The Clot Accelerating Effect of Dilution on Blood and Plasma. Relation to the Mechanism of Coagulation of Normal and Hemophilic Blood

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Contrary to a prevailing general impression, the coagulation of properly collected normal or hemophilic blood, native and citrated plasma is accelerated when moderately diluted with 0.85 per cent NaCl or other fluids. Stable plasma seems to owe its fluidity ex vivo to the presence of inhibiting agents, which lose much of their activity on dilution or contact with surfaces like glass. Among these inhibitors may be the lipid antithromboplastin separated from brain, blood, plasma and plasma fractions. It seems from evidence to be presented that with or without the aid of activating agents, hemophilic blood or plasma may, if adequately diluted, clot as rapidly as normal blood or plasma. When hemophilic euglobulin is prepared from sufficiently diluted plasma it is as active a clot accelerator as a similarly prepared normal euglobulin.

It is only by the employment of recently developed technics of blood collection and processing that it has been possible to observe these facts, and to have a full appreciation of the degree of fluidity of blood and how even slight dilution can disrupt the equilibrium that maintains the blood fluid.

Methods

Unless otherwise stated, all work was done on blood collected from normal men and from 10 subjects with hereditary hemophilia of various grades of severity. Over one-half of the hemophilies had not had transfusions for several years. When the blood of those who had transfusions in the recent but not immediate past was diluted, its response did not differ essentially from that of the blood of the other hemophilies. The blood was collected in silicone coated syringes, stored and tested in silicone coated tubes. Centrifugations were done in the cold (4 to 7 C.). The anticoagulant generally used was 19 per cent trisodium citrate placed in a syringe (0.2 ml. of the citrate solution to 10 ml. of blood). The blood was collected through 18 gauge needles, after a quick puncture of a tense, turgid vein; it was aspirated without bubbling, at a rate of no less than 0.5 ml. per second. It was centrifuged usually at 3,000 r.p.m. for one hour, and the upper three-fourths of the plasma carefully removed with silicone coated droppers, measured with siliconized graduated pipets and tested within an hour after collection. Native plasma was obtained by allowing blood to flow from the vein through a 16 gauge needle directly into chilled silicone coated tubes; the blood was centrifuged for 15 minutes at 3,000 r.p.m. and the upper three-fourths of the plasma removed.

In the calculation of the final percentage plasma concentration in a given clotting mixture, any or all of the following variables were taken into account: (1) the percentage plasma volume of the sample of blood (calculated from the hematocrit); (2) the total volume of anticoagulant added to the blood; (3) the total volume of the clotting mixture and the respective volumes of each of its ingredients, namely, plasma, recalcifying solution, acti-

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vating agent and diluting fluid. Example: 9.0 ml. of normal blood was aspirated into a syringe containing 1 ml. of 0.1 M sodium oxalate. The packed cell volume (hematocrit) of this mixture was 30 per cent. This meant that the 7.0 ml. of plasma in the sample was diluted by 1.0 ml. of the anticoagulant solution (or a dilution of 6:7). In preparing the initial clotting mixture, 0.5 ml. of the plasma was added to 0.05 ml. of thromboplastin followed at once by 0.05 ml. of 0.2 M CaCl₂ thus involving a further dilution of the plasma of 5:6; thus \( \frac{2}{3} \times \frac{1}{3} \times 100 = 71 \) or a final plasma concentration in the clotting mixture of 71 per cent. By using 19 or 38 per cent citrate solutions as anticoagulants and increasing the volume of plasma in the clotting mixture, it is possible to work with mixtures of higher plasma concentrations. Example: 10 ml. of blood is aspirated into a syringe containing 0.1 ml. of 38 per cent trisodium citrate. Let us say that the packed cell volume of the blood is 40. This means that the 6.0 ml. of plasma is diluted 59:60 by the anticoagulant. In preparing the clotting mixture, 1.0 ml. of plasma is added to 0.1 ml. of thromboplastin, followed at once by 0.05 ml. of 0.4 M CaCl₂. The plasma in the clotting mixture is therefore diluted 100:115. Thus \( \frac{1}{115} \times \frac{1}{100} \times 100 = 85 \), or a concentration of the plasma in the initial mixture of 85 per cent.

The optimal amount of calcium required to recalcify citrated or oxalated plasma was determined for each sample of undiluted plasma. On table 1 is an example of how the ingredients may be adjusted in each tube to obtain decreasing degrees of plasma concentration, while using optimal amounts of CaCl₂, constant volume and thromboplastin concentration. Unless the hematocrit of the sample was unusually high or low, plasmas citrated in the proportions above mentioned, required one-tenth of their volume of 0.2 M CaCl₂ solution for optimal recalcification (e.g. tube 1, table 1). As the plasmas were diluted, the amounts of CaCl₂ were adjusted to the actual quantity of plasma in the clotting mixture, using as standard the amount of CaCl₂ originally shown to have given the optimal clotting time in the undiluted plasma. A constant optimal ratio was thereby maintained in each clotting mixture between the actual amount of plasma in it and the quantity of calcium added.

**Definitions**

**Stable plasma:** Plasma collected with special precautions from normal subjects, using the silicone technic throughout. If centrifuged for one hour at 3000 r.p.m., 0.5 ml. of this plasma recalcified with 0.05 ml. CaCl₂ should not clot in silicone coated tubes in less than 1,000 seconds; when centrifuged for two hours at 16,000 r.p.m. it should remain fluid for eight hours or longer.

**Asbestos plasma:** Plasma that has been placed in contact with asbestos fibers (10 mg. asbestos fibers per 1 ml. plasma) for a fixed period of time.

**TABLE 1.—Adjustment of the Ingredients of a Clotting Mixture to Obtain Varying Concentrations of Plasma while Maintaining Constant the Thromboplastin Concentration, Total Volume and Optimal Calcium Content**

<table>
<thead>
<tr>
<th>Tube Number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma (ml.)</strong></td>
<td>0.5</td>
<td>0.35</td>
<td>0.25</td>
<td>0.055</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Thromboplastin (ml.)</strong></td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>0.85% NaCl (ml.)</strong></td>
<td>0</td>
<td>0.165</td>
<td>0.275</td>
<td>0.44</td>
<td>0.53</td>
</tr>
<tr>
<td><strong>CaCl₂ (ml.)</strong></td>
<td>0.05*</td>
<td>0.035*</td>
<td>0.025*</td>
<td>0.055†</td>
<td>0.01†</td>
</tr>
<tr>
<td><strong>Total Volume (ml.)</strong></td>
<td>0.65</td>
<td>0.65</td>
<td>0.65</td>
<td>0.65</td>
<td>0.65</td>
</tr>
<tr>
<td><strong>Plasma Concentration (per cent)</strong></td>
<td>75.6</td>
<td>53.0</td>
<td>37.8</td>
<td>8.3</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Blood hematocrit: 40. 20 ml. blood + 0.2 ml. 38 per cent trisodium citrate.

Dilution of plasma by anticoagulant 59:60.

* Molar concentration 0.2 M.

† Molar concentration 0.02 M.
Activated Plasma: Plasma to which thromboplastin, platelets, cephalin, glass particles, or similar activators have been added.

Nonactivated Plasma: Plasma to which no activator has been added.

Cephalin: The lipid extracted with ethyl ether from acetone dried brain and separated from the ether extract with cold absolute ethanol.

Purified Cephalin: The product obtained from at least five precipitations of the ether soluble lipid with cold absolute ethanol.

Thromboplastin: A clot accelerating, thermolabile, water soluble lipoprotein, extracted from acetone dried organs (human brain usually) with 0.85 per cent NaCl.

1. Effect of Dilution on the Rate of Coagulation of Normal and Hemophilic Blood, Native and Citrated Plasma

Blood was collected into chilled tubes from a normal and hemophilic patient, without anticoagulants, with the precautions already specified, centrifuged for ten minutes at 5 C. and the plasma separated with silicone coated pipets. Appropriate dilutions of blood or plasma with 0.85 per cent NaCl were made and the rate of coagulation of each mixture measured. Dilution accelerates the coagulation of both types of blood and plasma (fig. 1), the hemophilic more pronouncedly. Undiluted native plasma takes longer to coagulate than blood, but dilution seems to have a greater accelerating effect on native plasma than on blood. The shortest clotting times of normal blood are obtained between 50 and 70 per cent concentration, while in normal plasma they are between 30 and 50 per cent. This is perhaps because, owing to its red cell content, blood diluted to 60 per cent concentration actually has about the same amount of plasma, as plasma diluted to 30 per cent concentration. Most of the clot accelerating effect of dilution is evident in both types of blood and plasma at concentrations between 70 and 100 per cent. It seems that even slight dilution of blood or plasma is sufficient to
cause a pronounced change in its rate of coagulation. It has been pointed out by some workers\textsuperscript{5,6} that the rate of coagulation of plasma seems to be shorter than that of whole blood. This is because the test is usually done at plasma concentrations of about 30 per cent (0.1 ml. plasma, 0.1 ml. 0.85 per cent NaCl, 0.1 ml. CaCl\textsubscript{2}) or 50 per cent (0.1 ml. plasma, 0.1 ml. CaCl\textsubscript{2}). As shown in figure 1 both normal and hemophilic plasmas when tested in concentrations under 70 per cent have shorter clotting times than either of their respective undiluted bloods.

Dilution of stable, citrated, normal plasma produces results similar to those with native plasma (fig. 2). In this experiment, the plasmas were centrifuged for one hour at 3000 r.p.m. and each clotting mixture was diluted with 0.85 per cent NaCl immediately before recalcification. If well centrifuged normal plasma itself is collected with a painstaking technic, and a minimum of dilution with the citrate and calcium solutions is involved, it will often not clot in concentrations of about 80 per cent or above. Citrated hemophilic plasma diluted about five times with 0.85 per cent NaCl clotted at nearly the same rate as undiluted normal plasma. Ordinarily, in order to bring the coagulation of these two plasmas to about the same rate, the hemophilic plasma must be diluted ten or twenty times. When the plasmas are unusually stable, because of good collection technic, prolonged centrifugation and low platelet content, a greater dilution of hemophilic plasma is required before its rate of coagulation reaches that of undiluted normal plasma. These findings are incompatible with the explanation that the delayed coagulability of hemophilic blood is due to an insufficiency of an accelerator ("anti-hemophilic globulin")\textsuperscript{7,8} or the precursor of an accelerator ("Thromboplastinogen"). If this were so, dilution of either blood or plasma should accentuate the defect, and delay the rate of coagulation.
CLOT ACCELERATING EFFECT OF DILUTION ON BLOOD

2. Response to Human Brain Thromboplastin and Cephalin of Plasma at Different Concentrations

Solutions of aqueous extracts of human brain in various strengths were added to 0.5 ml. of either stable, normal or hemophilic plasma, the ingredients being so adjusted that the final concentration of the plasma in any test mixture was uniform, and not below 70 per cent. A difference in response between hemophilic and normal plasma is evident even with stronger thromboplastin solutions (fig. 3). This difference becomes more accentuated as the concentration of thromboplastin is reduced, and, eventually, the hemophilic plasma remains fluid while the coagulation of normal plasma is still being accelerated. If a strong thromboplastin solution of constant strength is used while the concentration of the plasmas is reduced by dilution, the resulting curves assume the familiar parabolic

character (fig. 4). The principal effect of an excess of thromboplastin on the rate of coagulation of normal plasma is in cutting down the initial sharply declining slope of the parabolic curve, best shown in nonactivated diluted plasma (fig. 2). The minimum clotting time is still observed between 20 and 30 per cent final concentration of the plasma. Regardless of the concentration of the thromboplastin, a significant difference is evident between hemophilic and normal plasma when both are tested in mixtures of high plasma concentrations; this difference is eventually effaced as the plasmas are diluted.

The response of activated plasma to dilution depends on the original stability of the plasma. On figure 5 is shown the response to dilution of four types of plasma activated by a strong thromboplastin. In clotting mixtures of high plasma concentration, thromboplastin is least effective on plasma containing the lipid anti-thromboplastin, next on the hemophilic, and most active on the asbestos
plasma. As the plasmas are diluted, however, while the concentration of the thromboplastin in the mixtures is maintained constant, the difference between the plasmas is gradually reduced until at or below 3 per cent plasma concentration, the four plasmas clot at about the same rate. The wide differences in response to thromboplastin between these plasmas can, therefore, be eliminated by dilution. Figure 5 serves to illustrate how potent the inhibitors are when permitted to act in clotting mixtures with a high plasma concentration. Since most one-stage “prothrombin time” methods are done in plasma concentrations of about 26 per cent, discrepancies are bound to result when plasmas of differing inhibitor content are being examined. The plasmas used in these tests all had a normal content of prothrombin and Ac-globulin, yet their “prothrombin times” varied widely.

![Graph](image)

**Fig. 5.—Effect of dilution (0.85 per cent NaCl) on the response to thromboplastin of normal, hemophilic and asbestos plasmas and plasma containing lipid antithromboplastin.**

Asbestos plasma: normal plasma after exposure for one hour to loose asbestos fibers (10 mg. asbestos per ml. plasma at 20 C.). Human brain lipid antithromboplastin added to normal plasma to give a concentration of 0.05 Gm. per cent of the lipid in the plasma. No significant difference in antithrombin content between the 4 plasmas.

What was found in regard to the response of normal and hemophilic plasma to thromboplastin was generally found to be true of cephalin. Purified human brain cephalin is much less active as a clot accelerator of hemophilic than of normal plasma but dilution equalizes the response of the two plasmas to a constant amount of cephalin, when plasma concentrations between 5 and 10 per cent are reached. Incubation of hemophilic plasma with a standard cephalin suspension greatly reduces its clot accelerating action an effect which, like the action of hemophilic plasma against thromboplastin, can be reduced or offset by simple dilution of the plasma.

3. **Response of Plasma to Dilution with Fluids other than 0.85 Per Cent NaCl**

The curves obtained after dilution of normal and hemophilic plasma with buffered beef fibrinogen, 5 per cent glucose solution, acacia, buffered 0.85 per
cent NaCl (pH 7.2) imidazole buffer solution (pH 7.4) were essentially similar to those obtained with plain 0.85 per cent NaCl. The effect of dilution on the coagulability of normal plasma cannot, therefore, be attributed to some property of the saline solution employed in most of these dilution experiments. It seems, therefore, that dilution of stable normal and hemophilic plasma releases prothrombin, accelerator globulin and even platelets from the restraining effect of inhibitors, thereby allowing faster conversion of prothrombin by the existing accelerators and consequently a prompter transformation of fibrinogen to fibrin by the formed thrombin. That this occurs in spite of the fact that prothrombin, Ac-globulin, platelets and fibrinogen are themselves diluted, is an indication of the strong role played by the inhibitors in undiluted plasma, where there is an obvious excess of these procoagulants.

4. Analysis of Dilution Curves.

The curves expressing the relation of clotting time to plasma concentration have a parabolic course, whether or not the plasma is activated, and regardless of the nature of the activating agent (figs. 2, 4). Three distinct phases may be detected in these curves. In the first or descending section of the curve, the rate of coagulation is accelerated, owing chiefly perhaps to partial release of procoagulants (prothrombin, platelets, Ac-globulin) from the retarding effect of anticoagulants (anticephalin, antithrombin). A point is reached (in the blood about 40 per cent, in the plasma about 20 per cent) when the clot enhancing effect of the reduction of inhibitors by dilution is offset by a progressive diminution in prothrombin, Ac-globulin, platelets and fibrinogen. This is represented by the flat or stationary section of the curve. After this, comes the ascending portion of the curve, the lengthening clotting time resulting chiefly from too great a diminution in prothrombin and other procoagulants. If a suitably strong thromboplastin is employed to activate the plasma (fig. 4) the resulting plasma dilution curve has the same three sections but, because of the excess of coagulant introduced into the system, both the ascending and especially the descending sections are depressed. These tentative explanations receive some support from the following experiments:

A. Heparin in various concentrations was added to stable normal plasma, and the effect of dilution on the activated clotting time of these heparinized plasmas was then compared with a control. Increasing amounts of heparin extend the first phase (descending section of the dilution curve fig. 6). When diluted, however, to a 1 per cent plasma concentration, the normal and heparinized plasmas clot at approximately the same rate, regardless of their original heparin concentration. When a lipid anticoagulant extracted from human brain is added to the plasma, a similar effect is observed on the first section of the dilution curve, but at 1 per cent concentration, the plasmas all clot at approximately the same rate (fig. 7). In spite of the fact that the mode of action of these two clotting inhibitors differs (heparin is antithromboplastic as well as antithrombic, while the lipid inhibitor is antithromboplastic only) they have a similar effect on the dilution curve. This is taken to support the interpretation that the first or descending section of the dilution curve is influenced principally by the content in inhibitors of the plasma. The higher the concentration of these inhibitors in the plasma, the more resistant the latter is to the clot accelerating effect of dilution.

B. Beef plasma was obtained from freshly slaughtered animals, the blood being collected in a siliconized glass pitcher containing 19 per cent sodium citrate. Dilution curves of the
platelet-poor plasma were then worked out, using each of the following diluting agents:
(1) 0.85 per cent NaCl, (2) buffered beef fibrinogen, (3) buffered beef plasma Ac-globulin,

FIG. 6

FIG. 7

FIG. 8.—Comparison of the effect of dilution on normal plasmas and plasma containing heparin. A small volume of 1 per cent Toronto Heparin solution is added to normal plasma to obtain the concentration shown.

FIG. 7.—The effect of added thromboplastin to diluted plasmas with varying content of lipid antithromboplastin. A small volume of a 10 per cent suspension of the lipid antithromboplastin (homogenized by exposure to supersonic waves) added to the stable normal plasma to obtain the concentrations shown.

FIG. 8.—Effect of dilution of activated beef plasma with various mixtures of clotting components. All ingredients originated from beef plasma. Purified prothrombin and fibrinogen supplied by courtesy of Dr. W. H. Seegers. Ac-globulin separated from beef plasma by Owen's salt fractionation technic. The results of using buffered purified prothrombin alone are identical with those using prothrombin and Ac-globulin together as shown. Silicone technic is used throughout except in the isolation of the purified clotting reagents.

(4) buffered purified beef prothrombin and Ac-globulin together and (5) buffered purified prothrombin alone. To each of these, the proper amount of plasma was added, followed by beef brain thromboplastin and calcium. As shown on figure 8 dilution of the plasma with these purified clotting components accelerates coagulation until a plasma concentration of
about 30 per cent is reached. Further dilution prolongs the rate, except in the mixture containing purified prothrombin, an indication that the rising, third phase of the parabolic dilution curve is principally due to prothrombin diminution, as might have been suspected.

Some of the clot accelerating effect of dilution must naturally be attributed to a diminution in antithrombin. There are reasons, however, to believe that this is not the principal cause of the change: (1) in poorly collected hypercoagulable blood with normal antithrombin content, but with low anticephalin activity, dilution delays the rate of clotting. If blood is collected in uncoated glass syringes and tested in glass tubes, dilution will usually not shorten coagulation, though antithrombin activity is unchanged. (3) Exposure of stable normal or hemophilic plasmas to asbestos for about two hours will shorten their rate of clotting, without affecting antithrombin activity. Yet dilution of such plasmas often delays the rate of clotting. (4) Plasmas to which a lipid antithromboplastin is added, display an accentuated response to dilution (fig. 7) though the antithrombin activity of such plasmas is usually less than those without added antithromboplastin. (5) Dilution of stable normal plasma with 24 hour old normal serum will, like 0.85 per cent NaCl, also accelerate the rate of clotting of the plasma, though serum is rich in antithrombin.

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**5. Platelets and the Response of Normal and Hemophilic Plasma to Dilution**

Platelets were isolated by fractional centrifugation from normal and hemophilic blood and washed three times with 0.85 per cent NaCl. They were then added to normal or hemophilic recalcified plasma in silicone coated tubes, the volume of each component of the clotting mixture being so adjusted that the final plasma concentration in each tube was 75 to 80 per cent. As shown in figure 9 addition of an increasing number of platelets accelerates the rate of coagulation of normal plasma, but even more so that of hemophilic plasma. The relation between clotting time and platelet concentration is approximately linear for both types of plasma between the limits of 0.001 and 0.8 M. platelets per cu. mm. The slopes of the two lines are, however, markedly different, the addition of platelets producing a steeper fall in the clotting time of the hemophilic than of the normal plasma.

If enough platelets are added, as shown by Eagle, hemophilic plasma will
clot as rapidly as normal plasma. Between five and ten times as many platelets as normally found in the blood are required to produce this effect. Below a certain platelet concentration (on figure 9, below 500 per cu. mm. of the mixture) the hemophilic plasma will not clot if a clotting mixture of about 80 per cent plasma concentration is maintained. The results of this experiment were the same, whether washed hemophilic or normal platelets were used. The results were interpreted to indicate that platelets supply a clot accelerator, which when in sufficient concentration, is capable of offsetting the action of an excess of an antagonistic inhibitor in hemophilic plasma. If this were so, however, it should be possible to clot hemophilic plasma and normal plasma at the same rate after adding an equal number of platelets to each, if the plasmas were suitably diluted. This was tried in the next experiment which consisted in comparing the effect of three times washed normal human platelets on the rate of coagulation of diluted normal and hemophilic plasma. As shown on figure 10, progressive dilution not only accelerates their rate of clotting but also narrows the original difference in the response of the undiluted plasmas to the same number of platelets, and eventually eliminates that difference. The plasmas have to be diluted to about 1 per cent concentration before that point is reached.

Addition of distilled water platelet extracts or of suspensions of disintegrated platelets (destroyed by supersonic waves) to diluted hemophilic and normal plasma does not alter significantly the curves shown. These findings are not only incompatible with the explanations mentioned above\(^7\)\(^-\)\(^9\) but they likewise do not support the hypothesis\(^1\)\(^3\) that hemophilic plasma is lacking or deficient in a plasma factor necessary for the utilization of the clot accelerating factor in platelets. If it were so, dilution of the plasma would naturally accentuate any existing deficiency and render the plasma even less able to mobilize or utilize the platelets. Moreover, the difference between the rate of coagulation of hemophilic and normal plasma would not be reduced by simple addition of an excessive number of platelets.

Further proof that the action of platelets is modified by the prevailing concentration of stabilizing inhibitors in the plasma is supplied by the following experiment: Stable normal and hemophilic citrated blood was collected and centrifuged in the cold at 5,000 r.p.m. for two minutes. The platelet rich plasma was removed and centrifuged for five minutes; this plasma was centrifuged for 20 minutes and subsequent ones for 60, 360 minutes and 24 hours. In addition, the platelet sediment at the end of one hour centrifugation of platelet rich plasma was re-suspended in approximately one-fourth of the original plasma volume, thereby increasing the platelet concentration in that specimen four fold. The number of platelets was counted and dilution studies were done in each of the 7 specimens (fig. 11). The numbers adjacent to each curve indicate the platelet content per cu. mm. of plasma. It may be seen at once, that the number of platelets influences the coagulability of the sample, principally in the clotting mixtures of high plasma concentrations (89 per cent).

For example, while 89 per cent normal plasma with 660,000 platelets per cu. mm. clotted at about 1,000 seconds, a mixture of normal plasma of the same concentration but with only 160 platelets took longer than 20,000 seconds, and that with 3,072,000 platelets clotted in about 400 seconds. Yet when these normal
plasmas with widely varying platelet content were diluted to about 30 per cent concentration, their rates of coagulation were brought close together. The same applies in an even more striking manner to hemophilic plasma (fig. 11). As the concentration of platelets increases, a tendency is evident for the right limb of the dilution curve to lose its sharp slope until, in the mixtures with the high platelet content, the parabolic character of the curve is almost lost. It is also seen that the clot accelerating effect of dilution is more striking in platelet poor plasmas. Moreover, hemophilic plasma can be made to clot within or near the normal range, and to display a dilution curve resembling that of the normal, by increasing its platelet content (fig. 11).

The principal role of platelets as clot accelerators seems to be therefore, as agents to offset the stabilizing effect of a first phase inhibitor, acting most effectively in plasmas of high concentration. When the plasma, and consequently

![Figure 11](image-url)

Fig. 11.—Rate of coagulation of serial dilutions (0.85 per cent NaCl) of normal and hemophilic plasma of varying platelet content.

the inhibitor, are diluted, the need for platelets seems to be lessened. It is also unlikely that the clot accelerating effect of dilution is mediated through a disrupting effect of the diluting fluid on the platelets, since normal and hemophilic plasmas, regardless of their platelet content, diluted to concentrations of about 10 per cent, clot much faster than when undiluted (fig. 11).

It has been pointed out that normal plasma centrifuged for five minutes at not over 1,000 r.p.m. clots at about the same rate as that centrifuged at 3,000 r.p.m. for five minutes. The tests were done, however, in glass tubes and at plasma concentration of less than 30 per cent. When silicone surfaces and mixtures of high plasma concentration are used, the speed and length of centrifugation markedly affect the clotting time of normal plasma and, even more so, of hemophilic plasma. Elsewhere, evidence shall be presented that prolonged high speed centrifugation of plasma renders its lower layers hypercoagulable, while its
topmost layers may be incoagulable. This inequality is due to displacement of lighter lipid inhibitor molecules upward as much as to packing of platelets in the lower sections of the plasma.

6. The Contacting Surface and the Response of Normal and Hemophilic Plasma to Dilution

If glass particles (chemically clean and washed ground glass, screened so that each particle passed through a No. 40 B. of S. sieve) are added to normal or hemophilic plasma, the rate of coagulation of both plasmas is accelerated. The curves (fig. 12) resemble those of normal and hemophilic plasma to which a thromboplastic agent has been added. If, however, the concentration of both plasmas is reduced by dilution while the quantity of glass particles is maintained constant, the rate of coagulation of the two plasmas gradually shortens, until it becomes the same, when a plasma concentration of about 2 per cent is reached (fig. 13).

Another example of the influence of contact with certain surfaces on the stability of plasma and its response to dilution is illustrated in figure 14. Stable normal and hemophilic plasmas were exposed to loose asbestos fibers (10 mg. asbestos per 1 ml. of plasma in a silicone coated tube, without agitation, at 20 C.) for varying periods of time (0 to 210 minutes). After the stipulated interval, the fibers were packed down at the bottom of the tube, the plasma removed and its response to dilution tested at once. Contact with the fibers (like the presence of platelets and thromboplastin) modified principally the rate of clotting of the samples tested at a high plasma concentration. Increasing exposure to asbestos depressed the first (descending) phase of the parabolic dilution curve. While stable normal and hemophilic plasma, not exposed to asbestos, tested in high plasma concentration (89 per cent), clotted in a longer time than when diluted.
ten fold, after the plasmas had been in contact with asbestos for 120 minutes, they had a longer rate of coagulation, when diluted to that extent. The concentrations of prothrombin and Ac-globulin in each of the samples of exposed plasma as measured by the two-stage method, were not altered by exposure to asbestos. Hemophilic plasma, which when tested in a mixture above 50 per cent plasma concentration did not clot before exposure to asbestos, clotted after 60 minutes of contact. Two hundred and ten minutes of contact were required for hemophilic plasma to reach the rate of coagulability of normal plasma exposed for only 120 minutes (fig. 14). As after addition of platelets, cephalin and thromboplastin, the rate of coagulation of normal and hemophilic plasma may also be equalized by appropriate exposure to a suitable contacting surface.

Fig. 14.—Effect of contact with asbestos fibers on the response of normal and hemophilic plasma to dilution. After exposure to asbestos for 210 minutes, the response of hemophilic plasma to dilution is like that of normal plasma exposed for 120 minutes.

7. Normal and Hemophilic Plasma Euglobulin Fractions

Ever since the work of Frank and Hartmann it has been known that the normal plasma euglobulin has a clot accelerating effect on hemophilic blood in vivo and ex vivo. Such fractions, separated by dilution and acidification of normal plasma, have been shown to exert a similar effect, while those derived from hemophilic plasma are much less effective. This seemed to support the explanation which has gained wide acceptance, that the delay in the inception of coagulation of hemophilic blood is due to a deficiency of a plasma factor variously termed “plasma thromboplastin,” “anti-hemophilic globulin,” “thromboplastinogen.” On table 2 are shown the results of addition of euglobulin solutions, separated from normal and hemophilic plasmas by the dilution and acidification method. At 1.0 Gm. per cent concentration, it is obvious that the normal euglobulin has a clear clot accelerating effect on normal plasma and an analogous, although slightly less pronounced effect, on hemophilic plasma. The hemophilic
euglobulin, on the other hand, though having a clear clot accelerating effect on normal plasma, has no significant accelerator action on hemophilic plasma.

Putting aside, for the moment, the little stressed finding that the hemophilic euglobulin itself accelerates the clotting of normal plasma, let us examine the effect of the two euglobulins, when tested at lower concentrations. As shown in table 2, when a 0.005 Gm. per cent concentration of euglobulin in the plasma is used, the previously inactive hemophilic euglobulin becomes as active an accelerator of the clotting of hemophilic plasma as the normal euglobulin. This led us to examine further the action of various concentrations of the two euglobulins on the two plasmas, and of fixed amounts of the two euglobulins on variable concentrations of the two plasmas. The citrated plasmas from which the euglobulins were derived were collected from normal and hemophilic subjects, using the silicone technic throughout, and stored in silicone coated tubes up to the point when they were diluted. From that point on, they were processed in uncoated glassware. The plasmas were diluted 1 to 10 with cold distilled water, 1 per cent acetic acid was added to bring the pH of the mixture to 5.9 and the resulting precipitate was separated by centrifugation, dried and weighed. A fairly uniform gravimetric yield was obtained from 100 ml. of normal and hemophilic plasmas (mean of 6 experiments, normal: 450 mg., hemophilic: 452). The residue was taken up in 0.85 per cent NaCl, the pH adjusted to 7.4 and the tests were carried out immediately after making the solutions. Silicone tubes and pipets were used throughout in preparing and testing the clotting mixtures. Recalcification was done with amounts of calcium designed to bring about optimal clotting times. In contrast with Fraction I (Cohn) the euglobulin precipitated by plasma dilution and acidification does not seem to contain citrate. When the euglobulin solution is added to plasma, the calcium required for optimal clotting time of the mixture is not altered by the presence of the euglobulin solution in concentrations of one per cent or below. On figure 15 are shown the results of these experiments.

On normal plasma, the hemophilic euglobulin is as effective a clot accelerator as the normal euglobulin, at concentration of 0.05 per cent or below (fig. 15, No. 1 and 3). The slight difference between the action of the two euglobulins on normal plasma appear at concentrations of 0.1 or above, when the hemophilic

<table>
<thead>
<tr>
<th>Type of Euglobulin</th>
<th>Gm. per cent in clotting mixture</th>
<th>Clotting Time (Secs.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.0</td>
<td>195</td>
</tr>
<tr>
<td>Normal</td>
<td>0.005</td>
<td>1164</td>
</tr>
<tr>
<td>Hemophilic</td>
<td>1.0</td>
<td>264</td>
</tr>
<tr>
<td>Hemophilic</td>
<td>0.005</td>
<td>1190</td>
</tr>
<tr>
<td>0.85% NaCl Control</td>
<td></td>
<td>1,374</td>
</tr>
</tbody>
</table>

Contents of each clotting mixture: 0.5 ml. plasma, 0.1 ml. euglobulin solution (or 0.85% NaCl), 0.05 ml. 0.2 M CaCl₂. silicone technic, 38 C.
euglobulin (and to a lesser extent the normal euglobulin) begin to behave in an apparently anomalous manner, i.e., greater concentrations of the euglobulin either do not change or actually prolong coagulation. This anomalous behavior is especially evident when the hemophilic euglobulin is added to hemophilic plasma (fig. 15, No. 1). At high concentrations of the euglobulin (above 0.1 per cent) the hemophilic euglobulin has no clot accelerating effect on hemophilic plasma, though it still has a significant effect on normal plasma. As the concentration of the hemophilic euglobulin is reduced a clot accelerating effect on hemophilic plasma itself appears, until concentrations are reached (below 0.01 per cent) where the hemophilic euglobulin is as effective as the normal euglobulin in accelerating the clotting of hemophilic plasma (fig. 15, No. 1 and 3).

**Fig. 15.—Response of normal and hemophilic plasmas to globulin fractions from normal and hemophilic plasma. Content of clotting mixtures for 1 and 3: 0.5 ml. plasma, 0.1 ml. euglobin solution (various concentrations), 0.05 ml. 0.2 M. CaCl₂. Actual plasma concentration of final clotting mixture 74 per cent. Content of clotting mixtures for 2 and 4: 0.5 ml. plasma (various dilutions), 0.1 ml. of 0.06 per cent euglobulin solution, 0.05 ml. CaCl₂ (various molar concentrations). Silicone tubes, 38° C.**

It is evident from these results that the euglobulins differ from each other not so much because of their content of coagulant material ("plasma thromboplastin"), but because of accompanying anticoagulants. Moreover, a deficiency in a clot accelerator in the hemophilic plasma does not seem to be the cause for its slower response to the normal or hemophilic euglobulin. If either hemophilic or normal euglobulin in a fixed concentration (0.06 per cent) is added to plasma of various concentrations, the two plasmas eventually respond alike to both euglobulins (fig. 15, Nos. 2 and 4). Though at high plasma concentrations (75 per cent in the charts) significant differences in the response of the two plasmas to the two euglobulins are observed, the responses become the same at a plasma concentration of 13 per cent for normal (fig. 15, No. 4), and of 6 per cent for
hemophilic euglobulin (fig. 15, No. 2). Incidentally, at 6 per cent plasma concentration and below, the hemophilic and normal euglobulin have the same activity, whether added to hemophilic or normal plasma (fig. 15, Nos. 2 and 4).

These and other points to be elaborated further in a study published elsewhere, seem to indicate that the differences in behavior between hemophilic and normal euglobulin may be traced not to a deficiency in a clot accelerating factor, but rather to the presence of inhibiting agents in greater concentration in the hemophilic euglobulin fraction and in the hemophilic plasma used as a substrate for the tests. Erroneous impressions are obtained by (1) testing the euglobulin fractions at a single fixed concentration, (2) using clotting mixtures of low plasma concentrations, (3) employing as a substrate hypercoagulable plasma in contact with glass surfaces.

**Discussion**

It is evident from the foregoing that the rate of coagulation of blood and plasma can be markedly shortened by simple dilution. The demonstration of the effect of dilution requires stable blood or plasma, obtained with a painstaking technic. Anything that contributes to disturb this stability—such as poor collection of blood, its exposure to glass, inadvertent dilution of the blood with anticoagulant solutions (citrate, oxalate), and of the plasma by complex buffer and recalcifying mixtures, may reduce or nullify the clot accelerating effect of dilution. Stable human plasma in silicone tubes seems to behave like the normal plasma of certain marine fish, which remains fluid indefinitely when in contact with glass but clots rapidly after addition of a suspension of porphyrized glass or by simple dilution with sea water.24-25

There are points of analogy between the effect of dilution on the coagulability of blood and on the peptic activity of gastric juice. Generally speaking, the higher the pepsin content of gastric juice, the more it must be diluted before it ceases to gain activity on further dilution.26 A 1 to 100 dilution is usually required to reach this point. Bucher et al.26 suggest that pepsin in gastric juice is present in equilibrium with an inhibitor (or inhibitors) forming a complex which dissociates upon dilution. Northrop27 has shown that impure pepsin is bound by inhibitors and that after dilution, it is dissociated into active pepsin. In order to determine the amount of pepsin present in solution, Northrop found it necessary to use such a dilution that the rate of digestion is directly proportional to the amount of enzyme solution taken. Likewise, it appears that in order that the rate of coagulation be proportional to the amount of prothrombin it is important to use a dilution at which inhibitors are no longer operative. This varies, of course, depending on whether or not thromboplastin is used to accelerate prothrombin transformation. It is significant that, if the plasmas are sufficiently diluted, the effect of the first phase inhibitor (anticephalin) is overcome, and the rates of clotting of the activated plasmas are the same, regardless of how much they might have differed when undiluted (figs. 5, 10). Considerations such as these probably forced those28 engaged in the measurement of prothrombin by the one-stage method, to use dilutions of plasma which yielded, after activation with a strong thromboplastin, a clotting time which varied inversely as the amount of prothrombin. Dilution as originally used in the two-stage prothrombin titration
method\textsuperscript{17} was, on the other hand, intended chiefly perhaps to overcome the effect of antithrombin, which, when in high concentration, destroyed thrombin before its activity could be measured; dilution must also, however, accelerate prothrombin conversion by sparing thromboplastin and Ac-globulin from the action of first phase inhibitors (antithromboplastin).

We have now reached the time when a tentative hypothesis may be presented to guide us in the understanding of the evidence presented in this paper and in developing future work. Normal circulating blood and plasma in contact with inert surfaces maintain their fluidity and coagulability by an equilibrium between clot inhibiting and clot promoting forces.\textsuperscript{29} Under normal conditions in the circulating blood, clot inhibiting forces are dominant, though procoagulant substances are present in excess, to be sure, of the amount required to bring about rapid clotting. The mode of action of these clot inhibitors or stabilizing substances may be mediated through either or both of the following ways: (1) the inhibitors may be bound to procoagulants, thereby preventing their transformation into coagulants, or virtually holding them in their precursor inactive form (antiprothrombin, antiaaccelerator globulin action). The clot accelerating effect of dilution on stable plasmas favors this explanation. (2) The inhibitors may reduce or inactivate the effect of released coagulants (antithromboplastin, antithrombin action). The reduced activity of cephalin, thromboplastin and thrombin after incubation with stable plasma or serum makes this explanation also plausible.

Clotting may then be initiated by release of the procoagulant into its active coagulant form, a change which may result from exposure of the blood or plasma to any force which will dissociate what is probably a loose conjugation between inhibitor and procoagulant. The present remarks are directed principally to the role of inhibitors in delaying or preventing the \textit{inception} of clotting, in other words, first phase inhibitors. Contact with certain surfaces, dilution of the blood, exposure to the by-products of blood and tissue cell disintegration, are some of the disturbing forces which may determine or help to determine these changes.

These findings and hypothesis reach some measure of reconciliation with Bordet’s theories and more recent developments. Bordet\textsuperscript{39} distinguished two types of prothrombin: one inactive, present in the plasma (proserozyme) slowly converted to thrombin by cytozyme; and an active prothrombin (serozyme) found in the serum and rapidly transformed to thrombin by cytozyme. Bordet considered that proserozyme may represent prothrombin combined to or protected by an inhibitor. He also showed that certain activating substances (substances “excitoproductrices”) released from the plasma during clotting were found in the serum and helped to convert prothrombin to thrombin. Expressing Bordet’s findings in the light of later developments and terminology, the inactive plasma prothrombin of Bordet may be looked upon as prothrombin in the presence of plasma Ac-globulin (factor V). His active prothrombin (serozyme) is prothrombin in the presence of serum Ac-globulin (factor VI). It seems not unlikely therefore, that the inhibitor which Bordet felt was responsible for the slow reactivity of proserozyme may be actually conjugated with plasma Ac-globulin and that contact or dilution dissociates the inhibitor from the Ac-globulin releasing the latter in the form of serum Ac-globulin.
What then of hemophilic plasma? It has been known for some time that this plasma has a normal prothrombin content but, as shown by several workers, its prothrombin is unusually slowly transformed into thrombin. Coagulation is delayed unless the plasma is diluted or brought into contact with asbestos or similar surfaces, or is acted upon by an excessive number of platelets, thromboplastin, cephalin or dissociated normal euglobulin (“antihemophilic globulin”). If the hemophilic plasma is diluted as well as acted upon by a strong thromboplastin and tested in glass tubes (as in the Quick one-stage “prothrombin time” method) little or no difference between the rate of prothrombin conversion of hemophilic and normal plasma will be observed. Likewise, if hemophilic and normal plasma are diluted 1 to 200 or more (as in the Smith two-stage prothrombin method) no differences in the rate of conversion between hemophilic and normal plasma will be observed, even when cephalin is employed to accelerate prothrombin conversion. Clearly, the cumulative effects of dilution, contact with glass and thromboplastin in excess, all tend to put the first phase anticephalin inhibitor at a disadvantage, and to hasten its dissociation, perhaps from the plasma Ac-globulin, and conversion of the latter into active serum Ac-globulin. That hemophilic plasma has a normal content of plasma Ac-globulin has been amply demonstrated. Yet “antihemophilic globulin” if prepared from a sufficiently diluted (1 to 200 or 500) hemophilic plasma, will have as strong a clot accelerating effect on normal and hemophilic plasma as when the euglobulin originates from 1 to 10 or 20 diluted normal plasma. Therefore, the differences between the clot accelerating effect of hemophilic and normal euglobulin may actually be the expression of a qualitative change in the Ac-globulin complex. Thus the hemophilic Ac-globulin may be conjugated with a greater amount of the inhibitor and therefore require greater dilutions and more prolonged contact with glass and similar surfaces to acquire a degree of clot accelerating potency comparable with the Ac-globulin obtained from normal plasma. This would make the conversion of plasma Ac-globulin (factor V) to serum Ac-globulin (factor VI) in nonactivated undiluted hemophilic blood or plasma unusually slow, thereby accounting for (1) the slow conversion of hemophilic prothrombin to thrombin, (2) the relatively poor content of serum Ac-globulin activity in fresh hemophilic serum. 

Summing up then, dilution (like contact or excessive thromboplastin) disrupts the stability of blood or plasma by dissociating an accelerator/inhibitor complex (Ac-globulin/anticephalin?). Release of the accelerator results in the rapid conversion of prothrombin to thrombin. In hemophilia, this conversion is slowed by the presence of an excess of inhibitor. Greater dilutions, more thromboplastin (or platelets) or a longer contact with certain surfaces are therefore required to free the accelerator from the inhibitor. Once this is achieved, the hemophilic accelerator is as active as that obtained from normal plasma. It becomes clear then, why the plasma Ac-globulin in hemophilic plasma has been found to be normal. The tests are carried out in either highly diluted plasmas in glass tubes or after addition of excess of thromboplastin; both procedures obviously offset the action of inhibitors and equalize the performance of the hemophilic and normal Ac-globulin, since, in the dissociated form, they are quantitatively and qualitatively alike.
CLOT ACCELERATING EFFECT OF DILUTION ON BLOOD

SUMMARY

Dilution with physiologic saline solution and other fluids accelerates the coagulation of properly collected normal and hemophilic blood and plasma in silicone coated vessels, with or without the aid of activating agents such as platelets, thromboplastin, cephalin, glass particles or plasma euglobulin fractions. When normal plasma is diluted under a concentration of about 20 per cent, its rate of clotting is prolonged, principally because of diminution in prothrombin.

Regardless of the activating agent used, the rate of coagulation of hemophilic plasma can be made equal to that of normal plasma by appropriate dilution. These findings speak against the existence in hemophilic blood or plasma of a deficiency in any procoagulant factor, and support the concept of the presence in excess of a stabilizing inhibitor which slows the conversion of prothrombin to thrombin by one or both of the following mechanisms: (1) reducing or inactivating the effect of released coagulants (antithromboplastin activity) (2) conjugation with a procoagulant thereby maintaining it in an inactive form (anti Ac-globulin activity).

REFERENCES


The Clot Accelerating Effect of Dilution on Blood and Plasma. Relation to the Mechanism of Coagulation of Normal and Hemophilic Blood

L. M. TOCANTINS, R. T. CARROLL and R. H. HOLBURN