IT HAS long been generally accepted that in the first stage of blood coagulation, prothrombin is converted into the active enzyme thrombin by the action of thromboplastin and ionized calcium. Recently, however, other agents influencing this reaction have been discussed, and there is a considerable volume of literature, all of which, more or less, points to the same general conclusion.\textsuperscript{1-22} Such factors have been derived from plasma, serum and platelets.\textsuperscript{19} In this laboratory the terms plasma Ac-globulin, serum Ac-globulin and platelet accelerator have been used. The properties of each show some characteristic differences from the others, but all accelerate the conversion of prothrombin to thrombin. Thus, thromboplastin requires the help of these other factors to bring about thrombin formation at the rate observed under physiologic conditions. Honorato\textsuperscript{10} has suggested, logically enough, that the action of the plasma accelerator can best be described as that of a co-factor of thromboplastin.

Plasma Ac-globulin is probably not an active material as it occurs in the blood stream. Ware and Seegers\textsuperscript{17} have shown that small amounts of thrombin will make this protein more active. The mechanism of thrombin action presumably is through activation of inactive Ac-globulin. When it is in the active form, it is identical with serum Ac-globulin. The latter has been prepared as a concentrate from bovine serum.\textsuperscript{17} Owren\textsuperscript{5-9, 11} discovered that the deficiency of factor V is responsible for a hemorrhagic diathesis. Without knowledge of Owren's work, due to conditions brought about by war, the same factor was again discovered, and called Ac-globulin because it acts as an accelerator in the activation of purified prothrombin.\textsuperscript{12}

Platelet extracts will potentiate the action of thromboplastin in prothrombin conversion. Thus, they also furnish thromboplastin co-factor. Recent studies have not confirmed the old view that platelets contain thromboplastin itself.\textsuperscript{18, 23, 24} It seems likely that at least a part of the hemostatic function of platelets is to supply thromboplastin co-factor.

Present evidence indicates that the platelet accelerator is present in the active form, and that it is an integral part of the platelets. Its activity in clotting experiments is identical with that of serum Ac-globulin, but some of the chemical
properties of the two differ. For example, the platelet material can be sedimented readily in a powerful centrifugal field, while the serum Ac-globulin cannot.

An interesting problem has arisen concerning the mechanism of the action of the substances responsible for acceleration of thrombin formation. The literature contains reports of experiments interpreted to show stoichiometric reactions of thromboplastin and calcium with prothrombin. Similar experiments can be arranged for the thromboplastin co-factors. There are several facts which do not permit the interpretation that these are stoichiometric reactions. For example, if the pH of a mixture of prothrombin, Ac-globulin, thromboplastin, and ionized calcium is varied, it appears as though the hydrogen ion also enters the reaction stoichiometrically. This is a highly unlikely circumstance. Also, thromboplastin is quantitatively recoverable after it has reacted with prothrombin to form thrombin. This would not be possible if some of it had become a part of the thrombin molecule or of some other reaction product. A crucial experiment is provided by the activation of prothrombin in a solution of sodium citrate. Thrombin is formed slowly by the process of autocatalysis under such conditions. This fact makes it unlikely that the activators of prothrombin contribute any material substance in the activation process.

The slow autocatalytic activation of prothrombin contrasts markedly with the rapid physiologic rate of thrombin formation. This emphasizes the fact that to understand how thrombin is formed physiologically we must study the action of these accelerating substances. An essential requirement in such a study is to determine which has thromboplastin activity in the traditional sense, and which has thromboplastin co-factor activity. With such a method available it can also be determined whether and to what extent one activator can be substituted for another. If such substitution can be made, a buffer mechanism is provided for assuring adequate thrombin formation. Thus, if there is a deficiency of thromboplastin co-factor, the possibility exists that it may be largely compensated for by thromboplastin, and vice versa.

In undertaking such a study, the experimental data must meet fairly exacting requirements. Points on the thrombin activation curves must be determined with the greatest precision possible. This requires that each of the experiments must be repeated enough times so that the best possible data are available on which to base an interpretation of the role of each of the activating substances.

In this paper further evidence is presented to show that thromboplastin is a catalyst of prothrombin activation just as is serum Ac-globulin. It is also shown that the substitution of Ac-globulin for thromboplastin, demonstrated by Owren, can be carried out on a quantitative basis. The conditions under which this substitution is possible permit the concept of compensation. The quantitative determination of thromboplastin or of thromboplastin co-factors must take these fundamentals into account. The similarity in reaction rates using serum Ac-globulin and platelet accelerator indicates that the accelerator material from platelets is present in an active form. Plasma Ac-globulin, in contrast, is probably inactive and is made more active by thrombin.
Materials

Prothrombin. Two sources of prothrombin were used. One was purified prothrombin prepared by the method of Ware and Seegers. It was free of Ac globulin. The other was prothrombin obtained by diluting, with saline, beef plasma which had been freshly defibrinated by addition of thrombin.

Standard Prothrombin Solution. A standard solution of prothrombin was prepared which contained 6.7 units per cc of prothrombin. This solution, when diluted by mixing with the other reagents and addition to fibrinogen, gave a final concentration of 1.34 units per cc. This amount of prothrombin, when fully converted to thrombin, gives 12 second clotting with a standardized fibrinogen solution.

Plasma Ac-globulin and Serum Ac-globulin. In most of the studies, purified materials prepared by methods previously described were used.

Platelet Accelerator. Platelet extracts were prepared in the manner described by Ware, Fahey and Seegers. Briefly, the method consists of separating the platelets from the other formed elements of the blood by multiple differential centrifugations. Finally, the platelets are washed with saline and packed by centrifugation. The packed platelets are then triturated with a small amount of saline, the extract so obtained from 0.2 cc of packed platelets is made up to 5 cc, and the macerated platelet fragments are removed by light centrifugation and discarded.

Thromboplastin. Crude thromboplastin prepared from bovine lung was centrifuged thirty minutes in a Sharples Super Centrifuge. The precipitate was resuspended in saline solution to the original volume, resedimented, resuspended in a small amount of saline and centrifuged at 2,000 rpm to remove gross particles. The resulting suspension was diluted ten times with saline for use in the analysis.

Acacia. Commercial preparations of acacia were purified by first precipitating calcium with oxalate and then precipitating the acacia with ethyl alcohol, followed by dialysis against distilled water to remove other inorganic ions. This purified material was then used as a 1 per cent solution in saline.

Calcium Chloride. A 1 per cent solution of anhydrous calcium chloride in saline was used.

Imidazole Buffer. This was prepared by dissolving 8.75 grams of imidazole in 90 cc of 0.1 N hydrochloric acid. The pH was adjusted to 7.25 by the addition of concentrated hydrochloric acid or sodium hydroxide and the mixture was then diluted to 100 cc volume with water.

Fibrinogen. The method of preparation described by Ware, Guest and Seegers was used. Only products in which at least 95 per cent of the total protein could be coagulated by thrombin were considered suitable. A 1 per cent solution of this fibrinogen was used.

Experimental

In all studies, the two-stage system of analysis was used. This approach offers opportunity to estimate the rate of thrombin formation and also indicates the quantity obtained. It has become apparent that the rate and amount of thrombin formed from a standard amount of prothrombin depend upon the amounts of each of two types of activators in the reaction mixture. One of these types of substances is represented by thromboplastin, the other by any one of the co-factors of thromboplastin. Thus, if one of the type substances is present in large quantity, prothrombin activation is slow, and the other will increase the rate. This increase in rate may be made a quantitative measure of the limiting factor.

Example of Multiple Two-Stage System of Analysis. The way in which an analysis for thromboplastin or its co-factors can be done is first described by an example. The reagents were mixed in the following proportions:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Proportions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard prothrombin solution</td>
<td>3 parts</td>
</tr>
<tr>
<td>Thromboplastin suspension</td>
<td>3 parts</td>
</tr>
<tr>
<td>Ac-globulin or platelet accelerator</td>
<td>2 parts</td>
</tr>
<tr>
<td>Calcium chloride solution</td>
<td>1 part</td>
</tr>
<tr>
<td>Acacia</td>
<td>2 parts</td>
</tr>
<tr>
<td>Imidazole buffer</td>
<td>1 part</td>
</tr>
<tr>
<td>Total</td>
<td>12 parts</td>
</tr>
</tbody>
</table>

This reaction mixture is placed in a constant temperature water bath at 28 C. At various time intervals,
Fig. 1.—Reaction mixtures consisting of standardized prothrombin solution, calcium, thromboplastin, accelerator, acacia solution, and imidazole buffer were allowed to stand at 28 C. At various times, indicated on the horizontal axis, 0.4 cc. of the reaction mixture was mixed with 0.1 cc. fibrinogen solution. The clotting time was noted and is represented on the vertical axis of the charts. The variables, which were studied one at a time, were placed in the reaction mixture in many different concentrations. These variables were serum Ac-globulin (upper right), plasma Ac-globulin (middle left), platelet accelerator (middle right), thromboplastin (lower left), and various combinations of limited amounts of thromboplastin and serum Ac-globulin.

0.4 cc. of the reaction mixture are added to 0.1 cc. of fibrinogen and the clotting time is determined. In this way it is possible to know simultaneously how much thrombin is formed and how rapidly it is being formed. The data can be plotted as shown by the curve in the upper left corner of figure 1.
In the example, after five minutes' incubation, the reaction mixture contained enough thrombin to clot fibrinogen in 32 seconds, thus giving point A on the chart. At points B, C, and D the incubation times were 6, 9, and 14 minutes and the clotting times were 24, 18, and 13 seconds respectively. At points E, F, G, and H, all the prothrombin had been converted, because 12 second clotting was obtained in each instance. The activation curve is only one of many which can be obtained by varying either the thromboplastin or accelerator concentration in the reaction mixture.

**EXPERIMENTAL RESULTS**

*Variations of Serum Ac-globulin Concentration.* The chart in the upper right hand corner of figure 1 was obtained by varying the serum Ac-globulin concentration in the reaction mixture. Each curve represents the effect of a different concentration of serum Ac-globulin. Curve 1 was obtained when 1/1000 of a unit of serum Ac-globulin was present in each cc. of the reaction mixture. The other curves of the figure correspond to successively higher concentrations of Ac-globulin. Thus, curve 800 was obtained with 8/10 units per cc. of serum Ac-globulin in the final reaction mixture. By increasing the concentration of serum Ac-globulin, the rate of thrombin formation is always increased. On the other hand, the yield of thrombin increased until about 6/100 of a unit of Ac-globulin was in each cc. of the reaction mixture. Greater concentrations did not increase the yield obtained from the standard prothrombin substrate.

*Variations of Plasma Ac-globulin Concentration.* The curves in the middle of the left hand column of figure 1 show the results when various concentrations of plasma Ac-globulin were used as co-factor of thromboplastin. Curve 1 represents the result when 1/1000 of a unit of plasma Ac-globulin was in the final reaction mixture. As with serum Ac-globulin an increase in plasma Ac-globulin concentration always increased the rate of prothrombin activation. The final yield of thrombin was constant, when 64/1000 or more units per cc. of accelerator were in the reaction mixture.

The more rectangular shape of the plasma Ac-globulin curves as compared with the serum Ac-globulin curve is of significance. It shows that the kinetics of activation for the two accelerators is not exactly the same. It is believed that the difference is due to the fact that plasma Ac-globulin exists in an inactive, or at least less active form, and must first be activated. This activation occurs in the incubation mixture and is probably due to thrombin.

*Variations of Platelet Accelerator Concentration.* The two curves in the middle of the right hand column of figure 1 were obtained when platelet extracts served as the only co-factor of thromboplastin. There are only two curves because the extracts were not stable enough for extensive precise work and because they also contain platelet factor 2 which interferes when high concentrations of the extract are employed. The extract contains very little thromboplastin.

The two curves are identical in shape with curves obtained when serum Ac-globulin is the source of accelerator. This indicates that this accelerator functions like the serum accelerator. The set of curves is incomplete, but there is suggestive evidence that an increase in platelet accelerator concentration always increases the rate of prothrombin activation, and the yield of thrombin probably increases only up to a certain point. Curve 2 and curve 8, both obtained with the platelet ac-
accelerator, superimpose exactly on curve 2 and curve 8 (serum Ac-globulin series), obtained by using the serum Ac-globulin accelerator. This exact correspondence makes it possible to measure the activity of either one in the same way; i.e., the same standard curves can be used.

Variation of Thromboplastin Concentration. In the experiments described thus far, thromboplastin was always in the incubation mixture in large quantity and the concentration of accelerator activity was varied. To reverse this procedure, serum Ac-globulin was chosen as the substance to have in high concentration, and lung extract thromboplastin was the variable.

By increasing the concentration of thromboplastin the rate of prothrombin activation was always increased (fig. 1, lower left hand chart). (With higher concentrations than shown on the chart inhibitory effects could be demonstrated.) Thrombin yield reached a maximum. The basic relationships which are found with varying the thromboplastin concentration are the same as those found when serum Ac-globulin concentration is varied. The Curves 2, 5, 8, 16, 80 and 800 represent quantitative concentrations of thromboplastin; they may also represent serum Ac-globulin concentration, for they can be superimposed exactly on the respective curves obtained when serum Ac-globulin concentration was varied and thromboplastin concentration was high. In other words, the curves of the lower left corner and upper right corner of figure 1 are identical in all respects, yet one set was obtained with high thromboplastin concentration plus progressively greater amounts of serum Ac-globulin and the other with a high concentration of serum Ac-globulin plus progressively greater amounts of thromboplastin.

Limited Concentrations of Both Ac-globulin and Thromboplastin. Because the curves obtained with thromboplastin as variable are equivalent to the curves obtained with serum Ac-globulin as variable it was suspected that one activator can substitute quantitatively for the other. To test this hypothesis a number of experiments were performed. They all confirmed the postulate. The curves shown in the lower right hand corner of figure 1 show a typical example of quantitative substitution of serum Ac-globulin for thromboplastin.

For the base curve B, the usual standard prothrombin solution was activated with concentrations of both serum Ac-globulin and thromboplastin known to be far less than the optimum amount required for complete conversion of the prothrombin. Curve C was obtained by using the same amount of serum Ac-globulin as for obtaining curve B, but the thromboplastin concentration of the base curve B was doubled. The thrombin yield and its rate of formation was increased. By doing the converse, i.e., keeping thromboplastin at the level of curve B and doubling the serum Ac-globulin concentration, curve C was also obtained. In other words, curve C can be obtained by doubling either the thromboplastin or Ac-globulin concentration. Finally, with the concentration of thromboplastin and serum Ac-globulin doubled simultaneously, a third curve, D, was obtained. It shows that the rate of thrombin formation and the quantity obtained is in excess of that which results from simply increasing the concentration of only one of the activators.

The three curves, B, C, and D, fit the contours of the curves obtained with
plasma Ac-globulin (fig. 1, middle left column). Regarding the quantitative relations, it is a remarkable fact that doubling the concentration of either accelerator, i.e., serum Ac-globulin or thromboplastin, to obtain curve C, was equivalent to doubling the concentration of plasma Ac-globulin in that series. Thus curve B corresponds to curve 6 (plasma Ac-globulin series), curve C corresponds to curve 12, and curve D corresponds to curve 24. Limited amounts of thromboplastin acting in conjunction with limited amounts of serum Ac-globulin are thus seen to activate prothrombin in a manner which is equivalent to the activation of prothrombin with large amounts of thromboplastin and limited quantities of plasma Ac-globulin.

DISCUSSION

It has been shown that purified prothrombin can be activated simply by dissolving the protein in a 25 per cent solution of sodium citrate. Under those conditions activation is by autocatalysis, thus indicating that prothrombin itself can contribute all the essential material required to have thrombin. Nothing needs to be supplied by the physiologic activators such as calcium, thromboplastin or accelerators. These are concerned with the rapid activation of prothrombin; for it requires many hours to activate prothrombin without them and such long intervals are incompatible with the ordinary requirements of effective hemostasis in the living organism.

The physiologic activators of prothrombin can be divided into two main classes. One is represented by the traditional thromboplastin and the other by the co-factors of thromboplastin or accelerators. Either class of activators alone does not activate purified prothrombin rapidly; the two must act together to accomplish this end. The accelerator groups of activators are represented by plasma Ac-globulin, serum Ac-globulin, and platelet accelerator.

Our work shows that the rate of thrombin formation may be increased and the quantity of thrombin obtained, within limits, may also be increased by adding one of the activators when the other is present in large quantity.

Any of the accelerator group of activators can substitute for thromboplastin provided a limited quantity of thromboplastin is already present. This concept confirms and extends the conclusion reached earlier by Owen, and demonstrates that buffer-like arrangements exist in the physiologic interactions of protein. A large amount of thromboplastin can compensate for a partial deficiency of Ac-globulin, and likewise Ac-globulin can compensate for a partial deficiency of thromboplastin. Our work was with in vitro systems but we see no reason why the conclusions cannot be applied directly to physiologic conditions.

The relationships which are represented in figure 1 can be applied to distinguish between thromboplastin and accelerator activity. Furthermore, each one can be measured quantitatively in exactly the same manner employed to produce the various families of curves in figure 1. In fact, the arbitrary numbers on each of the curves indicate relative concentration of the respective activator. For example, the numbers 2, 5, 8, 16, 80, and 800 (curves at lower left of figure 1) represent 2, 5, 8, 16, 80 and 800/1000 of a unit of thromboplastin per cc. of reaction mixture.
when thromboplastin is the variable or unknown being measured and accelerator is present in large quantity. This system of analysis makes it possible to know how much thrombin is formed at any given time and also how rapidly it is being formed, thus furnishing an opportunity to express thromboplastin or accelerator activity in quantitative terms. By this means one obtains considerably greater insight into the blood clotting mechanism than was possible previously. Heretofore it has been feasible to measure two activities quantitatively: (1) Thrombin, by rate of fibrinogen clotting, (2) prothrombin, by allowing complete activation and then measuring thrombin concentration. Now another activity can be measured; namely, that of the activators. This is accomplished by following the rate with which a standard prothrombin solution is converted.

The literature contains some isolated observations about platelets that can be correlated. Some of these are: (1) Platelets contain an accelerator of prothrombin activation. By using the silicone technic of Jaques et al. "platelet free" and spontaneously incoagulable dog plasma was obtained without the use of anticoagulants. (3) Platelets do not contain significant amounts of thromboplastin. (4) Unless platelets are present normal stable plasma is without effect on the clotting of hemophilia plasma. (5) In the presence of calcium, washed rabbit platelets plus a small amount of bovine globulin activated bovine prothrombin. Activation was much slower with platelets alone, and no activation was detected with globulin alone. We believe that these observations can be correlated with the view that platelets are essential for the rapid activation of prothrombin under physiologic conditions; that they furnish accelerator type activity by themselves; and, that in conjunction with plasma and in a manner as yet not known in detail, they furnish the equivalent of thromboplastin activity.

Summary

Activators of purified prothrombin may be divided into two groups: those which have thromboplastin activity, and those which have accelerator or thromboplastin co-factor activity. The two kinds of activators work together to convert prothrombin rapidly. Alone they are not very effective.

Experimentally, either thromboplastin or accelerator activity can be made the limiting factor in thrombin formation. Then, by increasing the concentration of the essential activator, the rate of prothrombin conversion is increased. The thrombin yield is at first increased, but later it is independent of activator concentration. This basic relationship describing rate and quantity of thrombin yield applies to thromboplastin, plasma Ac-globulin, serum Ac-globulin and very probably to platelet accelerator.

When both activators are present in limited quantity the rate of prothrombin conversion can be increased by adding either thromboplastin or accelerator. Under those conditions they are quantitatively interchangeable.

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312. PROTHROMBIN ACTIVATION


PROTHROMBIN, THROMBOPLASTIN, Ac-GLOBULIN AND PLATELET ACCELERATOR: QUANTITATIVE INTERRELATIONSHIPS

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