted out immediately before use. At 1 min intervals after mixing the thromboplastin reagents 0·1 ml. of the mixture is withdrawn into a gradutaed pasteur pipette and 0·1 ml. of M/40 $CaCl_2$ is withdrawn into a second pipette held in the other hand. The contents of the two pipettes are discharged into one of the tubes containing 0·1 ml. of platelet-poor plasma and the contents mixed. The clotting time of the mixture is recorded.

RESULTS

The formation of blood thromboplastin

When platelets, antihaemophilic globulin, factor VII and calcium chloride are mixed no thrombin is formed because no prothrombin is present in the reagents. Yet after incubation the simultaneous addition of the mixture and $CaCl_2$ to plasma may induce clotting in as little as 8 sec with an average in ten experiments of 12·1 sec (Table 1). The short clotting time of the plasma must

TABLE 1. Experiment to demonstrate the formation of thromboplastin. After incubating the reagents for varying periods of time 0·1 ml. of the incubation mixture and 0·1 ml. of M/40 $CaCl_2$ are added to 0·1 ml. of citrated platelet-poor plasma. The clotting times are recorded in seconds. The figures represent the average of the results of 10 similar experiments

Incubation mixture	Incubation time in minutes							
0·3 ml. platelets 0·3 ml. antihaemophilic globulin 0·3 ml. factor VII 0·3 ml. M/40 $CaCl_2$	1	2	3	4	5	6	8	16
	Clotting time in seconds							
	41·3	32·7	19·5	15·7	13·5	13·0	12·1	14·5

be due to the addition of thromboplastin to the plasma because no thrombin (detected by its ability to clot fibrinogen free from prothrombin) is found in the incubation mixture. The delay in thromboplastin formation is demonstrated in Table 1 and varied in different experiments from 3 to 8 min. When incubation of the thromboplastin reagents is prolonged for an hour or more the ability to clot recalcified plasma is markedly decreased. This may be due either to neutralization of thromboplastic effect or to its intrinsic instability. This instability of the blood thromboplastin accounts for the fact that no powerful thromboplastin can be demonstrated in normal serum. Attempts to separate this thromboplastin in a stable form have been unsuccessful.

The reagents tested separately have no appreciable thromboplastic activity and incubation of any three of the four reagents failed to produce a thromboplastin giving a clotting time of less than 48 sec with normal plasma.

The measurement of blood thromboplastin

Although the blood thromboplastin is not stable at 37° C its potency is maintained for several hours at 0° C. Samples of thromboplastin made as described above were serially diluted and the activity of the dilutions determined. From a number of different thromboplastin samples the curve shown in Fig. 1 was obtained. This curve is similar in shape to the curves obtained

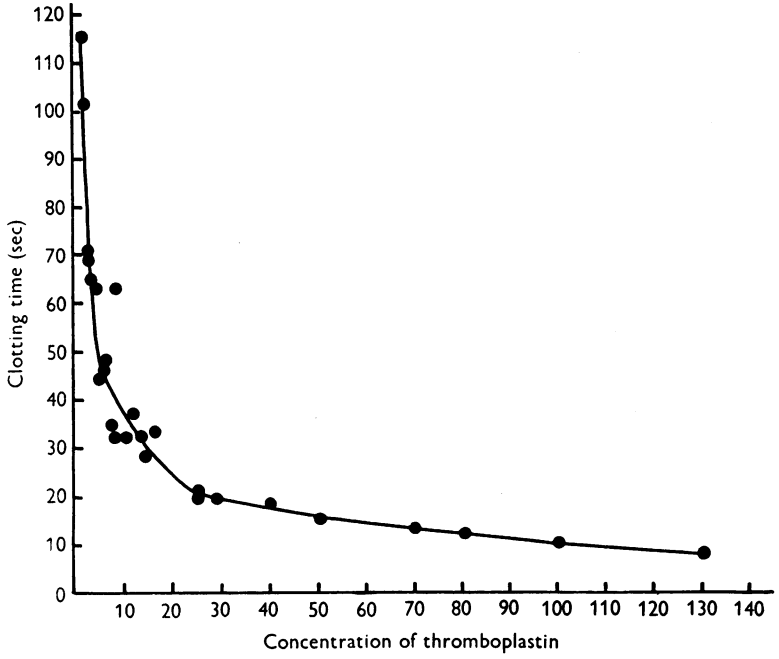

Fig. 1. Blood thromboplastin dilution curve. 0·1 ml. of different dilutions of blood thromboplastin were added to 0·1 ml. of platelet-poor citrated plasma and the mixture immediately recalcified with 0·1 ml. of M/40 $CaCl_2$. The clotting times in seconds are plotted against the relative concentration of thromboplastin.

using dilutions of brain thromboplastin though dilution of the blood thromboplastin has a bigger effect. Straight lines are produced if the values from the brain and blood thromboplastin activity/dilution curves are plotted on a double logarithmic scale.

Using the curve shown in Fig. 1 the observed clotting times can be expressed as relative thromboplastin concentrations where 100% thromboplastin represents a clotting time of 10 sec.

Blood thromboplastin and the concentration of platelets, antihaemophilic globulin and factor VII

Platelets. An attempt was made to count the number of platelets in platelet suspensions and the counts varied from 200,000 to 700,000/mm^3. The counts

were difficult to make because of the very large number of small fragments of refractile material present. These platelet preparations were diluted one in four so that in the mixtures forming thromboplastin it is probable that approximately physiological levels of platelets were used in the experiments.

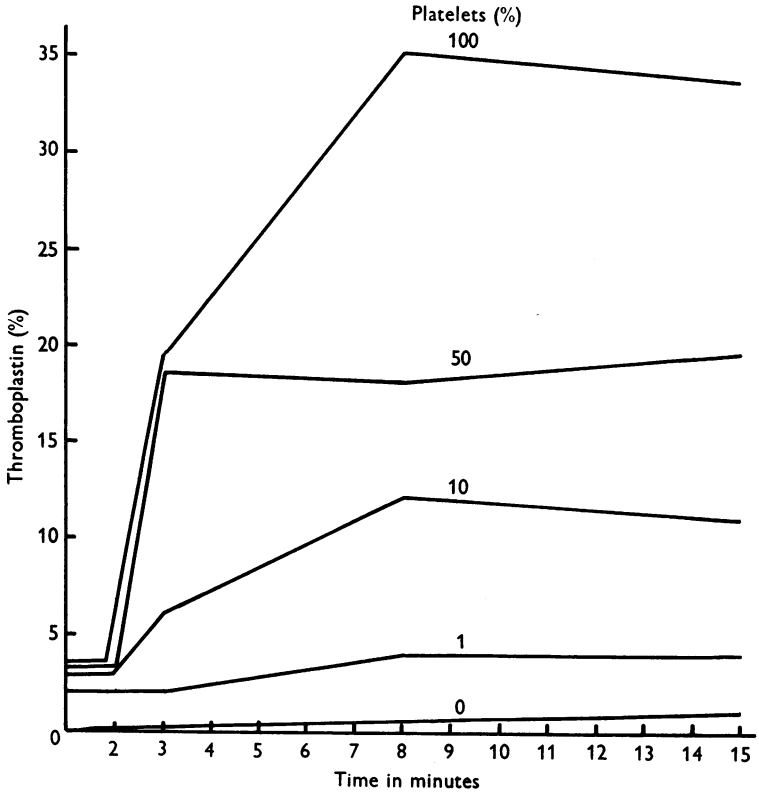

Fig. 2. Curves demonstrating the formation of blood thromboplastin in mixtures containing decreasing platelet concentrations. A suspension of platelets containing about 500,000 platelets/mm^3 was diluted 1/2, 1/10 and 1/100. The thromboplastin concentrations were deduced from Fig. 1 using the observed clotting times; in this and subsequent Figs. 100% thromboplastin represents a clotting time of 10 sec.

To determine the relation of platelet number to the formation of thromboplastin, different dilutions of platelet preparations were tested with the same preparations of antihaemophilic globulin and factor VII. In these experiments dilution of platelets considerably reduced the amount of thromboplastin formed but had little effect on the time at which thromboplastin formation began (Fig. 2). This result suggests that platelets are required quantitatively for the formation of blood thromboplastin and that reduction of platelets below the physiological level is likely to reduce the amount of thromboplastin formed.

Antihaemophilic globulin. Using $Al(OH)_3$-treated plasma from a haemophilic subject the amount of antihaemophilic globulin could be varied by adding known proportions of normal $Al(OH)_3$-treated plasma. The results of an experiment carried out in this way are shown in Fig. 3. The patient whose plasma was used for this experiment was a mildly affected haemophiliac whose whole blood clotting time and prothrombin consumption test were

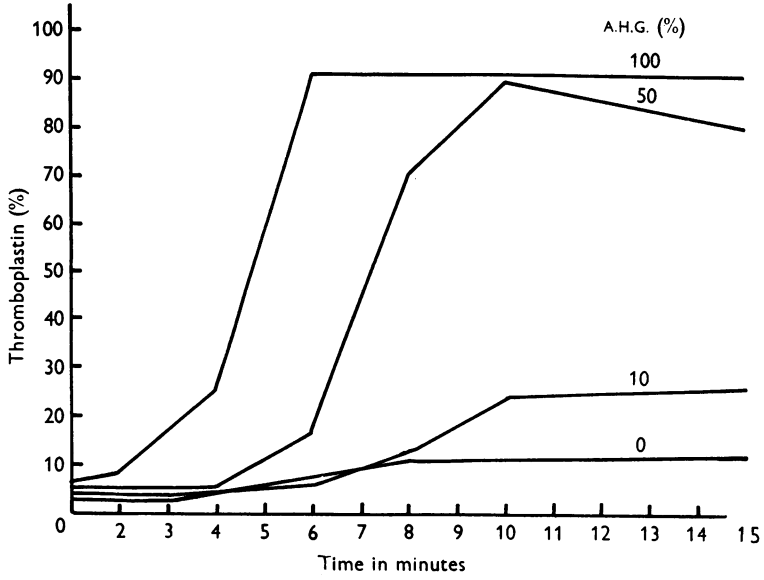

Fig. 3. Curves demonstrating the formation of blood thromboplastin in mixtures containing various concentrations of antihaemophilic globulin (A.H.G.). 100% A.H.G. represents normal $Al(OH)_3$-treated plasma. 50% A.H.G. represents a mixture of equal parts of normal and haemophilic plasma treated with $Al(OH)_3$. 10% A.H.G. represents a mixture of 1 part of normal with 9 parts of haemophilic plasma treated with $Al(OH)_3$. 0% A.H.G. represents haemophilic plasma treated with $Al(OH)_3$.

normal at the time that the test was made. These results suggest that the antihaemophilic globulin is required quantitatively for the formation of blood thromboplastin and that the concentration of antihaemophilic globulin influences the time at which thromboplastin formation begins. An interesting feature of this experiment is that although the presence of 50% of anti-haemophilic globulin was accompanied by the formation of a normal amount of thromboplastin the abnormal delay in thromboplastin formation was not eliminated.

Factor VII. The amount of factor VII present could be varied by using different dilutions of normal serum as a source of factor VII. When this was done the curves shown in Fig. 4 were obtained. From these results it will be seen that factor VII is present in considerable excess in normal serum. A reduction

from 50 to 6% of serum caused little decrease in the amount of thromboplastin formed though reduction below that level did diminish the amount. The concentration of factor VII also influences the speed of thromboplastin formation.

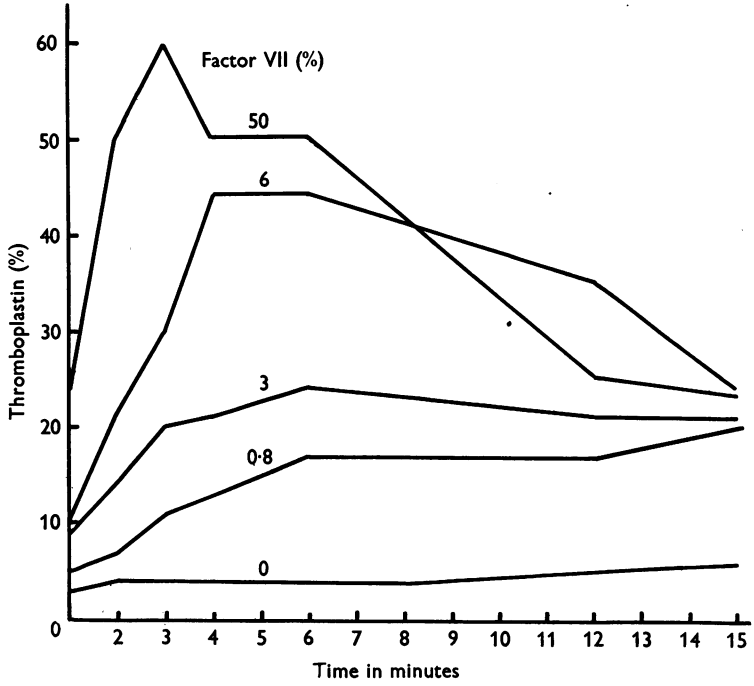

Fig. 4. Curves demonstrating the formation of blood thromboplastin in mixtures containing various concentrations of factor VII. The concentrations of factor VII were obtained by diluting normal serum. Thus 50% represents a 1/2 dilution of serum.

These experiments show that platelets, the antihaemophilic globulin and factor VII are all required quantitatively for the formation of blood thromboplastin. Within the limits of physiological variation, changes in the number of platelets have the greatest effect on the amount of thromboplastin formed. Factor VII normally appears to be present in considerable excess. The antihaemophilic globulin appears to have the greatest effect on the time at which thromboplastin formation begins and platelets the least effect.

The reaction between two components required for blood thromboplastin formation

In the interaction of these three components it is possible that a reaction between two components might precede a reaction of the product with the third component. To test this possibility the components were combined in

pairs with $CaCl_2$ and incubated for 10 min. After this time the third component was added. In no instance could any significant acceleration in the final speed of thromboplastin formation be demonstrated.

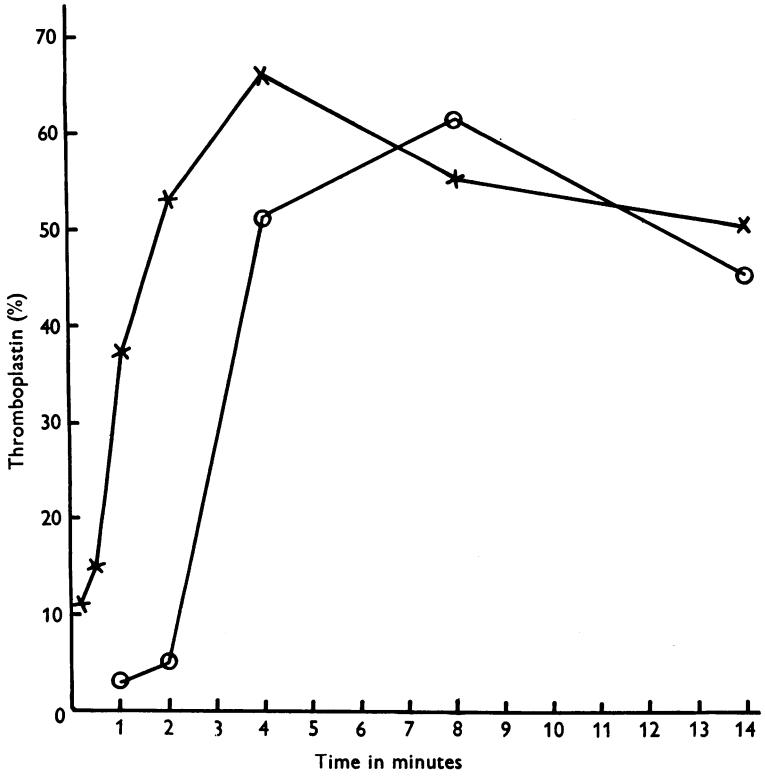

Fig. 5. Curves demonstrating the formation of blood thromboplastin from platelets, anti-haemophilic globulin, factor VII and $CaCl_2$ in the presence (X——X) and absence (O——O) of thrombin.

The effect of thrombin on the formation of blood thromboplastin

Thrombin has long been thought to have some accelerating effect on its own generation and this effect is readily demonstrated as described by Biggs & Macfarlane (1952). It was thought that thrombin might act by accelerating the formation of blood thromboplastin. To test this possibility thrombin was added to the usual reagents required for thromboplastin formation and the results were compared with parallel tests in which no thrombin was used. The findings are shown in Fig. 5. From these it is clear that thrombin accelerates the formation of blood thromboplastin. In this experiment the concentration of thrombin was very low; it clotted fibrinogen in about 150 sec and was too small in amount to clot plasma.

Blood thromboplastin and the formation of thrombin

When blood thromboplastin and calcium chloride are added to citrated plasma or to a mixture of prothrombin and factor V, separated from plasma as described by Biggs & Macfarlane (1952), thrombin is formed. The progress of thrombin formation is similar to that in experiments using brain thromboplastin. An experiment in which thrombin was formed in the presence of

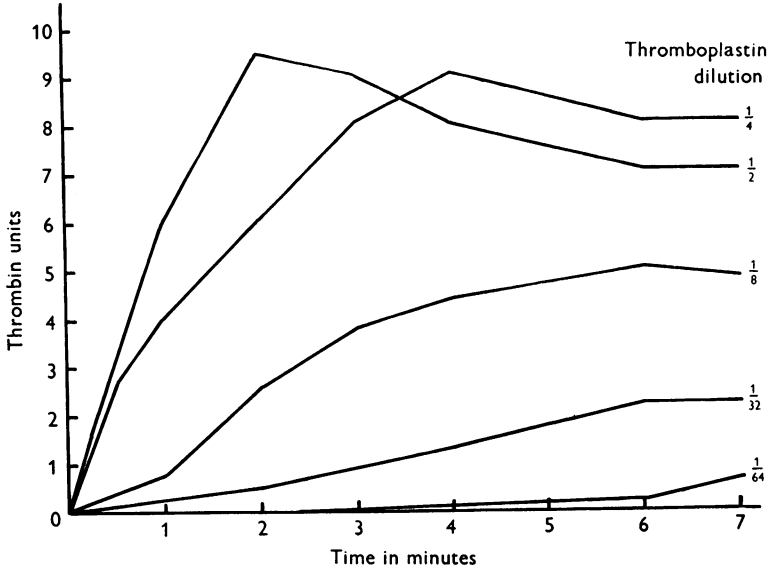

Fig. 6. Curves demonstrating the formation of thrombin from prothrombin in the presence of factor V, $CaCl_2$ and various dilutions of blood thromboplastin. The clotting times were deduced from a thrombin-fibrinogen dilution curve using the observed clotting times. By the method used a clotting time of 12 sec represents 10 units/ml. of thrombin in terms of a specific preparation of thrombin topical (Roche).

different dilutions of blood thromboplastin is illustrated in Fig. 6. In this experiment prothrombin and factor V were mixed with various dilutions of thromboplastin warmed to 37° C and $CaCl_2$ was added. At intervals, 0·1 ml. of the mixture was added to fibrinogen and the clotting times recorded. The fibrinogen clotting times were interpreted in terms of thrombin units using a thrombin-fibrinogen dilution curve. From this experiment it is clear that the concentration of blood thromboplastin has a marked effect on the speed of thrombin formation as do other thromboplastin preparations.

DISCUSSION

The experiments reported leave little doubt that the blood can generate a thromboplastin of great activity. When formed, it is capable of clotting plasma in 8–12 sec, and is thus more powerful than any tissue preparation so

far described, and is second only to Russell's viper venom acting together with lecithin.

The factors required for the production of this activity are platelets, antihaemophilic globulin, factor VII and calcium. In the absence of any one of these thromboplastin formation does not proceed. Contact with a foreign surface also seems to be essential. The nature of this reaction has not yet been determined, but it appears to require the simultaneous presence of all the factors. The amount of thromboplastin it produces is quantitatively related to the number of platelets available, and less critically, to the concentration of antihaemophilic globulin and factor VII. The time which elapses before thromboplastin begins to appear is most closely controlled by the concentration of antihaemophilic globulin and is scarcely affected by changes in platelet numbers over a certain minimum.

Antihaemophilic globulin seems to be consumed during the reaction since it cannot be found in serum. It is at present not known whether or not factor VII or platelets are consumed. Once formed, the blood thromboplastin has most of the functional characteristics of brain extract. It initiates thrombin formation in plasma without a delay phase. It is capable of clotting plasma from which the platelets have been removed, or plasma from a haemophilic subject, or plasma in silicone-coated containers. It differs from brain extract in being extremely labile; its dilution-activity curve is steeper.

These characteristics of the thromboplastin-forming system of normal blood explain a number of hitherto puzzling facts. Despite their relatively long clotting time, normal blood and recalcified plasma form clots suddenly and completely, an observation that is difficult to explain if only a weak thromboplastin were available but easily understood if a powerful thromboplastin appears suddenly after several minutes delay.

In haemophilia and thrombocytopenia there is a deficiency of one thromboplastin component, antihaemophilic globulin in haemophilia and platelets in thrombocytopenia. In both conditions there is delayed conversion of prothrombin to thrombin which would be expected from the formation of a low concentration of thromboplastin. In haemophilia there is characteristically a long clotting time whereas in thrombocytopenia the clotting time is usually normal. This difference is easily explained by the different activities of antihaemophilic globulin and platelets in the formation of blood thromboplastin. A deficiency of antihaemophilic globulin delays the time at which thromboplastin appears and therefore lengthens the clotting time. A reduction in platelet number has little or no effect on the time at which thromboplastin appears and therefore thrombocytopenia is seldom associated with delayed clotting.

The concept of blood thromboplastin and its origin from three precursor substances suggests methods for the study of clotting defects associated with

an abnormal thromboplastin system. The defect in haemophilia, for example, can be studied by using platelets from haemophilic blood and factor VII from normal serum; thromboplastin formation on the addition of various proportions of normal and haemophilic plasma treated with $Al(OH)_3$ can be observed. Using this technique it is possible to demonstrate the haemophilic defect even in mildly affected individuals whose clotting time and prothrombin consumption tests are normal. If it is suspected that the platelets are functionally abnormal as in 'thromboasthenia' (Hirsch, Favre-Gilly & Dameshek, 1950; Alexander & Landwehr, 1949), the patient's platelets may be compared with normal ones in their ability to form thromboplastin in the presence of the same preparations of antihaemophilic globulin and factor VII. If an inhibitor of the thromboplastin system is suspected then it is possible to discover whether the inhibitor prevents the formation of thromboplastin or its reaction when formed with prothrombin and factor V. These applications of the concept of blood thromboplastin presented in this paper will be considered in greater detail in a later communication.

The reasons why this thromboplastin-producing system has not been recognized are probably complex. The supposition that blood thromboplastin, even if it existed, was feeble has discouraged experiment and misled those who tried to demonstrate its activity The extreme lability of the material has also made investigation difficult because the thromboplastic activity can only be demonstrated in the absence of thrombin and by the time that thrombin is formed and neutralized in whole blood most of the thromboplastin has disappeared. The fact that three components are required for its production has also created experimental difficulties. Ferguson (1949) and Shinowara (1951) worked with a combination of platelets and antihaemophilic globulin, and Mann, Hurn & Barker (1951) used platelets and serum, but the complete system which is apparently required has not, to our knowledge, previously been investigated.

SUMMARY

1. The clotting time of normal human blood placed in small glass tubes lies between 5 and 10 min. This long clotting time is usually thought to be due to the feeble nature of the blood thromboplastin system. It can be shown that blood thromboplastin is not feeble but that it exists in a precursor form. The long clotting time is a measure of the delay in thromboplastin formation and not a measure of the strength of the thromboplastin when formed.

2. The factors necessary for thromboplastin formation are platelets, the antihaemophilic globulin, a serum fraction containing factor VII, and $CaCl_2$. When these factors are all present a thromboplastin is formed capable of clotting normal plasma in from 8 to 12 sec.

3. The amount of thromboplastin produced is quantitatively related to the

number of platelets available and, less critically, to the concentration of antihaemophilic globulin and factor VII. The time which elapses before thromboplastin formation begins to appear is most closely controlled by the concentration of antihaemophilic globulin and is scarcely affected by changes in platelet numbers over a certain minimum.

4. The presence of thrombin accelerates the formation of blood thromboplastin.

5. The concentration of blood thromboplastin has a marked effect on the speed of thrombin formation from factor V and prothrombin.

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