STUDIES ON PROTHROMBIN: PURIFICATION, INACTIVATION WITH THROMBIN, AND ACTIVATION WITH THROMBOPLASTIN AND CALCIUM*

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Prothrombin, thrombin, fibrinogen, thromboplastin, Ac-globulin, fibrinolysin, antifibrinolysin, antithrombin, antithromboplastin, antihemophilia globulin, and heparin are all factors which are known to be a part of the blood clotting mechanism. They function either to provide emergency hemostasis or to prevent intravascular clotting. In the laboratory their activities are recognized and measured, almost exclusively, either by the rate of fibrin formation or by the rate of fibrin disappearance. If such observations and measurements involve only the action of one factor, reliable conclusions can be made with ease. However, when two, three, or more variables are simultaneously involved in a single measurement, it is hazardous to attempt an answer to many of the important questions which require elucidation. Many of the uncertainties and controversies in the literature on this subject are due to the interplay of several variables. Usually when one of the clotting factors is obtained in purified form, experimental variables can be further eliminated; then a period of rapid progress follows and new horizons are seen.

The purification of prothrombin has presented many formidable difficulties, but it has been possible to report steady progress in an uninterrupted effort (1-4) which still requires further work. In the last report (4) material of high purity was obtained inconsistently. The variability in results could not be explained. Furthermore, the products were unstable in solution (5), thus defeating many of the objectives of the work. It is now known that the inconsistent results were due to the assay procedure and not to the method of preparation. The activation of purified prothrombin with thromboplastin and calcium ions can be accelerated by the addition of Ac-globulin (6-8), which, in small amounts, increases not only the rate of activation but also the final thrombin yield (7, 8). The removal of Ac-globulin during the purification processes results in products which are only partially activated in the two stage analytical procedure.

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1 A factor which accelerates the activation of prothrombin.
The prothrombin products obtained by this method (4) are, therefore, more potent than the reports indicate. Usually those preparations with the lowest apparent activity contain the least amount of Ac-globulin. With the aid of a modified two stage technique, in which adequate amounts of Ac-globulin are supplied, the last reported method (4) has been studied extensively. This method of prothrombin preparation appears to be the most reliable of numerous modifications which were attempted in a study involving the preparation of more than 200 prothrombin products. Improvements in the method, reported herein, have been made for the purposes of obtaining stable products and for removing the last traces of Ac-globulin. The modifications do not involve elimination of any further amount of inert material.

The present paper also includes experiments which show that low concentrations of thrombin destroy prothrombin, and medium concentrations of thrombin act on prothrombin in a diphasic manner; first, the prothrombin becomes refractory to thromboplastin and it later regains the ability to form thrombin. High concentrations of thrombin appear to have no effect on prothrombin. Experiments are also outlined which demonstrate that thromboplastin and calcium ions activate prothrombin in the complete absence of Ac-globulin.

Purification of Prothrombin

Prothrombin Product 2 is first prepared exactly as described (4). This entails, briefly, acid precipitation from diluted oxalated bovine plasma, adsorption on magnesium hydroxide, and elution with carbon dioxide under pressure. It was demonstrated that this product could be left overnight at room temperature without immediate loss of prothrombin, but it now appears that, under these conditions, small amounts of thrombin are produced which ultimately affect the stability of the final product. The appearance of this thrombin is avoided by cooling prothrombin Product 2 immediately to 0°C. The cooled product may be used at once or may be stored for a period up to 15 hours. Insoluble magnesium salts and fibrinogen are removed by straining through washed gauze. The clarified prothrombin eluate is then fractionated with saturated ammonium sulfate solutions exactly as described (4); this procedure eliminates almost all of the Ac-globulin. The precipitate obtained after saturation to 65 per cent is dissolved in 10 cc. of distilled water and dialyzed against distilled water at pH 7.0 (previous procedure, acidified water) until the specific resistance of the prothrombin solution is from 2000 to 3000 ohms when measured at 5°. This can be accomplished in 1 to 1½ hours by using the dialysis procedures described (4). Following the dialysis period the prothrombin is further purified by acid fractionation at 0°C. The pH
is brought to 5.4 by adding 0.1 N hydrochloric acid with constant stirring. A small precipitate which appears is removed by centrifugation. This precipitate contains the greater portion of the remaining Ac-globulin plus a small amount of prothrombin. Prothrombin is then precipitated from the supernatant fluid by adding acid until the pH reaches 4.6. It is removed by centrifugation and dissolved in 10 cc. of distilled water by cautiously adding 0.1 N sodium hydroxide to pH 7.0 to 7.5. The prothrombin may then be obtained in the dried form, without loss of activity, by drying from the frozen state. The products are almost invariably free of thrombin and as a result are stable in solution for periods of 24 hours or more at room temperature.

**Removal of Ac-Globulin Activity from Purified Prothrombin**

The prothrombin products described above contain less than 1 per cent of Ac-globulin by weight. All attempts to remove the remaining Ac-globulin by chemical means have failed. In order to eliminate completely the Ac-globulin activity, a method of heating has been employed. This follows the approach of Owren (11) for the removal of Factor V from prothrombin. It was reported in detail before that Ac-globulin is less stable to heat than prothrombin (8). At 53⁰, in neutral distilled water, prothrombin is fairly stable. Ac-globulin activity disappears precipitously under these conditions (8). In order to assure complete destruction of Ac-globulin activity in the above prothrombin preparations, the latter are subjected to a temperature of 53⁰ for a period of 2 hours. Except for an occasional loss in activity during the 2 hour heat treatment, the prothrombin does not appear to be damaged in any way. After 30 minutes of such treatment it is not possible to detect any Ac-globulin in the products by our method of analysis (8). By this method, 1.2 × 10⁻⁶ mg. of purified Ac-globulin can be detected in 1 cc. of test solution. Therefore, the possibility is considered remote than any Ac-globulin remains after the full 2 hour heat treatment.

On one occasion some prothrombin was dried from the frozen state prior to the 2 hour heat treatment. The snow-white dry material was then dissolved in saline and heated at 53⁰ for 2 hours. The Ac-globulin

2 The dry prothrombin products usually weigh about 75 mg. and contain approximately 300 units of Ac-globulin. The best Ac-globulin products average 330 units per mg. of dry weight (8). The latter were estimated to be 50 per cent pure. Therefore, it follows that an average prothrombin product contains 0.45 mg. of Ac-globulin or 0.6 per cent Ac-globulin by weight.

3 An assay procedure has been developed by which 1/2500 of a unit of Ac-globulin in 1 cc. of test solution can be detected. Since our best Ac-globulin products average 330 units per mg. of dry weight (8), it follows that 1.2 × 10⁻⁶ mg. of the preparation can be detected in 1 cc. of test solution.
was destroyed, as anticipated, but an unexpected drop in prothrombin activity was noted. Following that, it was found that prothrombin cannot be heated at that temperature in 0.9 per cent sodium chloride solution. A comparison of typical results obtained on heating prothrombin in distilled water and in 0.9 per cent sodium chloride is presented in Table I. Heating at 53° is possible in aqueous solutions but not in saline solutions.

Inactivation of Prothrombin with Thrombin

Purified prothrombin, as prepared previously, was not entirely free of thrombin and was not stable at room temperature (5). When dissolved in oxalated bovine plasma, it was stabilized (5). This was thought to be due, in part, to destruction of thrombin impurity by plasma antithrombin. It was, however, impossible to determine to what extent the instability

**Table I**

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>Distilled water</th>
<th>0.9 per cent NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7200*</td>
<td>7200*</td>
</tr>
<tr>
<td>1</td>
<td>7200</td>
<td>5100</td>
</tr>
<tr>
<td>3</td>
<td>7000</td>
<td>2300</td>
</tr>
<tr>
<td>5</td>
<td>6500*</td>
<td>1400*</td>
</tr>
</tbody>
</table>

* No detectable thrombin in solution.

could be attributed to thrombin, because prothrombin free of thrombin was not available. In the following experiments it is shown that as little as 1 unit of thrombin associated with 10,000 units of prothrombin is significant in causing prothrombin instability.

In all experiments, the purified prothrombin was dissolved in distilled water. The activity was measured by the modified two stage technique which supplies Ac-globulin (8). The thrombin added to the prothrombin was of high purity, comparable to the material described by Seegers and McGinty (12). This added thrombin activity was always measured and subtracted from the total prothrombin and thrombin activity.

In the first experiment (Fig. 1) the prothrombin, free of thrombin, was dissolved in water. It was stable for 24 hours. At 48 hours a significant decrease in activity had occurred, at which time a trace of thrombin was found to have appeared spontaneously in the solution. After 72 hours 1 unit of thrombin per cc. of solution was present, and the prothrombin concentration had decreased to less than 25 per cent of the original.
after it continued to decrease and the thrombin concentration increased until the respective concentrations of prothrombin and thrombin became 400 and 110 units per cc. on the 8th day.

In the second experiment (Fig. 2), 13,000 units of prothrombin were placed in solution with 10 units of thrombin. Within 2½ hours a significant drop in prothrombin concentration was found. The activity continued to decline and at 19 hours only 1900 units of prothrombin remained. After 72 hours the prothrombin had increased to 2700 units and the thrombin titer had increased to 165 units.

In the third experiment (Fig. 3) 13,000 units of prothrombin were placed in solution with 1000 units of thrombin. In 10 minutes a significant decrease in prothrombin activity was found. Only 3200 units remained at 2½ hours, but thereafter the activity began to increase; at 6 hours it was 5500 units, at 19 hours it was 9300 units, and thereafter it decreased slowly to 5450 units in 72 hours. At 19 hours there had occurred a 3-fold increase in the original thrombin added. It increased further to 5800 units, where it remained for the remainder of the experiment. This experiment has been repeated many times. The amount of prothrombin regeneration and the extent of spontaneous thrombin production vary proportionally, within limits, with the original quantity of thrombin added.

In the fourth experiment (Fig. 4) 6000 units of prothrombin were placed in solution with 500 units of thrombin. No significant change in prothrombin or in thrombin activity was found for a 72 hour period.
Activation of Prothrombin with Thromboplastin and Calcium Ions

It is of considerable theoretical importance to determine whether prothrombin can be activated in the complete absence of Ac-globulin. The prothrombin preparation described above has been shown to be free of this factor on the basis of heat inactivation curves and tests which are very sensitive to Ac-globulin. Thromboplastin, free of Ac-globulin, was prepared as follows by a modification of the sedimentation procedure of Chargaff et al. (13). 400 cc. of a crude saline extract of bovine lung (10) were centrifuged at 48,000 R.P.M. in a Sharples supercentrifuge for 30 minutes. The sediment was suspended in 200 cc. of saline and again sedimented by centrifugation at 48,000 R.P.M. for 40 minutes. The sediment was then resuspended in saline to make a total volume of 10 cc.

Our tests showed that this preparation was free of Ac-globulin. This thromboplastin preparation was used to activate various prothrombin solutions at a temperature of 28°. The prothrombin, having been heat-treated as described and dried from aqueous solution, was dissolved in a stock solvent, so that when 1 part of the thromboplastin preparation was mixed with 1 part of stock prothrombin, the resulting solution contained 0.15 per cent calcium chloride (approximately optimum), 0.9 per cent sodium chloride, 5 per cent imidazole buffer by volume, and a thromboplastin concentration approximately 20 times that in crude lung extract. The results are described below and are recorded in Table II.

Experiment 1—1 cc. of stock prothrombin solution containing 16,000
TABLE II
Formation of Thrombin from Prothrombin, Thromboplastin, and Calcium Ions at 28°

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Time</th>
<th>Prothrombin* units per cc.</th>
<th>Thrombin units per cc.</th>
<th>Thrombin per cent yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Stock thrombo-plastin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>8000</td>
<td>0</td>
<td>0</td>
<td>6.5</td>
</tr>
<tr>
<td>19 min.</td>
<td>0</td>
<td>520</td>
<td>800</td>
<td>11.1</td>
</tr>
<tr>
<td>32 &quot;</td>
<td>1550</td>
<td>1700</td>
<td>3500</td>
<td>44.0</td>
</tr>
<tr>
<td>3 hrs., 15 &quot;</td>
<td>3800</td>
<td>27 &quot;</td>
<td>2900</td>
<td>47.5</td>
</tr>
<tr>
<td>6 &quot;</td>
<td>0</td>
<td>14 min.</td>
<td>27 &quot;</td>
<td>36.0</td>
</tr>
<tr>
<td>0</td>
<td>8000</td>
<td>265</td>
<td>750</td>
<td>33.3</td>
</tr>
<tr>
<td>27 &quot;</td>
<td>1380</td>
<td>1 hr.</td>
<td>3000</td>
<td>38.0</td>
</tr>
<tr>
<td>6 &quot;</td>
<td>3800</td>
<td>3 hrs.</td>
<td>2900</td>
<td>47.5</td>
</tr>
<tr>
<td>27 &quot;</td>
<td>0</td>
<td>5 hrs.</td>
<td>Questionable</td>
<td>38.0</td>
</tr>
<tr>
<td>5</td>
<td>Stock thrombo-plastin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>300</td>
<td>7</td>
<td>7</td>
<td>2.3</td>
</tr>
<tr>
<td>20 min.</td>
<td>22</td>
<td>3 hrs., 10 min.</td>
<td>59</td>
<td>19.5</td>
</tr>
<tr>
<td>39</td>
<td>100</td>
<td>5 hrs.</td>
<td>105</td>
<td>33.3</td>
</tr>
<tr>
<td>27 &quot;</td>
<td>Trace</td>
<td>51</td>
<td>105</td>
<td>35.0</td>
</tr>
<tr>
<td>6</td>
<td>Stock thrombo-plastin diluted 25 times</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>300</td>
<td>2</td>
<td>8</td>
<td>2.7</td>
</tr>
<tr>
<td>20 min.</td>
<td>30</td>
<td>1 hr.</td>
<td>55</td>
<td>19.3</td>
</tr>
<tr>
<td>35 &quot;</td>
<td>51</td>
<td>3 hrs., 10 min.</td>
<td>51</td>
<td>17.0</td>
</tr>
<tr>
<td>135</td>
<td>70</td>
<td>5 hrs.</td>
<td>70</td>
<td>23.3</td>
</tr>
</tbody>
</table>

* The prothrombin concentration was measured with Ac-globulin supplied in the two stage assay procedure. Under these circumstances the preformed thrombin was subtracted from the total thrombin yield to give the prothrombin concentration.
units per cc. was mixed with 1 cc. of thromboplastin solution, giving an initial prothrombin concentration of 8000 units per cc. Thrombin was produced slowly. At the end of 3 hours and 15 minutes a thrombin yield of 3500 units per cc. was found. A prothrombin concentration of 1700 units per cc., obtained by analyzing in the presence of an excess of Ac-globulin, indicated that 2800 units per cc. had been destroyed. At 6 hours the thrombin yield reached its highest value of 3800 units per cc. (47.5 per cent yield); this decreased slightly in 27 hours when there remained only 600 units of prothrombin per cc. of solution.

Experiment 2—This experiment duplicated Experiment 1 except that the thromboplastin was first heated at 53° for 1½ hours. As compared with Experiment 1, there was no significant change in rate and amount of thrombin formed or in the amount of prothrombin destruction. Since Ac-globulin cannot exist at a temperature of 53°, this experiment is a further indication that the thromboplastin preparation was free of this factor.

Experiment 3—In order to determine the effect of concentrated thromboplastin on dilute prothrombin solutions, Experiment 1 was again repeated but this time the initial prothrombin concentration was adjusted to 5 units per cc. of solution. Thrombin production was quite slow. A trace was detected in 45 minutes. This increased to a 10 per cent yield in 1 hour and 15 minutes, and a 48 per cent yield developed during a 27 hour period. Only a trace of prothrombin remained at the 6 and the 27 hour intervals.

Experiment 4—This experiment is presented to demonstrate the interaction of the two proteins in dilute solutions. The prothrombin and thromboplastin were mixed in the same proportions as in Experiment 1, but in 1/1000 of the concentration. There was no thrombin production for 6 hours and at 27 hours only a trace was found. Only a part of the prothrombin could be accounted for at that time. This experiment is apparently comparable to the ones described by Owren (11), from which he concluded that thromboplastin and calcium ions cannot activate prothrombin.

Experiments 5 and 6—In these two experiments a prothrombin level was selected which is comparable to that in human plasma. In Experiment 5 concentrated thromboplastin was added. Thrombin was produced slowly, reaching a maximum 35 per cent yield in 5½ hours. In Experiment 6 the thromboplastin was diluted 25 times, so that the ratio of prothrombin and thromboplastin was again approximately the same as in Experiment 1. Thrombin production was somewhat slower than in Experiment 5 and the maximum yield (23 per cent) was somewhat less. In both experiments comparatively large amounts of prothrombin re-
mained at the end of 22 hours. However, as in the other experiments there was some destruction of the unconverted prothrombin.

**DISCUSSION**

Seegers, Loomis, and Vandenbelt (4) reported their most active prothrombin preparation to have 15,200 units per mg. of tyrosine. With prothrombin prepared according to their specifications, the amount of Ac-globulin in the preparations was insufficient to give maximum thrombin yields in the two stage prothrombin analysis. Such preparations, giving purity values from 13,000 to 14,000 units per mg. of tyrosine, were considerably more active when analyzed after supplying an excess of Ac-globulin. The specific activity values can be fixed somewhat between 20,000 and 25,000 units per mg. of tyrosine, but the exact activity must await further study. Work now in progress is intended to establish an absolute prothrombin unit on a sound basis. This work deals with stability of dry prothrombin, reference standards, optimum conditions of activation, seasonal variation in plasma prothrombin concentrations, etc.

Mertz, Seegers, and Smith (14) were the first to present evidence which suggested that thrombin destroys prothrombin activity. Owren (11) could obtain only slight evidence for the destruction of prothrombin by thrombin, and Ferguson (15) discounted any such possibility. Experiments reported by these investigators are not necessarily in conflict even though they are inadequate. The data recorded above show that much depends upon the relative quantities of thrombin and prothrombin in solution. Furthermore, the situation is complicated by prothrombin regeneration which is sometimes accompanied by spontaneous formation of thrombin in significant amounts. When 1000 units of thrombin, or less, are added to prothrombin of the strength used above, a fall in prothrombin titer occurs, which is followed by prothrombin regeneration. Spontaneous thrombin production appears to parallel prothrombin regeneration both in amount and in time of appearance. Therefore, it is probable that the regenerated prothrombin forms thrombin with greater ease than purified prothrombin under the conditions of these experiments. When 5500 units of thrombin are added, there is no great change in either prothrombin or thrombin concentrations. Apparently the prothrombin is destroyed and regenerated rapidly; spontaneous thrombin production does not take place to any extent because the reaction is in equilibrium. The generation of 5000 units of thrombin (Fig. 3), in the apparent absence of thromboplastin, Ac-globulin, and calcium ions, is a novelty to this field of research. It is not possible to say definitely that none of these three factors were present. Thromboplastin was certainly not present in appreciable quantities. Ac-globulin was absent from the prothrombin
and could have been present in the thrombin preparation only in traces if at all. Prothrombin and thrombin preparations were both dialyzed against demineralized water to a specific resistance of approximately 3000 ohms. Therefore, the calcium concentration was necessarily extremely low. It seems probable that the correct conditions have been found for the production of thrombin without Ac-globulin, without calcium, and without thromboplastin.

The experiments reported above show that thrombin is produced from prothrombin, thromboplastin, and calcium ions in the complete absence of Ac-globulin. Owren records experiments from which he concluded that thrombin production is not possible from the interaction of prothrombin, thromboplastin, and calcium ions alone (11). His experiments were carried out only at very low prothrombin concentrations for relatively short periods of time. It is not possible to determine from his data what concentrations of thromboplastin were used. If Owren, in addition to low prothrombin concentrations, used weak thromboplastin preparations, it becomes clear from our work why he arrived at his conclusions.

The question may be asked why only a 48 per cent yield of thrombin is obtained when thromboplastin alone is used as the activator. The chief reason appears to be that the reaction is so slow that side reactions produce an effect. One of these, the inactivation of prothrombin by thrombin, is described in this paper. Even in the presence of Ac-globulin, a maximum thrombin yield is not obtained unless an optimum amount of the accelerator is present (7, 8). Decreased thrombin yields apparently result when any factor slows the rate of interaction of prothrombin and thromboplastin. This was pointed out in 1944 (16) from studies which involved the effects of alterations in pH, calcium, electrolyte concentration, etc., on prothrombin activation.

On the basis of experiments which indicated that Ac-globulin catalyzes the reaction between prothrombin, thromboplastin, and calcium ions, it was decided to call this factor an accelerator (7). Since it was also found to be a globulin, the name accelerator globulin or Ac-globulin was proposed (7). The data reported here demonstrate that thrombin is produced in the absence of Ac-globulin. The latter speeds up the reaction (6–8). Therefore it is rightly designated as an accelerator and cannot be considered as a component of prothrombin.

SUMMARY

An extensive study of previously reported methods for the preparation of prothrombin has been made. Modifications are introduced which consistently give highly active preparations, stable in solution for more than 24 hours at room temperature. The products contain approximately
0.6 per cent Ac-globulin by weight. Efforts to remove this last quantity of Ac-globulin with fractionation procedures have repeatedly failed. However, the prothrombin is freed entirely of this activity by heating at 53° in aqueous solution. Heating in saline solution results in losses of both prothrombin and Ac-globulin.

Extremely small amounts of thrombin destroy prothrombin. Larger amounts of thrombin produce a diphasic effect upon prothrombin; there is first a destruction, followed by a regeneration of prothrombin activity. The regenerated prothrombin appears to be changed in such a way that it may form thrombin in the absence of thromboplastin, Ac-globulin, and calcium ions.

Thromboplastin and calcium ions activate prothrombin in the absence of Ac-globulin, but the reaction is slow and the final yield of thrombin is only about half of that produced in the presence of an optimum amount of Ac-globulin.

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