PURIFICATION OF PROTHROMBIN AND THROMBIN: CHEMICAL PROPERTIES OF PURIFIED PREPARATIONS*

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We wish to describe certain simplifications in the technique previously described (13, 14) for the purification of prothrombin and thrombin. With new adaptations it is possible to process large quantities of plasma and at the same time to obtain products 2 to 5 times as potent as those previously reported from this laboratory. A brief survey of the chemical properties of these newly obtained products is also given.

Preparation of Prothrombin

Beef plasma is diluted 10-fold with water and brought to pH 5.3 with 1 per cent acetic acid. The precipitate is redissolved and Mg(OH)$_2$ suspension is added. In our original technique (14) the prothrombin adsorbed on the Mg(OH)$_2$ was set free with CO$_2$ at atmospheric pressure. Improvement is achieved by using CO$_2$ at 4 to 6 atmospheres (by shaking in a metal chamber, with 8 to 10 volumes of water to 1 volume of Mg(OH)$_2$ paste). Following this elution, the CO$_2$ is allowed to escape, and variable amounts of magnesium carbonate settle out. As previously, the dissolved salts are then removed by dialysis. The use of increased pressure reduces the working volumes at least 90 per cent, and this simplifies enormously the procedure of dialysis.

A second improvement is the isoelectric fractionation of the dialyzed prothrombin-containing eluate; this is accomplished in two steps (at pH 5.6 and at 5.3). The first fraction (pH 5.6)

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usually contains mainly inert protein, whereas the second fraction (pH 5.3) contains much prothrombin of high potency. The latter is particularly potent if small amounts of Mg(OH)$_2$ are used in the adsorption process. If, however, a large excess of Mg(OH)$_2$ is used, there will be marked adsorption of inert protein, most of which will then appear in the first isoelectric precipitate. This voluminous precipitate carries the prothrombin with it, and thus defeats the objective of the fractionation. In order to obtain a combination of good yield and high purity, one should employ intermediate quantities of Mg(OH)$_2$. As an example, 100 cc. of crude prothrombin solution, containing 400 units (17) per cc., should be treated with approximately 15 cc. of 8 per cent Mg(OH)$_2$ suspension.

Preparation of Thrombin

The conversion of prothrombin into thrombin is carried out in a solution of 0.9 per cent NaCl containing 0.15 per cent Ca(NO$_3$)$_2$. The concentration of prothrombin can be allowed to vary within wide limits, but as a rule the final mixture is made up to contain 2000 to 7000 units of prothrombin per cc.

Thromboplastin, derived from lung, is consumed, apparently in stoichiometric quantities (9) during the formation of thrombin. We make it a practice to use a slight excess over the quantity theoretically required. The thromboplastin used is especially purified (8) to remove traces of antithrombin which are present in crude organ extracts. The pH of the reaction mixture is adjusted to approximately 7.3. It is advisable to add small amounts of imidazole buffer (7).

In such a mixture thrombin formation proceeds rapidly, but usually an hour is allowed for the reaction to come to completion. In order to eliminate electrolytes and to denature some of the protein impurities, the thrombin preparation is now precipitated with acetone, dried with acetone and ether, and then redissolved in water. An electrolyte-free solution can also be obtained by electrodialysis, without the use of acetone. In either case the aqueous solution is brought to pH 5.3 to 5.0 with acetic acid. The precipitate obtained contains inert protein, together with some thrombin carried down by adsorption and coprecipitation. Most of the thrombin, in highly purified form, still remains in solution,
for, unlike prothrombin, thrombin is highly soluble at this pH (see below). The thrombin can be obtained in dried form by acetone precipitation, or by distilling off the water at or below room temperature.

With these improvements in technique the potency is usually 300 units per mg. for prothrombin, and 600 units per mg. for thrombin. The most potent products, 520 and 950 units respectively, are 2 to 5 times as potent as the best previously reported from this laboratory (13, 14). The potency of the thrombin is such that less than 0.006 \( \gamma \) will eventually clot 0.3 cc. of purified fibrinogen solution; and 0.2 \( \gamma \) can be measured conveniently and precisely by our assay technique (17). Normal human plasma contains about 325 units of prothrombin per cc.; hence, its potential thrombin content is less than 37 mg. per 100 cc.

Recently Astrup and Darling (1) have reported work on the purification of thrombin. Their method is similar to our original procedure, except for the omission of the adsorption by Mg(OH)\(_2\). These workers report having obtained material with a potency of 10 Mellanby units per mg. 1 Mellanby unit causes clotting of 1 cc. of oxalated plasma in 30 seconds, whereas 1 of our units clots 1 cc. of purified fibrinogen solution in 15 seconds. The inhibiting action of oxalate and plasma antithrombin compensates almost exactly for the difference in the two clotting intervals. The two units are, therefore, almost equivalent, and we conclude that Astrup and Darling's thrombin was only slightly more than 1 per cent as potent as our 950 unit preparations.

Properties of Prothrombin and Thrombin

Solubility—At pH 7.0, prothrombin and thrombin are highly soluble, both in water and in 0.9 per cent NaCl. It is possible to prepare viscous solutions containing 60 per cent or more of the product in question. It is of interest that in aqueous solutions, low concentrations of a variety of salts, including those of Ba, Ca, Sr, and Al (approximately 0.005 M) cause precipitation of prothrombin, but not of thrombin. If the concentration of the salt is increased approximately 5-fold, the prothrombin precipitate redissolves.

When an aqueous solution of prothrombin was acidified with acetic acid, a precipitate appeared at pH 5.6 (Fig. 1). At pH 3.9
the precipitate went completely into solution. In the case of thrombin the precipitation range was pH 5.1 to 3.4. These data give presumptive evidence that the isoelectric point of thrombin is somewhat lower than that of prothrombin.

These same solutions were studied by titration technique to determine how much prothrombin and thrombin actually remained in solution at each pH. Prothrombin activity cannot be followed below pH 4.7 because of inactivation. However, it is evident from the two curves of Fig. 1 that at their respective points of minimum solubility thrombin is much more soluble than prothrombin. This difference in solubility is a fortunate circumstance, because prothrombin is always accompanied by inert prothrombin-like materials; and, when thrombin is formed, the alteration in solubility and in isoelectric point enables one to eliminate much of this inert material.

**Inactivation with Acid and Alkali**—Fig. 2 shows the effect of
allowing acid and alkali to act for 30 minutes on saline solutions of prothrombin and thrombin. In the case of thrombin, inactivation began at pH 4.1. At pH 3.5 the inactivation is irreversible but in the range pH 3.5 to 4.1 it is reversible. This can be shown by allowing the activity to regenerate for several hours at pH 7. For example, a solution allowed to stand 30 minutes at pH 3.55 possessed 55 per cent of its original activity the moment it was neutralized. This rose to the 70 per cent level within 2 hours, and to 95 per cent in 4 hours.

In the case of prothrombin, sensitivity to acid is even more marked. Fig. 2 shows that inactivation begins at pH 4.8, and is complete at about 3.5. The problem of reversibility has not been studied as thoroughly as in the case of thrombin. It is of interest that both prothrombin and thrombin are inactivated just on the acid side of their presumptive isoelectric points.

Both prothrombin and thrombin show some inactivation beyond pH 10, and both show marked inactivation beyond the range of pH 11. Fortunately the Mg(OH)₂ used as adsorbent in preparing prothrombin has a pH slightly below the beginning of the inactivation zone.

**Inactivation by Heating**—Heat inactivation studies were made by subjecting aqueous solutions of prothrombin and of thrombin, at pH 7.2, to various temperatures for 30 minutes (Fig. 3). The solutions were then cooled and the thrombin titrated at once (Curve A). The prothrombin solutions were mixed with optimal quantities of calcium and with an excess of thromboplastin. The amount of prothrombin present could then be plotted in terms of thrombin developed (Curve B). Curves A and B show that there was some inactivation of both prothrombin and thrombin at 40°, and at 60° the inactivation was almost complete.

Efforts to restore the activity of the prothrombin were unsuccessful when the product had been heated to 60° or more; but in the range of 40–60° some additional activity could be regenerated by allowing the heated prothrombin to react with calcium and thromboplastin for 30 minutes instead of the usual 10 (see Curve C). Within the zone of 40–60° part or all of the prothrombin is evidently made more sluggish in its reactivity to calcium and thromboplastin. These results correspond rather well with ones reported by Mellanby (5). His inactivation temperatures were
higher, because in his experiments the solutions were allowed to
stand for only 5 minutes at the various temperatures.

*Dialysis*—Neither prothrombin nor thrombin dialyzes through
the cellulose acetate membranes which we use (Visking casings).
If thrombin solution (1000 units per cc.) is placed on one side of
the membrane and plasma on the other, the latter does not clot

Fig. 3. Approximately 50 unit saline solutions were heated for 30
minutes. One series of prothrombin solutions was allowed to react with
optimal quantities of calcium and an excess of thromboplastin for 10
minutes (Curve B), and the other series for 30 minutes (Curve C). With
the concentration of thromboplastin used, 10 minutes are sufficient for the
complete conversion of unheated prothrombin into thrombin.

within 2 hours. This differs from the results of Mellanby (6), and
the discrepancy between his work and ours is probably due to the
difference in the quality of the membranes used.

We have undertaken to increase the sensitivity of this test
by replacing the oxalated plasma with antithrombin-free fibrinogen
solution. Nevertheless, no clot formed in 2 hours. It was pos-
sible to show that a solid clot would have formed if 0.01 per cent
of the thrombin had passed through the membrane. The failure
of the thrombin to diffuse shows that it is a large molecule, and the analysis reported below indicates that it is a carbohydrate-containing protein.

Chemical Analysis—Elementary analysis, performed by Dr. Carl Tiedke of New York, showed that the per cent composition of the thrombin product was N 13.23 (Dumas), C 46.37, H 7.35, and ash 3.98. The prothrombin contained N 14.03, C 48.53, H 7.31, and ash 2.03. Sulfur was present in both, but no quantitative determinations were made.

TABLE I

Relative Carbohydrate Content of Some Prothrombin and Thrombin Preparations

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Activity per mg. N</th>
<th>Carbohydrate content*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prothrombin 147</td>
<td>1100</td>
<td>3.8</td>
</tr>
<tr>
<td>Thrombin 147</td>
<td>4700</td>
<td>5.2</td>
</tr>
<tr>
<td>Prothrombin 77</td>
<td>1982</td>
<td>4.7</td>
</tr>
<tr>
<td>Thrombin 77</td>
<td>4600</td>
<td>5.1</td>
</tr>
<tr>
<td>Prothrombin 155</td>
<td>3620</td>
<td>4.3</td>
</tr>
<tr>
<td>Thrombin 155</td>
<td>3210</td>
<td>6.2</td>
</tr>
</tbody>
</table>

* Carbohydrate was estimated by the method of Tillmans and Phillipi (16) as modified by Sørensen and Haugaard (15). The standard was composed of equal quantities of galactose and mannose, and comparisons were made in a colorimeter (12). Glucosamine does not give the color reaction. For that reason these figures may be as much as 35 per cent too low.

The carbohydrate content of the preparations is rather high (Table I), and this helps to account for the fact that the nitrogen content is somewhat lower than for most proteins.

As stated above, when prothrombin is converted into thrombin, there are certain changes in solubility and in apparent isoelectric points which permit one to eliminate certain impurities from the thrombin preparation. The carbohydrate content of the thrombin preparation is thereby increased, indicating that it is associated with the active principle rather than with the impurities. Any remote possibility that the thrombin might be simply a carbohydrate, and the protein present as an impurity, would seem to be minimized by the fact that the activity of our thrombin and prothrombin preparations is readily destroyed by dilute solutions.
of HNO₂, at pH 5.5. This evidence would appear to indicate that the activity of the thrombin is dependent upon the integrity of α-amino groups.

Fibrinolysis—A number of workers (2–4, 10, 11) have reported the fact that their thrombin preparations not only cause clotting of fibrinogen, but that eventually they even dissolve the fibrin itself. We have made observations with fibrinogen prepared in a variety of ways, and have found that our purified thrombin preparations give no evidence of fibrinolytic activity over a period of many hours. It would seem likely that the fibrinolytic enzyme is a distinct substance which can be eliminated during the process of purification.

SUMMARY

Prothrombin possessing 300 units of activity per mg. of dry weight and thrombin of 600 units of activity per mg. of dry weight can be obtained routinely by the technique described. Products possessing as much as 520 and 950 units per mg. respectively have been obtained.

Evidence indicates that prothrombin and thrombin are carbohydrate-containing proteins.

Low concentrations of a variety of salts cause precipitation of prothrombin but not of thrombin.

In aqueous solutions prothrombin is relatively insoluble in the neighborhood of pH 4.9. In the case of thrombin the point of minimum solubility is near pH 4.3. When compared at their respective points of minimum solubility, thrombin is much more soluble than prothrombin.

Thrombin, in saline solution, is permanently inactivated by acid at pH 3.5, and reversibly inactivated in the zone pH 3.5 to 4.1. In the case of prothrombin, inactivation begins at pH 4.8, and is complete at pH 3.5.

On addition of alkali, inactivation of both prothrombin and thrombin begins at pH 10 and is quite marked above pH 11.

In aqueous solutions prothrombin and thrombin show partial inactivation after being heated for 30 minutes at 40°. At 60° inactivation is virtually complete.

Nitrous acid destroys the activity of both prothrombin and thrombin.
Our purified thrombin preparations do not show the phenomenon of fibrinolysis.

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