THE REACTION OF HAEMOPHILIC PLASMA TO THROMBOPLASTIN

BY

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Two well-known observations on the clotting defect of haemophilic blood would seem to be of basic importance. The first is that haemophilic blood responds quite normally to a strong suspension of tissue thromboplastin; the second, that it will clot in practically the normal time on the addition of a small proportion of normal plasma. The normal response to thromboplastin naturally suggests that the part of the clotting mechanism which is set in motion by thromboplastin is in normal working order. Certainly no defect has been demonstrated in haemophilic prothrombin, fibrinogen, or calcium, and the various factors recently described as being concerned in prothrombin conversion, such as Factor V (Owren, 1947) and “serum prothrombin conversion accelerator” (Alexander and de Vries, 1949), are stated by these respective authors to be normal in haemophilia.

The essential defect in haemophilic blood, therefore, seems to be its inability to clot normally in the absence of added thromboplastin, a defect which is largely corrected by the addition of a small amount of normal plasma. Since normal blood clots rapidly in glass, even when obtained with a minimum of contamination with tissue fluid, it must be supposed that there is some intrinsic mechanism for the production of thromboplastic activity. This matter has been discussed by many workers, and two current views are put forward by Brinkhous (1947) and Quick (1947) respectively. These authors agree in supposing that intrinsic thromboplastic activity develops on contact with a foreign surface, and depends on interaction between the platelets and a plasma factor. In Quick’s view it is the plasma factor, “thromboplastinogen,” that is the inactive precursor of thromboplastin and which is activated by a platelet factor. Brinkhous considers that the platelets themselves are the source of thromboplastin, and that they are activated by being broken up by the plasma factor, which is a “thrombocytolysin.” It is agreed by both that the platelets are normal in haemophilia, and that it is the plasma factor which is deficient in this condition. Normal plasma contains enough of this “antihaemophilic factor” to be able to correct the deficiency.

Ignoring the slight divergence of views as to the mode of action of the antihaemophilic factor, it seems to be accepted that its deficiency in haemophilia results in a deficient production of intrinsic thromboplastin, such thromboplastin presumably having a similar action to that of the more familiar tissue thromboplastin.

The normal response of haemophilic blood to added tissue thromboplastin is clearly important to the validity of this hypothesis. There is little doubt that with
strong suspensions of brain extract thrombin is formed in the same amount and at the same speed in haemophilic as in normal plasma or from haemophilic prothrombin and Factor V as compared with normal factors (Biggs, 1951b). Quick (1947) has claimed that haemophilic plasma reacts normally to even minute amounts of thromboplastin, but other workers would not agree with this statement. Addis, in 1911, showed that haemophilic plasma clotted more slowly than normal with added testis or kidney thromboplastin, and more recent observations suggest that its reaction to weak brain suspensions is less rapid than normal (Pavlovsky, Mittelman, and Castellanos, 1949; Dreskin and Rosenthal, 1950; Frommeyer, Epstein, and Taylor, 1950).

In view of this uncertainty it was considered that a study of the reactions of normal and haemophilic plasma and plasma fractions with different concentrations of thromboplastic agents might provide further information on the mode of action of the "antihaemophilic factor."

**Technique**

**Reagents.**—The following reagents were used:

*Brain Thromboplastin.*—Fresh human brain is collected in the post-mortem room, and all superficial vessels and meninges are removed. The substance is macerated with three or four times its volume of acetone in a mortar. The acetone is replaced four times, and the granular powder is dried by means of a suction filter. The dried brain can be stored for periods up to three months at room temperature with no special precautions.

For use 0.5 g. of the granular powder is suspended in 5 ml. of saline and incubated at 37°C for 15 minutes. The suspension is mixed once or twice during this time, and the coarse particles are allowed to settle by gravity. The supernatant fluid is removed and diluted 1/5 with 0.85% saline.

*Calcium Chloride.*—An M/40 solution of anhydrous calcium chloride is used.

*Fibrinogen* (Jaques, 1943).—First a 3 M phosphate buffer at pH 6.6 is made by dissolving 817 g. of anhydrous KH₂PO₄ in 1,000 ml. of distilled water. To this is added 750 ml. of 4N KOH. After warming, the volume is made up to 2,000 ml. and the solution is filtered.

Secondly, a preparation of Al(OH)₃ suspension (Bertho and Grassmann, 1938) is made up as follows: 100 ml. of 10% ammonium solution is poured into 600 ml. of water at 63°C containing 22 g. of ammonium sulphate, and the temperature is rapidly brought to 58°C. The mixture is stirred vigorously and poured, in one lot, into a solution of 76.7 g. of ammonium alum at a temperature of 58°C. The temperature rises to 61°C. Stirring is continued for 10 minutes, during which time the temperature is maintained above 58°C. The precipitate is separated by centrifuging. The precipitate is washed five times with 1,500 ml. of distilled water, separating the precipitate by centrifuging on each occasion. To the first washing water is added 0.44 ml. of 10% ammonia, and to the second 0.88 ml. of 10% ammonia. The whole procedure does not take much more than two hours. The precipitate is suspended in the least amount of distilled water that is required to make a solution that can be pipetted.

Thirdly, the fibrinogen is prepared. To 100 ml. of citrated plasma obtained from "bank" blood is added 2 ml. of Al(OH)₃ suspension to remove the prothrombin. The Al(OH)₃ is removed by centrifuging and discarded. To 100 ml. of this prothrombin-free plasma is added 100 ml. of 2 M phosphate buffer at pH 6.6. After centrifuging, the precipitate is washed with 100 ml. of molar phosphate buffer and dissolved in 75 ml. of M/4 phosphate buffer. The precipitation is repeated twice and the final precipitate is
dissolved in 50 ml. of 0.85% saline containing 0.0075 M sodium citrate. The solution is dialysed for 12 hours against citrate saline. The concentration of fibrinogen is usually about 400 mg. %.

**Collection of Blood.**—Blood was collected from normal and haemophilic subjects by venepuncture, and coagulation prevented by mixing 9 parts of blood with 1 part of 3.8% sodium citrate. The blood was centrifuged at 2,000 r.p.m. for 15 minutes. The plasma was separated for use.

**Preparation of Fractions by Acid Precipitation.**—The following methods were used:

"**Globulin Fraction.**"—Plasma was dialysed overnight against 0.38% sodium citrate. The pH was then adjusted to 5, using 10% acetic acid; the resulting precipitate was removed by centrifuging and dissolved in 0.85% saline, containing 1 part of 3.8 sodium citrate to 9 parts of saline. The volume of the final solution was made about half that of the original plasma. If samples from normal and haemophilic blood were compared, care was taken that the concentrations of globulin were as nearly comparable as possible. Before use the pH was adjusted to 7 with 2% sodium carbonate.

"**Albumin Fraction.**"—The supernatant remaining after acid preparation of the globulin fraction was neutralized with 10% sodium carbonate and constituted the albumin fraction. This fraction contains a large amount of protein precipitate by 50% saturation with ammonium sulphate and thus contains protein other than albumin.

**Preparation of Fractions by Ammonium Sulphate Precipitation.**—This procedure entailed preparing three fractions.

**Fibrinogen Fraction (Fraction I).**—Plasma, 10 ml., was largely freed from platelets by centrifuging for half an hour at 3,000 r.p.m. To the clear plasma were added 2 g. of solid ammonium sulphate. The mixture was well shaken to ensure the complete solution of the ammonium sulphate and the precipitate removed by centrifuging. The precipitate was dissolved in 5 ml. of 0.85% saline and dialysed overnight against saline containing 1/10 of its volume of 3.8% sodium citrate.

**Globulin Fraction (Fraction II).**—A further 1 g. of solid ammonium sulphate was added to the supernatant from the preparation of Fraction I. The precipitate was collected by centrifuging and dissolved in 5 ml. of 0.85% saline and dialysed against citrate saline.

**Albumin Fraction (Fraction III).**—The supernatant from the preparation of Fraction II was dialysed against citrate saline.

**Technical Methods**

**One-stage Prothrombin Time.**—Plasma, 0.1 ml., is mixed with 0.1 ml. of thromboplastin, and the mixture warmed to 37° C.; 0.1 ml. of M/40 calcium chloride is then added and the coagulation time recorded.

**Two-stage Prothrombin Test.**—A modification of the method of Herbert (1940) and Owren (1947) was used. Plasma, 0.3 ml., and 0.3 ml. of saline were mixed with 0.3 ml. of brain emulsion, and the mixture was warmed to 37° C. Then 0.3 ml. of M/40 calcium chloride was added, and at various intervals from one to 30 minutes 0.1 ml. samples were removed and added to 0.4 ml. amounts of fibrinogen in small tubes previously prepared and placed in the water bath. The coagulation times of the fibrinogen were recorded. The concentration of fibrinogen was adjusted to approximately 100 mg. %.

If thrombin formation was followed in the globulin fraction instead of whole plasma, 0.3 ml. of globulin fraction was mixed with 0.3 ml. saline or 0.3 ml. of the "albumin fraction" and 0.3 ml. of brain emulsion. Then 0.3 ml. of M/40 CaCl₂ was added, and thrombin generation was followed as before.
The clotting times obtained by the two-stage method record the concentrations of thrombin in the incubation mixture. The concentrations can be expressed as relative units of thrombin using a calibration curve prepared by adding decreasing concentrations of a solution of thrombin containing 1,000 units per ml. to 0.4 ml. samples of fibrinogen and recording the clotting times. For the graphical representation of results the clotting times have been converted to arbitrary thrombin units by this method.

**Experimental Results**

**Experiments on Whole Plasma.**—A few experiments were made using the one-stage method. Using normal and haemophilic plasma samples, the one-stage method was carried out with dilutions of brain emulsion varying from 1/10 to 1/50,000. The clotting time obtained with each sample of haemophilic plasma was then plotted against the clotting time of the corresponding normal plasma obtained with the same thromboplastin preparation. From Fig. 1 it will be seen that when the thromboplastin preparation was diluted to give with normal plasma a clotting time of more than 40 seconds, usually the haemophilic plasma had a longer clotting time than the normal. Thus, with quite strong suspensions of thromboplastin, the reaction of haemophilic plasma was abnormal.

This abnormality might be attributed to an initial deficiency of thromboplastin in the haemophilic plasma due to its supposed inability to generate intrinsic thromboplastin activity. If this is the true explanation, then an appropriate addition of thromboplastin to the haemophilic plasma should correct the defect. Enough thromboplastin was added to haemophilic plasma to make its clotting time the same as that of the normal plasma to which no thromboplastin had been added. The amount required was a 1/10,000 concentration of brain emulsion. Further additions of thromboplastin were then made to both normal and haemophilic plasma. From Fig. 2 it will be seen that the further additions of thromboplastin had less effect on haemophilic than on normal plasma. The defect in haemophilic plasma therefore cannot be attributed to a simple deficiency in thromboplastin.

The long clotting time of haemophilic plasma might be due to its neutralization of thromboplastin. A number of samples of normal and haemophilic plasma were incubated with brain thromboplastin for one hour, and at intervals samples were recalculated. The clotting time of both normal and haemophilic samples lengthened slightly, but there was no striking difference in their behaviour on incubation, though the clotting times of the haemophilic plasma samples were consistently longer than those of the normal (Fig. 3). In a further experiment one normal and two samples of haemophilic plasma were incubated at 37°C for one hour with two strengths of brain thromboplastin. At the end of this time the samples were used undiluted and diluted 1/10 as a source of thromboplastin for the one-stage method. The samples were tested on the same normal and the same two samples of haemophilic blood in all combinations. The results of this experiment are set out in Tables I and II. Incubation of brain thromboplastin with the normal plasma in this experiment led to rather more inactivation of thromboplastin than did incubation with either of the haemophilic plasmas. From these experiments it seems probable that progressive inactivation of brain thromboplastin by plasma is not an essential feature of haemophilic plasma.
Fig. 1.—Normal and haemophilic plasma samples were tested by the one-stage method using various dilutions of brain emulsion as thromboplatin. In the diagram the clotting times obtained with normal plasma samples are plotted against the clotting times obtained with the same thromboplatin preparations with haemophilic plasma samples. With concentrated preparations of thromboplatin which give clotting times with the normal plasma of less than 40 seconds, the clotting times of the haemophilic samples are often the same as normal; with weaker suspensions of brain emulsion the clotting times of the haemophilic samples are longer than those of the normal plasma samples.
The results of the one-stage method carried out with various dilutions of brain emulsion varying from 1/10 to 1/10,000 with (1) normal plasma (●), (2) haemophilic plasma (×), (3) haemophilic plasma containing a 1/10,000 concentration of brain emulsion (○).

Fig. 3.—Samples, each 0.1 ml., of normal and haemophilic plasma were incubated at 37° C. for periods up to one hour with various concentrations of brain emulsion. The clotting times of these mixtures on recalcification were tested. In any particular experiment the normal and haemophilic plasma samples were incubated with the same preparation of brain emulsion. The results represent the average for seven independent experiments. The upper curve shows the clotting times of the haemophilic plasma samples and the lower curve the clotting times of the normal plasma samples.
REACTION OF HAEMOPHILIC PLASMA TO THROMBOPLASTIN

TABLE I
CLOTTING TIMES DERIVED FROM ONE-STAGE TEST (Q.V.) OF NORMAL AND HAEMOPHILIC PLASMAS WITH THROMBOPLASTIN USING DIFFERENT PLASMAS AS SUBSTRATES

<table>
<thead>
<tr>
<th>Dilution of Brain Emulsion Incubated with Plasma</th>
<th>Type of Plasma</th>
<th>Clotting Times (secs.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>1/5</td>
<td>Normal</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Haemophilic I</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Haemophilic II</td>
<td>21</td>
</tr>
<tr>
<td>1/50</td>
<td>Normal</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Haemophilic I</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Haemophilic II</td>
<td>35</td>
</tr>
</tbody>
</table>

Three plasma samples, one normal and two from haemophilic patients, were incubated at 37°C for one hour with an equal volume of brain emulsion diluted 1/5 and 1/50 with 0.85% saline. At the end of the hour the mixtures were used as a source of thromboplastin.

TABLE II
CLOTTING TIMES DERIVED FROM ONE-STAGE TEST (Q.V.) OF NORMAL AND HAEMOPHILIC PLASMAS USING THE SAME PLASMAS AS SUBSTRATE

<table>
<thead>
<tr>
<th>Dilution of Brain Emulsion Incubated with Plasma</th>
<th>Type of Plasma</th>
<th>Clotting Times (secs.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>1/5</td>
<td>Normal</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Haemophilic I</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Haemophilic II</td>
<td>38</td>
</tr>
<tr>
<td>1/50</td>
<td>Normal</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Haemophilic I</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Haemophilic II</td>
<td>58</td>
</tr>
</tbody>
</table>

Three plasma samples, one normal and two haemophilic, were incubated at 37°C for one hour with an equal volume of brain emulsion diluted 1/5 and 1/50 with 0.85% saline. At the end of the hour these samples were diluted 1/10 with 0.85% saline, giving final dilutions of brain emulsion of 1/50 and 1/500 and used as a source of thromboplastin.

It is possible that haemophilic plasma contains an inhibitor of thromboplastic action which requires the presence of calcium or which is inhibited by sodium citrate. This possibility was not investigated, because it is difficult to study the destruction of thromboplastin in a system which is forming thrombin and a serum accelerator of thrombin formation.

In the majority of experiments thrombin formation was followed by the two-stage method in a system containing the albumin fraction which has a potent antithrombic action. Thrombin was therefore destroyed progressively as it was formed, and it is important to appreciate the factors which influence the concentration of thrombin in a system of this sort. The amount of thrombin available depends on the relative speeds of its formation and destruction.

The effect of varying the speed of formation may be shown in an experiment in which the amount of thromboplastin added to plasma in the two-stage method is varied. The stronger suspensions of thromboplastin lead to rapid thrombin generation, and a high level of thrombin is detected. With slow speeds of generation much less thrombin can be demonstrated (Fig. 4), although the same total amount is probably produced.
FIG. 4.—The curves represent the progress of thrombin formation and disappearance followed by the two-stage method in the same sample of normal plasma using brain preparations of varying strength. The curve ○ represents the reaction with brain emulsion diluted 1/10; the curve × the reaction with brain emulsion diluted 1/50; the curve ● the reaction with brain emulsion diluted 1/100.

FIG. 5.—The curves represent the progress of thrombin formation followed by the two-stage method in normal plasma diluted 1/10 with 0.85% saline (upper curve) and in the same plasma diluted with 0.85% saline to which various concentrations of plasma adsorbed with Al(C₂H₃O₂)₃ had been added.

FIG. 6.—The curves represent the progress of thrombin formation in the presence of dilute brain thromboplastin followed by the two-stage method in normal plasma (continuous line) and three samples of haemophilic plasma from different patients.
REACTION OF HAEMOPHILIC PLASMA TO THROMBOPLASTIN 453

The speed of production may be kept constant and the activity of the anti-thrombin may be increased by adding to a saline dilution of plasma plasma adsorbed with Al(OH)₃ to remove prothrombin. When plasma prepared in this way is tested by the two-stage method the curves in Fig. 5 result. Similar curves result from the addition of heparin to plasma. The amount of thrombin detected is controlled both by the speed of thrombin formation and the speed of its destruction.

In a number of samples of haemophilic plasma thrombin formation was studied by the two-stage method, using a 1/100 or 1/1,000 dilution of brain thromboplastin. In Fig. 6 the results in three individual haemophilic plasmas are compared with the average for four normal plasmas. From the previous discussion it is probable that the difference between the haemophilic and normal plasmas lies mainly in a difference in the speed of thrombin formation.

The addition of normal plasma to haemophilic whole plasma greatly improves the reaction of haemophilic plasma to weak suspensions of brain thromboplastin. In Fig. 7 are shown the effects on thrombin formation of adding 1/10 and 1/2 proportion of normal plasma to haemophilic plasma. These results are the mean values for eight experiments on the blood of different haemophilic and normal people. It will be seen that 1/10 volume of normal plasma considerably increases the speed of thrombin formation, but that even 50% of normal plasma does not restore thrombin formation to normal. The effect of 1/10 dilution of normal plasma is worth emphasizing. In previous experiments it was found that a concentration of 1/10,000 brain emulsion was required to bring the one-stage clotting time of haemophilic plasma to the same as that of normal plasma. Therefore, if the defect in haemophilic plasma is due to a deficiency in thromboplastin, normal plasma must contain an excess of thromboplastin in comparison with haemophilic plasma, and this excess is approximately equal to a brain concentration of 1/10,000. The addition of 1/10 proportion of normal to haemophilic plasma would therefore be equivalent to the addition of 1/100,000 dilution of brain thromboplastin. This concentration of brain thromboplastin is practically inactive with haemophilic plasma. This experiment therefore confirms the view that the defect in haemophilia cannot be a simple lack of thromboplastin.

Experiments on Plasma Fractions.—It has been shown previously (Biggs, 1951b) that prothrombin and Factor V isolated from haemophilic plasma have a normal reaction to platelet thromboplastin. It is therefore probable that the abnormal reaction of whole haemophilic plasma to thromboplastin is a feature of whole plasma, which is lost on fractionation. An attempt to identify the cause of the abnormality was made by a more simple fractionation of the plasma. Whole normal plasma and whole haemophilic plasma were dialysed against distilled water, and the globulin fraction precipitated by bringing the pH to 5. This fraction was dissolved in saline and neutralized. The supernatant plasma was neutralized and called the albumin fraction. In fact, this fraction was a crude mixture of albumin and globulin. These fractions were tested in various mixtures by the two-stage method, using a weak suspension of brain thromboplastin. The experiment was carried out on five haemophilic and five normal subjects.

The formation of thrombin in the globulin fraction alone given as an average for the five experiments shows no difference between the normal and haemophilic fractions (Fig. 8). This results suggests that the factor causing the abnormal reaction
Fig. 7.—The curves represent the progress of thrombin formation in the presence of dilute brain thromboplastin followed by the two-stage method in (1) normal plasma (○), (2) haemophilic plasma (△), (3) haemophilic plasma to which 1/10 of its volume of normal plasma had been added (●), (4) a mixture of haemophilic and normal plasma in equal parts (■). The results are the mean values for eight independent experiments on different samples of normal and haemophilic plasma.

of haemophilic plasma to weak thromboplastin lies in the albumin fraction or in the reaction between factors in the albumin and globulin fractions.

Thrombin formation was then studied in mixtures of the albumin and globulin fractions of normal and haemophilic plasma in all combinations. The speed of thrombin formation was increased by the addition of the normal albumin fraction to the normal globulin fraction and to the haemophilic globulin fraction (Figs. 9 and 10). The addition of haemophilic albumin to the normal globulin also caused an increased speed of thrombin formation (Fig. 9). The addition of haemophilic albumin to haemophilic globulin gave no increase in the speed of thrombin formation. The results illustrated in Figs. 9 and 10 represent the average for five independent experiments on different haemophilic and normal plasmas. The proportions of the different fractions used were constant in all the experiments.

The interpretation of these results is not simple. The method of fractionation failed to separate the better recognized plasma constituents at all specifically. The "globulin fraction" from normal plasma probably contained prothrombin, Factor V, fibrinogen, and some antihaemophilic factor. In two experiments the normal albumin fraction contained the antihaemophilic factor in addition to antithrombin.
Fig. 8.—Thrombin formation in the presence of dilute brain thromboplastin in (1) the globulin fraction from normal plasma (O), and (2) the corresponding fraction from haemophilic plasma (×). The curves in Figs. 8, 9, and 10 are the average values for five independent experiments.

Fig. 9.—Thrombin formation and disappearance in (1) the globulin fraction from normal plasma mixed with the albumin fraction from normal plasma (O), and (2) the globulin fraction from normal plasma mixed with the albumin fraction from haemophilic plasma (×). Thrombin formation in the normal globulin fraction alone is given for comparison.

Fig. 10.—Thrombin formation and disappearance in (1) the globulin fraction prepared from haemophilic plasma mixed with the albumin fraction from normal plasma (O), and (2) the globulin fraction from haemophilic mixed with the albumin fraction from haemophilic plasma (×). Thrombin formation in the haemophilic globulin fraction alone is shown for comparison.
The fact that the so-called globulin fractions from normal and haemophilic plasma behaved in the same manner, and the fact that the speed of thrombin was accelerated if both the antihaemophilic factor and the albumin fraction were present, suggest that the full effectiveness of the antihaemophilic factor depends in some way on its interaction with some substance in the albumin fraction.

An attempt was made to obtain a more specific separation of important components by ammonium sulphate fractionation. In this way fractions precipitated at 33% and 50% saturation with ammonium sulphate and a supernatant from the 50% saturation were isolated. The 33% fraction (Fraction I, or fibrinogen fraction) from normal plasma contained the fibrinogen and antihaemophilic factor, Fraction I from the haemophilic plasma contained no antihaemophilic factor, the 50% fraction (Fraction II, or globulin fraction) contained prothrombin and Factor V, and the supernatant from the 50% fraction (Fraction III, or albumin fraction) contained antithrombin. The only fraction which showed any ability to shorten the clotting time in haemophilic plasma was the 33% fraction from normal plasma. These fractions were tested for their ability to form thrombin, and in general the results confirm the findings obtained with the acid fractionation. Some of the experiments are illustrated in Figs. 11 and 12.

When tested alone Fractions I and II from normal and haemophilic plasma behaved similarly. In Fig. 11 the three normal fractions were tested together and gave a rapid speed of thrombin generation. When the normal Fraction I was replaced by that from haemophilic blood there was a considerable delay in thrombin formation. When in addition the haemophilic albumin fraction replaced the normal albumin fraction a further reduction in the speed of thrombin formation occurred.

In Fig. 12 similar experiments with the haemophilic fractions are shown. When all three fractions from haemophilic plasma were used thrombin formation was poor. When the haemophilic Fraction I was replaced by that from normal plasma the speed of thrombin formation was accelerated. The further replacement of the haemophilic albumin fraction by the corresponding normal fraction led to a second increase in the speed of thrombin formation.

The fact that the globulin fractions from haemophilic and normal plasma behaved similarly, and that the normal albumin fraction accelerated the speed of thrombin formation from the normal globulin fraction, suggests that the full effectiveness of the antihaemophilic factor depends on its co-operation with a factor in the albumin fraction.

The inhibitory effect of the haemophilic albumin fraction in this experiment may have been due to the presence of an inhibitory substance as a result of immunization following repeated transfusions (Frommeyer et al., 1950), or the haemophilic albumin may have lacked some substance present in normal plasma. The inhibitory effect of the haemophilic albumin fraction is not a consistent finding, because it was observed in one only of the experiments carried out on fractions prepared by acid precipitation.
Fig. 11.—The curves represent the progress of thrombin formation followed by the two-stage technique using dilute brain thromboplastin in mixtures of plasma fractions prepared by ammonium sulphate precipitation from haemophilic and normal plasma. In the curve marked O the normal fibrinogen, globulin, and albumin fractions were used (Fractions I, II, and III). In the curve marked × the haemophilic fibrinogen fraction was mixed with normal globulin and albumin fractions. In the curve marked † the haemophilic fibrinogen fraction was mixed with normal globulin and haemophilic albumin.

Fig. 12.—The curves represent the progress of thrombin formation followed by the two-stage method using dilute brain thromboplastin in mixtures of plasma fractions prepared by ammonium sulphate precipitation from normal and haemophilic plasma. In the curve marked O the fibrinogen, globulin, and albumin were derived from haemophilic plasma. In the curve marked × the normal fibrinogen fraction was mixed with globulin and albumin from haemophilic plasma. In the curve marked † the haemophilic globulin fraction was mixed with fibrinogen and albumin from normal plasma.

Discussion

From the foregoing experiments it would seem that the defect in the clotting mechanism of haemophilic blood is associated with a failure to react normally to small amounts of thromboplastin. There is no evidence that there is a deficiency of thromboplastin in haemophilic blood, compared with normal, nor of any abnormal inhibition or destruction of thromboplastin. Since the so-called antihaemophilic factor largely corrected the defective response of haemophilic plasma to thromboplastin, it appears that this factor is concerned in some way in potentiating or accelerating thromboplastic action.

The antihaemophilic factor is not essential for the thromboplastic action of brain, since strong suspensions of thromboplastin will clot haemophilic blood as readily as normal blood, but its accelerating effect becomes marked as the concentration of thromboplastin is reduced.
The mode of action of the antihaemophilic factor is difficult to investigate without fractionation of the plasma, yet this fractionation itself introduces serious difficulties. In the first place simple methods of fractionation do not always give a clear-cut separation, even of such well-recognized plasma constituents as fibrinogen and antithrombin. One experiment is never exactly comparable with another. In addition it is never possible to be certain that fractionation does not separate artificially components which, in the body, react as one physiological unit. Conclusions drawn from fractionation experiments must always be made cautiously, and regarded only as provisional hypotheses until more reliable methods of verifying the results are available.

The fractionation experiments recorded in this study appear to show that the Factor V and prothrombin of haemophilic and normal plasma react to weak brain suspensions in the same manner. The accelerating effect of the antihaemophilic factor on the haemophilic fractions appears to depend on the reconstitution of a whole plasma containing the albumin fraction in addition to the other components.

It is probable that in natural conditions the coagulation of the blood is largely dependent on such intrinsic thromboplastin as it possesses. The platelets may provide some or all of this, since it has been shown (Biggs, 1951a) that they have definite thromboplastic activity, despite previous contrary views (Ware, Fahey, and Seegers, 1948). Measured in terms of brain thromboplastin this intrinsic thromboplastin is relatively feeble, being equivalent only to a 1/10,000 suspension of brain extract, and it is at such levels that the action of the antihaemophilic factor becomes of great importance. Under these conditions a deficiency of this factor will result in such a slow prothrombin conversion that the rate of thrombin generation barely exceeds the rate of its destruction, and the concentration required to react with fibrinogen may not be achieved for a considerable time, if at all.

**Summary**

When dilute brain thromboplastin was added to haemophilic and normal plasma the haemophilic plasma had a longer clotting time on recalcification than normal.

The abnormal reaction of haemophilic plasma to thromboplastin could not be corrected by the addition of thromboplastin.

Haemophilic plasma had no specific ability to destroy thromboplastin.

In the two-stage method, using dilute thromboplastin, haemophilic plasma caused the formation of thrombin more slowly than normal plasma.

When the globulin fractions of normal and haemophilic plasma were separated from their respective albumin fractions, and tested by the two-stage method, using dilute brain thromboplastin, the speed of thrombin formation was the same in the two fractions.

The slow speed of thrombin formation which characterized whole haemophilic plasma was obtained when the haemophilic albumin and globulin fractions were mixed. The haemophilic albumin fraction had no inhibitory effect on the normal globulin fraction.
REACTION OF HAEMOPHILIC PLASMA TO THROMBOPLASTIN 459

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Rosemary Biggs and R. G. Macfarlane

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