SOME PROPERTIES OF PURIFIED PROTHROMBIN AND ITS ACTIVATION WITH SODIUM CITRATE

By Walter H. Seegers, Ph.D., Robert I. McClaughry, M.S., and John L. Fahey, M.S.

The blood clotting mechanism affects the welfare of an individual in more than one important way. It serves as a protection against hemorrhage and participates in wound healing. It is the immediate cause of a variety of vascular disorders, and with increasing knowledge it is becoming evident that a study of the clotting phenomenon contributes broadly to our understanding of the mechanism of living processes. In order to help elucidate this important mechanism the number and nature of the substances which participate must be known. With purified materials in hand many of the uncertainties expressed in the literature concerning blood clotting can be eliminated.

Prothrombin occupies a central postion in the clotting mechanism, thus making it the most desirable substance to have in purified form. More than a decade of persistent effort by one laboratory alone has been required for the development of methods for preparation of prothrombin in sufficient quantity and purity for scientific study. This work has been hampered because of the small amounts in plasma, by the sensitivity of prothrombin to the usual laboratory reagents used for separation of protein, and because a reliable method for quantitative estimation was not available until recently.

In this paper we shall describe several properties of the purified materials obtained by methods already described; however, the main discussion is concerned with the activation of these preparations with sodium citrate. This is accomplished by dissolving the purified prothrombin in a 2.5 per cent solution of sodium citrate and allowing the mixture to stand at room temperature. After about five hours, measurable amounts of thrombin appear. Soon thereafter activation of prothrombin is rapid and the activation curve follows the course of an autocatalytic activation reaction. Activation by this means makes it possible to avoid the introduction of calcium, inadequately purified Ac-globulin preparations, and thromboplastin with its conjoined parts. Thus we are assured that the phenomenon studied is not complicated by addition of complex activators as well as impurities associated with these preparations. These activators are indispensable for normal physiologic activation of prothrombin and it is, therefore, important to understand their function. This may be studied by substituting molecules of known structure for the complicated ones. The results which these compounds produce in activating prothrombin must be essentially like those produced by the natural activators, and much is known about the chemical action of these simple molecules.

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Aided by a grant from the United States Public Health Service. Parke, Davis and Co. supplied large quantities of plasma required for the preparation of prothrombin.
Methods

Preparation of Prothrombin. Prothrombin was prepared as described previously. 
Briefly, this consists of diluting especially oxalated bovine plasma 16-fold and acidifying to pH 5.1. The precipitate is dissolved in oxalated saline and the prothrombin is adsorbed on magnesium hydroxide, from which it can be eluted by decomposing the hydroxide with carbon dioxide. The eluate is fractionated with concentrated ammonium sulfate and the prothrombin is finally precipitated from aqueous solution near the isoelectric point.

Prothrombin Analysis. Prothrombin activity was measured quantitatively by the modified two-stage procedure. 
This modification of the original method of Warner, Brinkhous and Smith supplies Ac-globulin to insure maximum activation of prothrombin.

Thrombin Analysis. Thrombin activity was measured by the method of Seegers and Smith.

Tyrosine and Tryptophane Analysis. A method for the quantitative determination of these amino acids in proteins has been developed by an associate of this laboratory, D. Maxwell Teague. It is based on spectrophotometric technics and the method is to be described in detail in a separate paper.

Some Properties of Prothrombin Preparations

In previous work, emphasis was placed on the development of methods for the preparation of prothrombin, and no extensive chemical studies were made of the products. It is believed that any impurities which might still be in the preparations are now present in relatively low proportions so that the chemical properties of such preparations more closely approximate the properties of prothrombin itself.

Elementary Composition. An elementary microanalysis, done in duplicate, gave the following results for percentage composition:

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>S</th>
<th>Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>44.1</td>
<td>6.4</td>
<td>13.6</td>
<td>0.9</td>
<td>3.8</td>
</tr>
<tr>
<td>Averages</td>
<td>44.1</td>
<td>6.5</td>
<td>13.7</td>
<td>0.9</td>
<td>3.7</td>
</tr>
</tbody>
</table>

The ash analysis was repeated on another sample which had been dialyzed against water more thoroughly before drying and the result was 0.36 per cent. No phosphorus was found.

Tyrosine and Tryptophane Content. Three different preparations of prothrombin were analyzed for their tyrosine and tryptophane content. The results were as follows:

<table>
<thead>
<tr>
<th>Prothrombin No.</th>
<th>Tyrosine per cent</th>
<th>Tryptophane per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>484505</td>
<td>4.74</td>
<td>3.35</td>
</tr>
<tr>
<td>481007</td>
<td>4.60</td>
<td>3.30</td>
</tr>
<tr>
<td>481116</td>
<td>4.41</td>
<td>3.35</td>
</tr>
<tr>
<td>Average</td>
<td>4.58</td>
<td>3.33</td>
</tr>
</tbody>
</table>

Specific Activity. In early work on the purification of prothrombin it was found convenient to express the specific activity in terms of units of prothrombin per milligram of tyrosine. In the determination of tyrosine the method of Folin-Ciocalteu was employed, and although it does not give the true tyrosine value, as recorded above, we continued to use that method. During the purification work it was discovered that Ac-globulin is essential for securing the maximum titer for the prothrombin. 
This factor increased the specific activity of the product entirely on the basis of results for prothrombin analysis and not as the result of further purification. In recent years repeated modifications of methods of purification have not increased the specific activity. In general, the highest can be expected to be 2,000 units per milligram of tyrosine. It is now essential to translate this value into an expression based on the dry weight of the protein and the correct tyrosine value.

Comparisons of specific activity must depend upon the reproducibility of results. This may be insured by use of a reference standard. Oxalated bovine plasma may be used for such comparison. It contains 349 ± 29.8 units of prothrombin per cc. of plasma when standard reagents and procedures are employed. 
Actual tests show that the prothrombin concentration of bovine plasma is the same from year to year.
The plasma prothrombin is stable for long periods of time in deep freeze; in fact, it is quite stable in an ordinary ice box. These are all desirable properties of a reference standard.

To take these considerations into account and characterize the specific activity of the purified prothrombin products, two of the latter were selected. They were of the highest activity which could be produced; namely, 23,000 units per milligram of tyrosine. The preparations were thoroughly dialyzed against water to remove salt, dried from the frozen state, and further dried in a desiccator. The specific activity was 1,470 and 1,340 units per milligram of dry weight or in round numbers an average of 1,400. The analytical reagents and same technics of handling these reagents showed oxalated bovine plasma to contain 32.9±2.9.8 units per cc. of prothrombin. On the basis of the tyrosine value of 4.58 per cent of the protein the specific activity is 33,000 units per milligram of tyrosine. The value of 1,450 units per milligram dry weight is slightly lower than the specific activity of 1,500 reported by Seegers, Loomis and Vandenbelt. This does not necessarily mean that their product was of equal or better quality because all of the data required for comparison are not available.

Stability of Prothrombin Preparations. In neutral aqueous solutions purified prothrombin is stable for periods of months provided it is stored in a deep freeze and provided no thrombin is in the solution. Prothrombin can be dried from the frozen state without loss of activity. Nevertheless, the procedure apparently produces a deleterious effect, because such dried preparations may lose activity rapidly when allowed to stand in a desiccator at room temperature. Wide variations in this property were encountered. For example, one preparation lost no activity in ten days, another lost 52 per cent in fourteen days, while a third lost 67 per cent of the original activity in two hundred days. Prothrombin in the dry and stable form can be obtained by precipitating and drying it with cold acetone.

There are some peculiarities associated with this loss of activity which are not as yet understood. In the assay procedure, done by the modified two-stage method, calcium, Ac-globulin, and thromboplastin are supplied in optimal concentration. But recovery of the lost prothrombin activity can be forced if given sufficient time and high Ac-globulin and thromboplastin concentrations are used. One can also force activation with sodium citrate after the manner described below. The loss in sensitivity to the conditions of the two-stage procedure is thus not an absolute loss of reactivity. Our working hypothesis in current experiments is that an inactive derivative of prothrombin is produced by drying it from the frozen state with subsequent standing in a desiccator. Thus, quite apart from Ac-globulin governing the rate of activation of prothrombin, the purified prothrombin preparation itself may undergo changes which slow its rate of activation.

Electrophoretic Analysis. Electrophoresis in the Tiselius apparatus of material with a specific activity of...
1,400 units per milligram of dry weight resulted in the pattern shown at the top of figure 6. The main component comprised A 87 per cent, D 95 per cent of the total.* Two other components appeared, moving at a slower rate. This result has been repeated with several preparations. The mobility curve for prothrombin is represented in figure 1. Although high, it was found to be slightly less than described in a preliminary report in 1944." In general it may be said that the mobility is between albumin and α1-globulin.

It was necessary to extrapolate for estimating the isoelectric point (fig. 1) because the solubility is very low at that pH. The result for the isoelectric point is approximately pH 4.2 as contrasted with a value of pH 4.8 given in the preliminary work.14 In general, it may be said that the mobility is between albumin and α1-globulin.

In efforts to obtain materials with a high specific activity many products were made and the protein was usually examined by electrophoresis. The purity, as measured by specific activity, was not always in agreement with the purity as measured by electrophoresis. Sometimes a product with a specific activity of 1400 units per milligram of dry weight would indicate greater purity in its electrophoretic pattern than one with a specific activity of 1400 or more units per milligram of dry weight. This could mean that the activity analyses were not sufficiently accurate, which is doubtful because they were done with great care and a reference standard was used. There is suggestive evidence that the impurities which were most difficult to remove in purification work were actually derivatives of prothrombin.

Activation of Prothrombin with Sodium Citrate

During the course of purification, difficulties were encountered with the stability of aqueous solutions of prothrombin.15 It was eventually possible to show that small amounts of thrombin were appearing in the products and that they were responsible for the instability of the prothrombin. It was reasoned that sodium citrate could be used effectively to block the formation of these small amounts of thrombin and the desired stable prothrombin could then be obtained. However, when this salt was added to the prothrombin, activation resulted instead of an inhibition of activation. The artificial activation of prothrombin was studied further.

Concentration of Sodium Citrate. Figure 2 shows that a 5 per cent solution of sodium citrate will produce some activation, a 10 per cent solution is slightly more effective but good results are obtained only when the concentration is 25 per cent. There is first an induction period when no thrombin is formed, then it appears rapidly, and later slowly again, thus giving a sigmoid curve. Activation does not occur to any significant extent in the usual anticoagulant concentrations.

With less highly purified prothrombin preparations, erratic results may be obtained in attempts at activation with sodium citrate. The reason for this is unknown. Perhaps inhibitors of the activation are associated with the prothrombin.

Activation of Prothrombin with Other Salts. Sodium citrate is not the only salt which can be used as an activator of prothrombin. Others that give good results, when added in high concentration with prothrombin, are ammonium sulfate, magnesium sulfate, sodium sulfate, potassium citrate, potassium oxalate and dipotassium hydrogen phosphate. In sodium chloride, potassium chloride, ammonium chloride, or magnesium chloride solution there is practically no activation. In fact, these salts seem to have the opposite effect; they tend to stabilize

* A 87 per cent means the percentage calculated from data of the ascending boundary pattern and D 95 per cent refers to the descending boundary pattern. This applies throughout the paper. The reason for these differences is not known.
prothrombin. The activity of the anion of the salt seems to be the determining factor in the activation mechanism.

**Electrophoresis of Prothrombin, before, during and after Activation with Sodium Citrate.**
In order to study the activation by sodium citrate in greater detail, a stockpile of prothrombin was made available by pooling a number of preparations. These were all of high specific activity but not necessarily of the best quality which could be produced. From this source, two solutions of prothrombin were prepared: one in saline to serve as a control, and one in 2.5 per cent sodium citrate. Samples were taken periodically from the latter solution for analytical work, including electrophoresis, thrombin activity, and prothrombin activity. For electrophoresis the material had to be transferred from the 2.5 per cent citrate solution to barbital buffer of 0.1 ionic strength. This was accomplished by dilution with water and precipitating the protein with ammonium sulfate at 66 per cent saturation. The precipitate was then taken up in the buffer for electrophoresis and dialyzed for proper equilibration. The prothrombin could be examined immediately or stored in the deep freeze until analyzed.

Activity in the control (fig. 3) remained practically the same throughout the experiment, showing that the prothrombin was stable in physiologic saline solution. It contained no thrombin. In the citrate solution the prothrombin plus thrombin curve showed a decline during the first five hours. This lost activity reappeared during the next five hours. Thrombin activity appeared in appreciable quantity at the end of three hours and then followed the course of the theoretic curve resulting from an autocatalytic reaction. In autocatalytic activation the end product of the reaction, when added to the parent compound should at any time influence the activation rate of the latter. To test the prothrombin with respect to this theory, a series of separate experiments on this same solution of prothrombin

![Graph](https://via.placeholder.com/150)

**Fig. 2.**—Activation of purified prothrombin with various concentrations of sodium citrate.
in sodium citrate was performed. Thrombin was added at various times and in all instances the formation of thrombin was hastened.

The results of the electrophoretic analyses are shown in part in figures 3 and 4. In figure 3 the presentation is intended to simplify visualization of the relationship of the electrophoretic work to the activity of the thrombin and prothrombin, whereas in figure 4 the electrophoretic patterns may be compared with each other to good advantage. The first curves obtained were with a sample of prothrombin not acted upon with sodium citrate. The prothrombin concentration was A 76 per cent, D 86 per cent of the total protein and the impurity was represented by two components. The first sample from the sodium citrate solution was taken twenty minutes after the prothrombin had been dissolved. The major component represented A 67 per cent, D 84 per cent, and the first minor component had increased from A 19 per cent, D 11 per cent to A 24 per cent, D 14 per cent. The next sample was taken from the sodium citrate solution at three and three-quarters hours. At that time, thrombin was appearing in appreciable quantity and the prothrombin plus thrombin curve was coming to its lowest point. Of the original prothrombin curve only A 12 per cent, D 44 per cent remained, and the original minor component could now be considered the major component and represented A 82 per cent, D 47 per cent. Further samples were taken at 6, 16½ and 51 hours, and the changes in the patterns were progressive. At 16½ hours a new and faster moving component had appeared, which was not detectable in the samples taken at 0, ½, 3½ and 6 hours. This boundary was also present at 51 hours and was most...
Fig. 4. (Left)—Electrophoresis patterns of purified prothrombin before, during and after activation in 25 per cent sodium citrate solution. I = control prothrombin. II, III, IV, V and VI were obtained at 1, 32, 6, 16g and 5t hours respectively after addition of prothrombin to 25 per cent sodium citrate solution. Electrophoresis was carried out for one hundred and fifty minutes in barbiturate buffer, ionic strength 0.1, and pH 8.6.

Fig. 6. (Right)—Electrophoretic patterns of prothrombin and of thrombin formed by activation with 25 per cent sodium citrate. The top pattern is that of a prothrombin product of high specific activity. The second pattern is an analysis of the thrombin resulting from sodium citrate activation. The third protein was the insoluble material when 780 mg. of prothrombin were dissolved in 13 cc. of 47 per cent ammonium sulfate. The bottom pattern shows the results of the electrophoresis of the soluble material resulting from the ammonium sulfate fractionation above. All electrophoresis measurements were carried out with barbiturate buffer of ionic strength 0.1, at pH 8.3. The time was one hundred and fifty minutes.

Fig. 5.—Electrophoretic mobility of purified bovine thrombin in barbiturate, phosphate, and acetate buffers of 0.1 ionic strength. The mobilities were calculated from observations of the descending boundary pattern.

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evident on the descending boundary pattern. After complete activation the patterns were too complex for detailed interpretation, but they give evidence of certain important facts. The degradation of prothrombin involves the formation of three components. Two of these, present in high proportion, have a mobility lower than prothrombin itself while a small quantity of another derivative possesses a very high mobility.

Activation of Prothrombin with Calcium, Thromboplastin, and Ac-globulin. It seemed desirable to compare thrombin products obtained with sodium citrate as the activator with those obtained with calcium, Ac-globulin and thromboplastin. To do this prothrombin was prepared by procedures which would supply an abundant quantity of Ac-globulin in the preparation. It was necessary only to modify the regular procedure in a few respects. The modifications consisted of collecting prothrombin in a fraction between the limits of 45 to 67 per cent of saturation with ammonium sulfate instead of the limits 50 to 67 per cent. This prothrombin was then activated to the extent of 65 per cent by calcium and purified thromboplastin, the latter having been obtained from lung extract by repeated centrifugal sedimentation at 20,000 G. The prothrombin-thrombin-Ac-globulin-thromboplastin mixture was then centrifuged at 20,000 G. to remove thromboplastin, and fractionated with ammonium sulfate. At 51 per cent of saturation the precipitate was discarded and at 66 per cent of saturation the protein was collected by centrifugation, dissolved in water, and dialyzed to a specific resistance of 14,000 ohms. After acidification to pH 5.5 the precipitate was discarded and the supernatant was used for electrophoresis.

The electrophoretic patterns usually showed two main components together with other minor representations. One of these main components possessed the mobility of prothrombin (fig. 5, curve I) and the isoelectric point was pH 4.1. The other protein, which was very likely thrombin, was isoelectric at pH 4.8. At all pH values its mobility was much lower than that of prothrombin and equal to that of the thrombin obtained with sodium citrate. In these experiments there was no evidence that the material of low mobility might be separating into two parts, whereas that was definitely found in the citrate experiments.

Preparation of Thrombin and Properties of the Product

In the experiment of figure 3, all of the prothrombin activity appeared as thrombin. Unfortunately it was not always possible to obtain such good results; commonly the yield was found to be only 75 per cent or thereabouts. In order to study thrombin and to see whether other derivatives are obtained through the action of sodium citrate on prothrombin it was not only important to produce activation with sodium citrate but also to obtain complete activation. Fortunately, after much experimentation it was possible to arrange the conditions so that essentially all of the prothrombin activity would appear as thrombin activity. By adding a small amount of 3-methyl-4,6,4'-triaminophenyl sulfonyl to a solution of prothrombin in 25 per cent sodium citrate virtually complete activation of prothrombin results. Further studies of the action of this sulfonyl and related compounds are still in progress. The compound is sparingly soluble and usually a saturated solution is used in prothrombin activation.

Preparation of Thrombin. Prothrombin with a specific activity of 1,400 units per milligram of dry weight was first prepared. 3-Methyl-4,6,4'-triaminophenyl sulfonyl was added to 15 cc. of 1 per cent prothrombin in 25 per cent sodium citrate solution. This was allowed to stand at room temperature, and at the end of twenty-four hours the yield of thrombin was complete; i.e., practically all of the activity
appeared as thrombin and none as prothrombin. The thrombin, in citrate solution, was diluted to 100 cc. with water, and precipitated by slowly adding 100 cc. saturated ammonium sulfate solution. The temperature of the thrombin was kept at 0 degrees C. The precipitate was then dissolved in 10-15 cc. of water and dialyzed against cold water to remove ammonium sulfate. When, in about one and one-half hours, the specific resistance of the solution was near 6,000 ohms it was dried from the frozen state. There was no loss of activity during the drying operation and in contrast to prothrombin the dry material is apparently stable. The specific activity of products obtained by this method is usually 17,000 to 19,000 units per milligram tyrosine (Folin-Ciocalteu).

**Electrophoresis of Thrombin.** The thrombin was analyzed by electrophoresis and the patterns are shown in figure 6. There was clear evidence of two components comprising the major portion of the protein. A component represented in a small percentage possessed high charge density. This was most evident in the descending boundary patterns where this component is prominently ahead of all the others. The remaining protein seemed to be impurity carried over from the prothrombin preparation. This was judged from the amount and mobility of this material.

**Solubility Experiments.** The determination of solubility in the presence of increasing amounts of solid phase gives important information concerning the purity of a protein preparation. In work reported by Seegers and McGinty, solubility data indicated that thrombin activity is possessed by two different proteins. That thrombin, obtained by activating prothrombin with sodium citrate, would give similar results was a likely possibility, because the electrophoretic patterns indicate the existence of two major components. Preliminary experiments showed that a suitable solvent could be prepared as follows: 47 cc. saturated ammonium sulfate plus 53 cc. of a mixture composed of 90 cc. water plus 10 cc. imidazole buffer. Various amounts of dry thrombin were added to one cc. of this solvent. The temperature was kept at 0 degrees C., and after allowing four hours for approximate equilibrium to be established the two phases were separated by centrifugation, and both were analyzed for thrombin activity and tyrosine (Folin-Ciocalteu) content.

It was found that about 0.5 milligram of protein could be dissolved completely. By adding more solute about half of the thrombin would dissolve and the specific activity of the thrombin in solution was, within experimental error, equal to that of the thrombin which did not dissolve. This nearly exact separation into soluble and insoluble thrombin continued until 60 milligrams of dry material had been added to 1 cc. of solvent. At that point the more soluble component stopped going into solution. The latter was now saturated with respect to this thrombin. Even by adding 72 milligrams of dry material to 1 cc. of solvent the total activity in solution as well as the total protein in solution was about the same as when 60 milligrams of dry material were used. These results could be interpreted as indicating that there are two separate proteins with thrombin activity.

If two proteins possess thrombin activity it should be possible to separate the two. From the solubility data it seemed promising to do this by duplicating the conditions at which the thrombin which is presumed to be more soluble, stopped going into solution. In order to examine the two fractions by electrophoresis a large scale separation was required. 780 milligrams of thrombin were suspended in 13 cc. of the solvent used in solubility studies and the insoluble phase was separated from the soluble phase. The solution had about the same amount of total and specific thrombin activity as the insoluble material. Upon electrophoresis there was some discernible difference in the electrophoretic patterns, but the results were not decisive. The more soluble material seems to contain more of the protein of slightly higher mobility. It is quite evident that it will be difficult to separate the two proteins by this approach to the problem.

**DISCUSSION**

Owren has reviewed the question whether an autocatalytic reaction takes place in the process of blood coagulation and concluded that the literature is not decisive on this point. The difficulty, of course, has always been that the experiments which were interpreted as showing autocatalytic activation of prothrombin were conducted with complex mixtures of clotting factors. Such experiments always have the limitation of being influenced to an unknown extent by one
or another of many variables. For this reason we feel that the autocatalytic activation of purified prothrombin described in this paper is the first unequivocal demonstration of prothrombin activation involving this mechanism. From our experiment, however, it is not possible to conclude how important autocatalysis is under physiological conditions. Certainly the activators calcium, thromboplastin, and Ac-globulin have an important role in activation. Simply to say that autocatalysis explains in part the physiologic activation of prothrombin, because it works in vitro, is, however, not permissible. Therefore, it is only possible to say that autocatalysis may be a part of the mechanism of prothrombin activation under physiological conditions.

A somewhat more definite conclusion can be reached regarding the function of calcium, thromboplastin, and Ac-globulin. It appears that they are catalysts in the sense that they contribute nothing in the way of material substance during the activation of prothrombin, but contribute to the speed of the reaction. The latter contains all of the structural material required in the thrombin molecule. This must be so because sodium citrate activates without added calcium, thromboplastin, or Ac-globulin. These activators do not require certain definite atoms or groups of atoms to be exchanged in an interaction with prothrombin. They need only to bring the proper force to bear on the proenzyme, and that can also be supplied by snake venoms, citrate, oxalate, thrombin, etc.

The nature of the substances produced when prothrombin becomes activated is not as yet clearly known from our experiments. Tentatively we may conclude that at least three substances are derived. Two have an electrophoretic mobility which is less than that of prothrombin. Probably these two substances each have thrombin activity. The other substance has a high electrophoretic mobility and there is no clue to its composition or special biological properties. The view that prothrombin is degraded during activation is in harmony with the tentative conclusion that the molecular weight of thrombin is approximately half that of prothrombin.18

When sodium citrate activates prothrombin there are very marked electrophoretic changes long before thrombin activity can be found to any appreciable extent. After these electrophoretic changes occur the prothrombin is refractory to the action of calcium, thromboplastin, and Ac-globulin at the usual concentrations used in analytical work (fig. 3). This indicates that an intermediate or an inactive derivative of prothrombin forms during the activation process. It is not impossible that this same altered prothrombin is formed after freeze drying operations. On the other hand, they may be different inactive derivatives of prothrombin, and further experiments are needed. For the present it will suffice to point out that prothrombin can be changed to make it less sensitive to the interaction of calcium, thromboplastin, and Ac-globulin. Only a few years ago much thought was given to the possibility that the reactivity of prothrombin could be altered. That was one of the possible explanations for the slow conversion rate of purified prothrombin. When it was discovered that Ac-globulin would in large measure restore the activation rate of purified prothrombin, less emphasis was placed on the possibility of a true alteration of prothrombin itself. But it does occur.
Milstone\textsuperscript{10} made a special point of the fact that his prothrombin preparations became activated spontaneously under certain conditions. He emphasized that calcium ion was not necessary, but his data did not permit a decision as to whether it was the prothrombin itself or some accompanying factor which was responsible for this phenomenon. From our experiments it is evident that the concentrated ammonium sulfate which he used was the factor responsible for the prothrombin activation.

We also use ammonium sulfate in the preparation of prothrombin, but the proper conditions for activation do not exist very long. However, much of the impurity remaining in our prothrombin preparations may represent a derivative of prothrombin itself produced by the action of ammonium sulfate. The derivative produced by ammonium sulfate would very likely be the same as produced by sodium citrate and, therefore, would need to be refractory to the action of calcium, Ac-globulin, and thromboplastin, and have a mobility less than that of prothrombin. The bulk of the impurity conforms to that description and it is our belief that this explanation is valid.

In the experiments of Seegers, Loomis and Vandenbelt\textsuperscript{6} an attempt was made to examine their prothrombin product by solubility technics. A curve representing that required of pure prothrombin was obtained. In their work it was not possible to obtain the material in quantity and try to repeat the experiment. Upon repetition of the work in this laboratory it has not been possible to reproduce the curve; indeed, the curves which we have obtained do not show exact correspondence from one to another. Active material invariably appears in the solid phase before the point of saturation is reached, and the specific activity of the material in the solid phase is usually equal to that in solution.

\section*{Summary}

Prothrombin preparations were examined by electrophoresis and found to contain approximately 90 per cent of the protein in one component. Such products possess a specific activity of 1,400 units per milligram of dry weight when analyzed with reagents and technics that give a value of 32.9 ± 30 units of prothrombin per cc. of oxalated bovine plasma. The electrophoretic mobility is approximately equivalent to that of $\alpha_1$-globulin. That of thrombin is less. The isoelectric point in 0.1 ionic strength salt solution is near pH 4.2, and for thrombin it is approximately pH 4.8. Analysis of dry preparations indicated 0.96 per cent sulfur, 13.57 per cent nitrogen, 4.58 per cent tyrosine, and 3.33 per cent tryptophane, and no phosphorus was found. The purified material could be activated to thrombin by dissolving the prothrombin in concentrated solutions of sodium citrate, ammonium sulfate, magnesium sulfate, sodium sulfate, potassium citrate, potassium oxalate, and dipotassium hydrogen phosphate. Concentrated solutions of sodium chloride, potassium chloride, ammonium chloride or magnesium chloride do not produce activation. Difficulties were encountered when attempts were made to activate less highly purified prothrombin preparations with sodium citrate. The activation with sodium citrate is autocatalytic, and the yield of thrombin can be increased to a maximum by adding a small amount of 3-methyl-4,6,4'-triaminodiphenyl sulfone to
the activation mixture. The sodium citrate concentration must be high, preferably near 2.5 per cent. A new method for preparing thrombin is based on the use of these principles. During activation the electrophoretic properties of prothrombin change long before appreciable amounts of thrombin activity are found. These changes in electrophoretic properties are associated with a prothrombin derivative which is refractory to the action of the activators calcium plus thromboplastin plus Ac-globulin. When activation with sodium citrate is complete almost none of the material which had electrophoretic mobility of prothrombin is found in the electrophoresis boundary patterns. The major portion of the pattern is comprised of two components having mobilities less than prothrombin while a third component with high mobility is especially evident in the descending boundary pattern. Solubility studies indicate that two proteins may possess thrombin activity, but the evidence is not conclusive. An attempt was made to separate the proteins in the thrombin preparation by fractionation with ammonium sulfate, and it was found that their properties are so similar that this procedure is not effective. Thrombin obtained by activation of prothrombin with sodium citrate may be dried from the frozen state and is stable. Prothrombin is not stable after drying in this manner, however, it can be dried with acetone. It is reasoned that since sodium citrate and other salts can produce thrombin from prothrombin, the latter must contain all the structural material needed for the thrombin. For that reason calcium, thromboplastin, and Ac-globulin are regarded as catalysts of prothrombin activation and not as activators which must contribute material substance in prothrombin activation.

REFERENCES

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