PURIFICATION OF THROMBIN

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The coagulation of blood is customarily considered to proceed in two stages. During the first stage a series of reactions results in the activation of prothrombin to thrombin. In the second stage thrombin brings about the conversion of fibrinogen to fibrin, which composes the solid framework of the blood clot. The mechanism of these reactions has yet to be unraveled, despite a great deal of work with crude materials. Consequently, recent years have seen increasing attention devoted to the purification of the central factors in the clotting system (1-7).

The purpose of this paper is to present a new method for the purification of thrombin, which regularly yields a product the specific activity of which is 100-175 times the potential specific activity of whole plasma.

Preparation of Prothrombin

The first two steps of this method were derived from the procedure of Mellanby (1). The present modification has yielded consistently good results when employed on fresh plasma from carefully collected blood.

Oxalated beef plasma which was no more than 4 days old was fractionated as outlined in Table I. The euglobulins were precipitated from diluted plasma at pH 5.2. From the precipitate, which contained prothrombin, thrombokinase, and fibrinogen, most of the prothrombin was extracted by dilute calcium bicarbonate. During the earlier work with this procedure, it occasionally happened that the finely suspended globulin would flocculate heavily upon the addition of the calcium bicarbonate, and thus render the extraction inefficient. It appeared that the success of the operation depended on extracting the prothrombin before the fibrinogen had progressed too far toward coagulation. Therefore the time limits recorded in Table I were closely observed. Only scrupulously clean apparatus was used, since extraneous matter often accelerates coagulation.

The prothrombin was precipitated from the extract between 0.28 and 0.55 saturated ammonium sulfate. It was then made up to approximately 1 mg. protein nitrogen per ml. and twice reprecipitated by 0.45 saturated ammonium sulfate. The final precipitate, dissolved in a small quantity of distilled water, gave an amber, opalescent solution, the pH of which was 5.4.

* Commonwealth Fellow.
TABLE I

Preparation of Prothrombin and Conversion to Thrombin

<table>
<thead>
<tr>
<th>No.</th>
<th>Units</th>
<th>mg. P.N.</th>
<th>mg.</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml. plasma</td>
<td>ml. plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxalated beef plasma</td>
<td>1,900</td>
<td>11</td>
<td>170</td>
<td></td>
</tr>
</tbody>
</table>

In 4 aluminum vats: 3.5 liters of 1 per cent acetic acid stirred with 201.4 liters distilled water. 10.6 liters plasma added, stirred gently. Allowed to settle 2 hrs. at 20°C. Supernatant siphoned off.

Euglobulin precipitate

No. 1 suspended in remaining mother liquor and packed by centrifugation. Cake ground in mortar and suspended in 20 liters distilled water at 20°C. Suspension distributed among seven 6 liter Erlenmeyer flasks. 3 liters dilute calcium bicarbonate added to each flask which was immediately closed by a rubber stopper and mixed by inversion. Solid kept in suspension by occasional inversion of flask. After 20 min. at 20°C. flasks kept in water baths at 6°C. for 75 min. Supernatant decanted through coarse folded filters at 6°C. Sediment packed by centrifuge in rubber stoppered tubes. Supernatant poured into filters.

Combined filtrates = extract

39 liters No. 2 stirred by motor while 13.6 kg. solid ammonium sulfate added slowly. Stirred 2.5 hrs. at 20°C. Plus 80 gm. Filtercel. Filtered with suction. Filter cake stirred 15 min. with 1 liter 0.9 per cent sodium chloride and filtered with suction.

Filter cake

Filtrate and washings

No. 3 stirred 15 min. with 350 ml. 0.9 per cent sodium chloride. Filtered with suction.

Filtrate and washings

No. 4 and No. 5 combined, 1,550 ml. Plus 600 ml. saturated ammonium sulfate to approximately 0.28 saturated. Centrifuged. Precipitate discarded. Supernatant plus 600 ml. saturated ammonium sulfate to approximately 0.45 saturated. Supernatant discarded. Precipitate

No. 6 dissolved in distilled water to 1 liter of solution. Plus 818 ml. saturated ammonium sulfate, centrifuged. Precipitate

No. 7 dissolved in distilled water to 1 liter of solution. Plus 818 ml. saturated ammonium sulfate. Centrifuged. Precipitate

No. 8 dissolved in distilled water to 90 ml. solution. Concentrated prothrombin

No. 9 dialyzed against 3.5 liters 0.09 saturated ammonium sulfate at 6°C. 1 week at 20°C. then stored at 1°C. Crude thrombin

The underlined figures are average values from six preparations.

The concentrated prothrombin was set aside to change to thrombin. The preparation up to the point of dialysis was completed within 24 hours.
"Spontaneous" Activation of Concentrated Prothrombin

The conversion of prothrombin to thrombin under these conditions proved to be particularly interesting, especially when compared to the behavior of the extract from which it had been concentrated. The pH of the extract was usually 6.8. When stored in the ice box it lost about 10 per cent of its prothrombin per week, but produced no detectable thrombin. Even when calcium and physiologic saline were added, it did not develop thrombin unless an activator such as cephalin or lung extract were included.

In contrast, the concentrated prothrombin changed to thrombin without any added activator. Furthermore, ionized calcium was unnecessary. Quantitative determinations on the concentrate showed the calcium to be 0.002 m; and this was probably bound to protein. The essential condition for "spontaneous" activation seemed to be the concentration of the biologic material. Indeed, activation could be retarded merely by dilution of the concentrate. The data on hand do not permit a decision as to whether it was the prothrombin itself or some accompanying factor which was responsible for this phenomenon.

In a series of experiments performed at pH 7.4 it was found that 0.026 m oxalate failed to retard activation. Although the conversion was accelerated by crystalline trypsin (cf. 8) it was unaffected by crystalline trypsin inhibitor. Conditions which favored rapid activation did not necessarily lead to better yields of thrombin. For example, activation was much faster at pH 6.5 than at pH 5.4 but thrombin was destroyed more rapidly at the higher pH, resulting in a lower final yield. Conditions for optimal yield were found to be 0.1 saturated ammonium sulfate at pH 5.4 and the temperature variations mentioned in Table I.

Fractionation of Crude Thrombin

Preliminary experiments had shown that thrombin was soluble in 0.45 saturated ammonium sulfate, whereas crude prothrombin was not. In the fractionation of prothrombin (Table I) particular care had been taken to eliminate protein which was soluble in 0.45 saturated ammonium sulfate. Therefore, proteins insoluble in that solvent constituted almost all the impurities which were present in the concentrated prothrombin, and consequently, in the crude thrombin. On the other hand, a large proportion of the thrombin itself would be expected to remain dissolved in 0.45 saturated ammonium sulfate when the crude thrombin was fractionated. Such was the case, as is shown in Table II, where the most soluble fraction, 9/15 III, contained almost half the total activity, but only 3 per cent of the total protein. When 9/15 III was again treated to remove protein soluble in 0.45 saturated ammonium sulfate, the specific activity rose from 15,100 to 22,000. From there on fractionation was difficult but clearly showed that the protein was not yet homogeneous. As may be seen from the data in Table II, the thrombin was
TABLE II

Fractionation of Thrombin

<table>
<thead>
<tr>
<th>No.</th>
<th>Per cent Crude Thrombin</th>
<th>Total mg. F.N.</th>
<th>Units mg. F.N.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude thrombin from 3 preparations plus distilled water to 3 liters plus 2,450 ml. saturated ammonium sulfate and 75 gm. Filter-cel. Filtered. Filtrate and washing ...</td>
<td>1</td>
<td>0.45</td>
<td>500</td>
</tr>
<tr>
<td>Cake stirred in 3,300 ml. distilled water. Plus 2,700 ml. saturated ammonium sulfate. Filtered. Filtrate and washing ...</td>
<td>2</td>
<td>0.45</td>
<td>4,420</td>
</tr>
<tr>
<td>No. 1 and No. 2 combined. Vol. 11.1 liters. Plus 2.3 kg. ammonium sulfate and 24 gm. Filter-cel. Filtered. Filtrate discarded ...</td>
<td>9/15 I</td>
<td>52</td>
<td>5,300</td>
</tr>
<tr>
<td>Cake extracted twice with 900 ml. 0.45 saturated ammonium sulfate. Residue ....</td>
<td>9/15 II</td>
<td>0.65</td>
<td>122</td>
</tr>
<tr>
<td>Combined extracts plus 1,030 ml. saturated ammonium sulfate. Centrifuged. Supernatant discarded ...</td>
<td>9/15 III</td>
<td>15,100</td>
<td></td>
</tr>
<tr>
<td>9/15 III dissolved in 35 ml. distilled water. Vol. 48 ml. Plus 24 ml. saturated ammonium sulfate. Centrifuged. Supernatant ...</td>
<td>3</td>
<td>0.45</td>
<td>27</td>
</tr>
<tr>
<td>Residue extracted twice with 150 ml. 0.45 saturated ammonium sulfate. Combined extracts ...</td>
<td>4</td>
<td>0.45</td>
<td>2,900</td>
</tr>
<tr>
<td>No. 3 and No. 4 combined. Vol. 370 ml. Plus 212 ml. saturated ammonium sulfate. Centrifuged. Supernatant ...</td>
<td>10/2 I</td>
<td>0.65</td>
<td>69</td>
</tr>
<tr>
<td>10/2 II dissolved in 70 ml. distilled water. Vol. 77 ml. Plus 30 ml. saturated ammonium sulfate plus 130 ml. (0.99 saturated ammonium sulfate, 0.04 M pH 5.2 acetate). Centrifuged. Supernatant discarded ...</td>
<td>10/2 II</td>
<td>0.70</td>
<td>22,000</td>
</tr>
<tr>
<td>Precipitate extracted 3 times with 180 ml. (0.5 saturated ammonium sulfate, 0.08 M pH 5.2 acetate). Residue ...</td>
<td>10/20 I</td>
<td>0.70</td>
<td>20</td>
</tr>
<tr>
<td>Combined extracts plus 366 ml. (0.99 saturated ammonium sulfate, 0.04 M pH 5.2 acetate). Centrifuged. Supernatant discarded ...</td>
<td>10/20 II</td>
<td>0.70</td>
<td>57</td>
</tr>
<tr>
<td>10/20 II dissolved in 70 ml. (0.35 saturated ammonium sulfate, 0.08 pH 5.2 acetate). Vol. 76 ml. ...</td>
<td>11/10 A</td>
<td>0.49</td>
<td>20,000</td>
</tr>
<tr>
<td>Plus 16.5 ml. (0.98 saturated ammonium sulfate, 0.08 M pH 5.2 acetate). Centrifuged. Precipitate ...</td>
<td>11/10 B</td>
<td>13.5</td>
<td>15,500</td>
</tr>
<tr>
<td>Supernatant plus 2 ml. 0.98 SAS.* Centrifuged. Precipitate ...</td>
<td>11/10 C</td>
<td>6.3</td>
<td>25,600</td>
</tr>
</tbody>
</table>
accompanies by at least two other proteins, one of which was slightly less soluble (in 11/10 A and 11/10 B) and the other slightly more soluble (in E1–E17) than thrombin.

It should be mentioned that the fractionation of Table II was the best of several. In other fractionations the specific activity stopped between 17,000 and 30,000. In one case a sample was treated by several successive additions of saturated ammonium sulfate and gave twelve successive precipitates with a specific activity about 17,000. The reason for this may be surmised from a consideration of the closely similar solubilities of thrombin and the final impurities. The proportion of these impurities in the crude thrombin might determine at what point fractionation would become impracticable. It must be left to future work to determine to what extent the final impurities might be derived from thrombin itself, and whether there are varieties of thrombin molecules with different specific activities (cf. 9).

**Some Properties of the Thrombin Preparations**

From the data in Table II it can be calculated that 70 per cent of the activity present in sample 9/15 III could be accounted for in the 32 fractions derived from it in the course of the next 2 months. In another experiment, on a sample having a specific activity of 13,000, it was found that thrombin was most stable at pH 5.2. Solutions containing 1 mg. protein nitrogen per ml. of 0.35

<table>
<thead>
<tr>
<th>Table II—Concluded</th>
<th>No.</th>
<th>Percent</th>
<th>Total</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant plus 1 ml. 0.98 SAS. Centrifuged. Precipitate</td>
<td>11/10 C</td>
<td>3.7</td>
<td>30,500</td>
<td></td>
</tr>
<tr>
<td>Supernatant plus 1 ml. 0.98 SAS. Centrifuged. Precipitate</td>
<td>11/11 A</td>
<td>3.4</td>
<td>29,700</td>
<td></td>
</tr>
<tr>
<td>Supernatant plus 30.5 ml. 0.98 SAS. Centrifuged. Supernatant discarded</td>
<td></td>
<td>0.62</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Precipitate extracted 10 times with 10 ml. portions of (0.55 saturated ammonium sulfate, 0.08 M pH 5.2 acetate). Total for 10 extracts</td>
<td>E1–E10</td>
<td>0.55</td>
<td>8.1</td>
<td>12,300</td>
</tr>
<tr>
<td>Residue extracted with 10 ml. portions of (0.53 saturated ammonium sulfate, 0.08 M pH 5.2 acetate). Total for extracts</td>
<td>E11–E15</td>
<td>0.53</td>
<td>4.3</td>
<td>16,800</td>
</tr>
<tr>
<td></td>
<td>E16–E17</td>
<td>1.4</td>
<td>26,100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E18–E22</td>
<td>2.5</td>
<td>31,000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E23–E25</td>
<td>0.9</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>Residue</td>
<td></td>
<td>4.0</td>
<td>22,000</td>
<td></td>
</tr>
</tbody>
</table>

* 0.98 SAS (0.98 saturated ammonium sulfate, 0.08 M pH 5.2 acetate).
saturated ammonium sulfate at pH 5.2 lost less than 10 per cent of their activity in 4 days at room temperature.

Solutions having 1 mg. protein nitrogen per ml. and a specific activity of 20,000 or more, were water-clear and had an almost imperceptible yellow tinge. Dr. Alexandre Rothen kindly performed electrophoretic tests on several samples. On Fig. 1 is shown the best pattern obtained, from a sample having 29,000 thrombin units per mg. protein nitrogen. Solubility curves on several samples were of the rounded, solid solution type indicating inhomogeneity (10).

A few tests for proteolytic action on casein (pH 7.6) were performed with a thrombin sample having a specific activity of 9,000. In the standard test (11) 0.1 mg. protein nitrogen caused no change in the viscosity of casein in 1 hour. The effect of thrombin on the formol titration of casein was about 1/10,000 that of crystalline trypsin. Of course, these tests do not exclude the possibility that thrombin has some proteolytic action of fibrinogen. As noted also by Seegers (5) purified thrombin has no fibrinolytic activity.

**DISCUSSION**

If it be assumed that 1 molecule of prothrombin is converted to 1 molecule of thrombin having approximately the same molecular weight it follows that the same number would designate the specific activity of pure thrombin and the potential specific activity of pure prothrombin. From the results herein presented, that number would be above 30,000. 2,000 prothrombin units per ml. plasma would then represent less than 0.067 mg. prothrombin nitrogen. Since there are about 11 mg. protein nitrogen per ml. plasma, prothrombin comprises less than 0.6 per cent of the plasma proteins.

By the present assay method normal beef plasma contains 2,000 prothrombin units per ml. and human plasma, 1700 units per ml. By the Iowa method of assay, there are 314 Iowa units of prothrombin per ml. beef plasma, and 325 Iowa units per ml. human plasma (3, 5). Thus, the present unit is 1/6 the...
Using this factor to convert the assay values of Seegers to the present unitage it can be calculated that the usual thrombin preparation obtained by Seegers had 28,000 units per mg. protein nitrogen, and that his best preparation had a specific activity of 43,000. The highest single assay value obtained during the present work was 36,000. Further comparison of the two methods might prove fruitful since their strong features are quite different.

Materials and Methods

Buffer for Fibrinogen.—This was a 0.9 per cent solution of sodium chloride containing 0.0067 m phosphate buffer pH 7.4 and 0.013 m potassium oxalate.

Buffer for Assays.—50 ml. 0.1 m sodium diethylbarbiturate were mixed with 36 ml. 0.1 m hydrochloric acid and diluted to 250 ml. with oxalated saline. The final concentration of oxalate was 0.0008 m.

Calcium Chloride.—0.11 m.

Calcium Bicarbonate.—Distilled water was shaken with excess CaO. The filtered solution was diluted tenfold with distilled water. CO2 was rapidly bubbled through the 0.1 saturated Ca(OH)2 until the solution was yellow to brom thymol blue. Air was then bubbled through until the solution was orange to phenol red. The bottle was closed tightly with a rubber stopper.

Beef Plasma.—Beef blood was obtained at the slaughter house. The blood gushing from a severed vessel was caught directly into 150 ml. of 10 per cent potassium oxalate in a wide mouthed 4 liter jar. The mouth of the jar was not allowed to touch the wounded tissue and any blood which flowed over the wound was rejected. Within a few seconds the jar was J/4 full and it was quickly stoppered and shaken. After the blood had cooled in the ice box the plasma was collected by centrifugation and stored at 6°C.

Fibrinogen.—17 ml. of saturated ammonium sulfate were slowly added to 50 ml. of beef plasma. 15 minutes later the mixture was centrifuged lightly for 5 minutes and the precipitate was immediately stirred thoroughly with 200 ml. 0.25 saturated ammonium sulfate. After 15 minutes the residue was collected by centrifugation and dissolved in 75 ml. of buffer. Kept in the ice box these solutions were fairly stable and as will be seen below were sufficiently uniform for assay purposes. The small portions which were warmed to room temperature for each assay were not returned to the cold stock solution since long exposure to room temperature affects the clotting response.

Lung Extract.—0.5 gm. dried beef lung was extracted by grinding with 10 ml. of 0.9 per cent sodium chloride.

Assay of Thrombin Activity.—All reagents were at room temperature. The thrombin solution was diluted with buffer and to 1.0 ml. of the diluted thrombin was added 0.2 ml. calcium chloride. Then 0.1 ml. of this mixture was blown directly into 0.3 ml. of fibrinogen in a 10 × 75 mm. pyrex tube. The contents were immediately mixed by rapid oscillation. The clotting time was taken as the interval between the addition of the thrombin and the instant when the clot could hold its position with the
Purification of Thrombin

tube inverted. It was determined empirically that the clotting time was inversely proportional to the concentration of thrombin under these conditions. A unit was defined as the amount which gave a clot in 300 seconds. The dilutions were usually arranged so that 3 to 10 units were estimated. Example: A solution of thrombin was diluted 1/16, then 3/10, and the calcium chloride added. 0.1 ml. of this produced a clot in 50 seconds. The original solution contained \(10 \times \frac{1}{3} \times \frac{12}{10} \times 10 \times \frac{300}{30} = 3340\) thrombin units per ml.

A standard solution was prepared by precipitating thrombin with ammonium sulfate and dissolving the precipitate in glycerin. Kept in the ice box this solution maintained its titer for 8 months. During this time the titers obtained with eight fresh preparations of fibrinogen varied from 11,700 to 13,500 units per ml. Only one fresh fibrinogen preparation (from anemic blood) had to be discarded because it gave values outside this range. When a fibrinogen solution had aged to the extent that it gave low titers for the standard thrombin it was replaced by a fresh preparation.

Prothrombin Assay.—0.9 ml. of a buffer dilution of prothrombin was mixed with 0.1 ml. lung extract and 0.2 ml. calcium chloride. At intervals the activation mixture was assayed for thrombin, the plateau value being used to calculate the potential activity of the prothrombin.

Protein Nitrogen.—Turbidity method (9). The turbidity curve was standardized by Kjeldahl determination on a sample having a specific activity of 18,000.

Tests for Proteolysis.—Northrop and Kunitz (11).

SUMMARY

1. Under certain conditions crude prothrombin changes to thrombin without the addition of extraneous activators and in the absence of ionic calcium.
2. Thrombin is soluble in 0.45 saturated ammonium sulfate, whereas crude prothrombin is not.
3. Partially purified thrombin is comparatively stable in concentrated ammonium sulfate solutions at pH 5.2.
4. A method based on the above facts yields a thrombin preparation the specific activity of which is 100 to 175 times the potential specific activity of whole plasma.

This work was carried out under the guidance of Dr. John H. Northrop. The author is also indebted to Mr. J. F. Gettemans who helped work up 400 liters of blood.

REFERENCES