Studies on the Antithrombin and Heparin Co-factor Activities of a Fraction Adsorbed from Plasma by Aluminum Hydroxide

By Frank C. Monkhouse, Ph.D., Evelyn S. France, M.S. and Walter H. Seegers, Ph.D.

A chemical method has been devised for the quantitative determination of plasma antithrombin. A concentrate of antithrombin has been prepared from plasma. It also contains heparin cofactor activity. The two distinct activities could not be separated and may quite possibly be the properties of a single substance. When the concentrate is combined with heparin the capacity of the concentrate to destroy thrombin is not increased but there is interference with the thrombin fibrinogen interaction. In a mixture of the concentrate and thrombin there is simultaneous destruction of antithrombin, and thrombin activities. Small amounts of the concentrate significantly prolong the clotting time of blood.

In all contemporary considerations related to the mechanisms for maintaining the fluidity of the blood, heparin, heparin cofactor and antithrombin have occupied a high rank. Numerous efforts have been made to elucidate the chemical mechanisms with which these substances are involved. Nevertheless, it is not known whether heparin cofactor and antithrombin are one substance. It seems likely than an effort to concentrate the heparin cofactor and antithrombin of plasma would now be most helpful in determining whether or not they are two separate substances. Moreover, since heparin is already available in purified form success in obtaining concentrates would make it possible to perform incisive experiments designed to relate its function to the cofactor and antithrombin mechanisms. The concentrates of heparin cofactor and antithrombin could, furthermore, be valuable source material for achieving their complete purification. From this there would logically follow more precise physical chemical studies.

A recent summary from this laboratory classifies the known antithrombin effects into four categories as follows: Antithrombin-I consists of the adsorption of small amounts of thrombin on the fibrin clot. Antithrombin-II refers to the heparin cofactor found in plasma and serum. In conjunction with heparin the cofactor is believed to interfere with the interaction of thrombin and fibrinogen. The term interference implies that reactive sites on the thrombin molecule are not neutralized by this mechanism. Their action is merely inhibited without destroying thrombin. Antithrombin-III relates to the destruction of thrombin. Presumably this involves direct combination of thrombin and antithrombin. Heparin apparently is not needed for the interaction nor does it alter the kinetics appreciably if it happens to be present. Antithrombin-IV is an effect observed during the activation of prothrombin. Apparently there is unusually rapid destruction of thrombin at the time of its formation, by mechanisms that are not understood in detail.

In this paper we show that the heparin cofactor and antithrombin can be adsorbed on aluminum hydroxide. The active material can be further concentrated from an eluate by fractionation with ammonium sulphate. The several fractions obtained during the purification procedures always possess heparin cofactor and antithrombin activity in exactly corresponding proportions. Neutralization of one activity also neutralizes the other. It is shown that the concentrate, with the aid of heparin, interferes with the thrombin fibrinogen...
interaction. Without heparin the concentrate destroys thrombin activity. Although heparin cofactor activity and antithrombin activity clearly correspond to the requirements for two distinct chemical reactions they seem to involve the same molecule. One of the great difficulties in clarifying our concepts of the antithrombin mechanisms has been the dependence upon clotting tests for the quantitative measurement of thrombin activity. In the work described below we used chemical methods and thus were able to examine critically questions of controversy not easily resolved by clotting experiments.

**Materials and Methods**

Aluminum Hydroxide. This was prepared as the C alpha product according to the method of Bertho and Grossman.¹

Heparin. A preparation of bovine heparin (Connaught Laboratories, Toronto) was used. The strength of the stock solution was 1000 units per ml.

Phosphate Buffer. 55.2 Gm. of Na₂HPO₄·H₂O were dissolved in 1 liter of distilled water. Enough 0.4 N NaOH was added (approximately 500 ml.) to bring the pH to 7.8. The volume was then made up to exactly two liters with distilled water.

TAME (p-toluenesulfonyl-arginine-methyl ester). This was a product prepared by the H. M. Chemical Co., Santa Monica, Calif. A 0.4 M solution was prepared each day.

Tris Buffer. 15.8 Gm. of trimethylol amino-methane were dissolved in 1500 ml. of distilled water and the pH was adjusted to 8.5 by the addition of 1 N. HCl (approximately 50 ml.). The volume was then made up to exactly 2 liters.

Defibrinated Plasma. Bovine plasma was defibrinated by rapidly heating to 53 C and maintaining that temperature for 4 min., then it was cooled rapidly to room temperature. The fibrinogen was removed by filtering through gauze.

Thrombin. The thrombin was a product prepared from purified bovine prothrombin by autocalysis in 25 per cent sodium citrate solution as previously described.⁷ For thrombin assay the chemical method recently developed by Sherry and Troll was used. This method is based on the observation that thrombin splits the methyl ester linkage of TAME (p-toluenesulfonyl-arginine-methyl ester). Within limits the number of carboxyl groups formed is directly proportional to the strength of the thrombin solution. By titration with NaOH to neutrality, a quantitative measure of thrombin concentration can be obtained. Throughout the course of this investigation thrombin potency was estimated with reference to a standard curve which relates quantity of NaOH used for titration to units of thrombin (fig. 1). As with previous methods⁸ a unit of thrombin is defined as that amount which will clot 1 ml. of standardized fibrinogen solution in 15 seconds.

The following is a typical analysis: 0.2 ml. of thrombin solution were mixed with 0.2 ml. TAME and 1.6 ml. tris buffer. The mixture was allowed to stand at 37 C for 30 min. At the end of this time 2 ml. of neutral formaldehyde and 6 drops of 0.01% phenol red were added. The mixture was titrated with 0.025 N NaOH to give a pink color. The volume of NaOH used was 0.90 ml. From figure 1 this corresponds to 15 thrombin units for the reaction mixture and hence the strength of the original thrombin sample was 75 U per ml.

**Results**

Measurement of Antithrombin Activity. Although Seegers, Miller, Andrews and Murphy⁷ made a thorough study of variables in the antithrombin reactions and developed a valuable quantitative method for measuring antithrombin activity we wanted a chemical method and thus it was necessary to devise a new procedure.

We define the unit of antithrombin as the amount which will neutralize one unit of thrombin in 1 hour at 28 C. Since the amount of thrombin destroyed by antithrombin varies with the concentration of the thrombin present at the beginning of the interactions,⁷ we used a constant thrombin substrate and varied the amount of antithrombin added. An example of the analytical procedure is given.

Two hundred units of thrombin (0.1 ml. of
a 2000 units per ml. solution of thrombin) are placed in each of a number of siliconized test tubes. Varying amounts (0.05-0.80 ml.) of the antithrombin solution to be assayed are then added. The volumes are adjusted to 1 ml. by addition of tris buffer. The tubes are then allowed to incubate at 28 C for 1 hour. During that time the interaction of thrombin and antithrombin is completed. To perform a quantitative analysis for the remaining thrombin 0.2 ml. aliquots of each solution are placed in test tubes containing 0.2 ml. TAME solution. The volumes are adjusted to 2 ml. with tris buffer and the tubes incubated at 37 C for 30 minutes. The extent of TAME hydrolysis is then determined and thus also the strength of the thrombin solution as described above. The total amount of thrombin destroyed is calculated for each test. A curve is then made by plotting the volume of antithrombin solution against units of thrombin neutralized. To estimate the potency of an antithrombin preparation the point on the curve which represents 100 units of thrombin destroyed (50 per cent destruction) is located and a perpendicular is dropped to the abscissa. This gives the volume of solution containing 100 units antithrombin and by simple multiplication the potency, in terms of units of antithrombin per ml., is calculated. This scale is adequate for all solution with a potency greater than 50 per cent of normal defibrinated plasma. Solutions with antithrombin potency less than this are estimated by comparing their strength with 0.2 ml. of normal defibrinated plasma.

Preparation of Antithrombin Concentrates. Many experiments were performed, and eventually a suitable adsorbant was found on which antithrombin could be adsorbed, and from which it could be eluted. An example of one preparation is given and the analytical data on various fractions are recorded by means of table 1. Five hundred ml. of defibrinated plasma were mixed with 50 grams of BaCO₃ to adsorb prothrombin. The adsorbant was removed by centrifugation. The volume was now 465 ml. Four hundred ml. of this adsorbed plasma were thoroughly mixed with 200 ml. of Al(OH)₃ and at the end of 15 minutes at room temperature the mixture was centrifuged. The precipitate was washed twice with 200 ml. quantities of distilled water and then mixed with 200 ml. of phosphate buffer. This eluate was designated 'first eluate,' and represented approximately a 32 per cent yield of antithrombin while approximately 90 per cent of the protein was deleted. The precipitate was mixed with a 100 ml. of phosphate buffer, to obtain the 'second eluate,' which represented a yield of about 12 per cent.

For purposes of further fractionation the first eluate was transferred to a stainless steel beaker and placed in an ice salt bath. One hundred and twenty ml. of saturated (NH₄)₂SO₄ were slowly added. The precipitate was removed in a refrigerated centrifuge at 4000 g.

![Fig. 2. A comparison of the potencies of antithrombin concentrates with defibrinated plasma. Varying quantities of the antithrombin solutions were incubated for 1 hr. at 28 C with 200 units thrombin. Residual thrombin was measured.](http://circres.ahajournals.org/)

### Table 1—Antithrombin Activity of Plasma Fractions

<table>
<thead>
<tr>
<th>Material</th>
<th>U/ml Solution</th>
<th>% Protein</th>
<th>U/mg Protein</th>
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<tbody>
<tr>
<td>Defibrinated plasma</td>
<td>250</td>
<td>6.17</td>
<td>4</td>
</tr>
<tr>
<td>After BaCO₃ treatment</td>
<td>230</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>After Al(OH)₃ treatment</td>
<td>&lt;25</td>
<td>3.75</td>
<td>—</td>
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<tr>
<td>First eluate</td>
<td>200</td>
<td>0.95</td>
<td>21</td>
</tr>
<tr>
<td>2nd eluate</td>
<td>150</td>
<td>0.60</td>
<td>25</td>
</tr>
<tr>
<td>Fraction I</td>
<td>&lt;50</td>
<td>2.32</td>
<td>—</td>
</tr>
<tr>
<td>Fraction II</td>
<td>500</td>
<td>1.49</td>
<td>33.5</td>
</tr>
</tbody>
</table>

**Dr. M. Burstein, Central National de Transfusion, Paris, suggested by personal communication, that antithrombin activity can be adsorbed on tricalcium phosphate. We were able to confirm this observation.**
for 15 minutes. The precipitate was dissolved in 25 ml. of tris buffer (0.05 M), placed in a special dialyzing apparatus and dialyzed in the cold for 3 hours against saline containing 0.05 M tris buffer. This product is referred to as Fraction I. It had a volume of 40 ml., contained much protein, and very little antithrombin. To the supernatant solution a further 360 ml. of saturated (NH₄)₂SO₄ solution was added and the precipitate was collected by centrifugation and dissolved in 25 ml. of tris buffer and dialyzed for 3 hours as described for Fraction I. This product is referred to as Fraction II.

Fraction II had a volume of 35 ml. and contained 34 antithrombin units per mg. protein as compared with 4 units per mg. protein found in bovine plasma. The concentrate was dried from the frozen state without loss of activity. The protein was examined in the electrophoresis apparatus and no albumin was found, consequently we are unable to support the assertion that antithrombin is an albumin.

Concentration of Heparin Cofactor in Plasma Fractions. It has long been discussed whether antithrombin and heparin cofactor represent one and the same molecule or whether there are two distinct entities. The two kinds of activities are certainly different. To distinguish cofactor activity from plasma antithrombin activity we made use of the fact that heparin plus cofactor immediately inhibits the interaction of thrombin and fibrinogen. To 0.2 ml. of a standard fibrinogen solution the following were added in the order given: 0.5 units of heparin in 0.1 ml., 0.1 ml. of saline or various dilutions of plasma fractions and finally 0.1 ml. of thrombin solution. The data are summarized in table 2. By comparing tables 1 and 2 it is apparent that heparin cofactor activity and antithrombin activity are not separated by any of our methods used to obtain these fractions. Furthermore, concentration of one activity is invariably accompanied by a corresponding concentration of the other.

According to Lyttleton, the antithrombin content of defibrinated plasma may be reduced by incubation with thrombin, and the cofactor activity is simultaneously reduced. These observations were of great interest to us and an attempt was made to confirm the observations by means of the following experiment: To 2 ml. of Fraction II were added 1000 units of thrombin and the volume was adjusted to 4 ml. with tris buffer. It had been determined by a previous experiment that these quantities of thrombin and antithrombin would neutralize each other. As a control 2 ml. of Fraction II were diluted to 4 ml. with tris buffer. These solutions were allowed to remain at room temperature overnight and the following morning they were assayed for antithrombin and cofactor activity according to the methods already described. It was found that both activities were destroyed by interaction with thrombin. The control tubes contained the full titre of cofactor and antithrombin activity. Moreover, additional stability studies indicate that our concentrate is remarkably stable. We were thus able to confirm the important work of Lyttleton, which showed that thrombin may destroy heparin cofactor and antithrombin.

Heparin and Thrombin Inactivation. On the basis of clotting experiments the view has evolved that heparin and the cofactor interfere with the thrombin fibrinogen interaction, and that the rate or quantity of thrombin de-

<table>
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<th>Dilutions</th>
<th>F.S.</th>
<th>1:2</th>
<th>1:3</th>
<th>1:4</th>
<th>1:5</th>
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<th>1:10</th>
<th>1:15</th>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Defibrinated plasma</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>60</td>
<td>45</td>
<td>40</td>
<td>34</td>
<td>30</td>
<td>100</td>
<td>50</td>
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<td>First Eluate</td>
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<td>—</td>
<td>—</td>
<td>100</td>
<td>50</td>
<td>40</td>
<td>33</td>
<td>29</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction I</td>
<td>25</td>
<td>25</td>
<td>—</td>
<td>24</td>
<td>—</td>
<td>—</td>
<td>100</td>
<td>50</td>
<td>35</td>
<td>28</td>
<td></td>
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<tr>
<td>Fraction II</td>
<td>—</td>
<td>—</td>
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<td>—</td>
<td>100</td>
<td>50</td>
<td>35</td>
<td>28</td>
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* Control Clotting Times: Saline + Fibrinogen + Thrombin 15 sec.; Heparin + Fibrinogen + Thrombin 25 sec.
destroyed by antithrombin is not altered by heparin. We have been able to uphold these viewpoints with experiments in which the chemical method for the determination of thrombin activity was used. We had a product containing both activities in concentrated form, thus it was possible to determine if the addition of heparin to this product would alter its ability to destroy thrombin.

Tubes were set up to obtain varying quantities of antithrombin and also varying quantities of heparin. At intervals of 5, 20, 35 and 90 minutes aliquots of the incubation mixtures were added to TAME and assayed for thrombin as previously described. The results, at 2 of these intervals; namely 5, and 90 minutes, are shown on figure 3. In no instance did the addition of heparin increase the amount of thrombin destroyed. Contrariwise there was actually a decrease in the amount of thrombin destroyed, thus indicating that heparin may, to a certain extent, inhibit antithrombin activity rather than facilitate it as was commonly believed in the past.

**Antithrombin as Anticoagulant.** With concentrates of antithrombin at our disposal it was possible to determine, for the first time, what happens with respect to the clotting time of whole blood when antithrombin is added. Table 3 illustrates the prolongation in clotting time following the addition of concentrated antithrombin to dog’s blood. The antithrombin used was a Fraction II concentrated by lyophilization and again dissolved so that 0.1 ml. was equal in antithrombin strength to 0.5 ml. of defibrinated bovine plasma (fig. 2). The blood was withdrawn from the vein of an anesthetized dog, into a siliconed syringe. One ml. portions of the blood were immediately placed in pyrex tubes (13 x 100 mm.) containing varying amounts of the antithrombin. The clotting time was significantly prolonged both in ordinary glass and in the siliconed tubes. For example, clotting occurred in 20 minutes (glass) when the antithrombin equivalent of 2.4 ml. of bovine plasma was added to one ml. of blood.

**DISCUSSION**

The separation of the antithrombin and heparin cofactor activities of plasma by adsorption on aluminum hydroxide has made it possible to obtain a product which is eight times as concentrated per mg. of protein as plasma itself. One of the original objectives of this investigation was to see whether we could separate heparin cofactor and antithrombin, but we did not find that possible. When one activity was concentrated the other was also concentrated and to an equal degree. When one was destroyed the other was also destroyed. Particularly interesting was the finding that the destruction of antithrombin by means of incubating it with thrombin was also accompanied by the neutralization of cofactor activity. Lyttleton\(^\text{12, 13}\) takes the view that the two activities are due to different substance. Nevertheless it is our opinion that his data could be used equally well for regarding the two activities as being associated with one substance. It may eventually be possible to show conclusively that two distinct phenomena can be ascribed to the activity of one substance and

<table>
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<th>ml Antithrombin</th>
<th>Clotting Time (min.)</th>
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<tr>
<td></td>
<td>Silicided Tubes</td>
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<tr>
<td>0.00</td>
<td>8</td>
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<tr>
<td>0.10</td>
<td>14</td>
</tr>
<tr>
<td>0.20</td>
<td>18</td>
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<tr>
<td>0.30</td>
<td>34</td>
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</table>

**Fig. 3.** The effect of heparin on the thrombin neutralizing potency of antithrombin concentrates. Varying amounts of antithrombin plus heparin and of antithrombin alone were incubated for 1 hour at 25 C with 200 units thrombin. Residual thrombin was measured.
perhaps even to the same group on the molecule. Meanwhile the interference with the thrombin fibrinogen interaction (heparin cofactor phenomenon or antithrombin-II) is an activity so distinctly different from thrombin inactivation (antithrombin-II) that an apparent dualism will always present itself. A cofactor is required for inhibiting the activation of prothrombin with heparin. Eventually it may also be found that our concentrate is concerned with the inhibition of prothrombin activation, and that might represent a third function of the same substance.

By considering a single substance in these mechanisms we may speculate about the practicality of a combination of concentrated antithrombin and heparin as a useful anticoagulant. Even the concentrate, without heparin, has anticoagulant properties. With further concentration this might be made sufficiently powerful to prevent the blood from clotting. This makes it desirable to do experimental work to determine whether alterations in clotting time can be brought about in vivo.

The fact that our antithrombin concentrate destroys thrombin and simultaneously loses its own antithrombin activity as well as its own heparin cofactor activity may be taken as an indication that interactions occur in combining proportions. Quite possibly an addition compound forms between thrombin and antithrombin. This would also account for the observation that the antithrombin concentration of serum is lower than that of plasma.

Summary

A method is described for the quantitative determination of antithrombin concentration. This method depends upon the hydrolysis of p-toluenesulfonyl-arginine-methyl ester by thrombin. A fraction has been prepared from bovine plasma by adsorption on Al(OH)₃ followed by (NH₄)₂SO₄ fractionation that contains antithrombin and heparin cofactor activities in concentrations equal to 8 times their plasma concentrations. Electrophoretic studies indicate that the concentrate contains very little if any protein with mobility equal to albumin. Incubation of the concentrate with thrombin simultaneously destroys heparin cofactor, antithrombin, and thrombin activities. The addition of heparin to this fraction does not increase its capacity to destroy thrombin nor does it increase the rate at which thrombin is inactivated. This supports the view that heparin plus cofactor interferes with the thrombin fibrinogen interactions. Relatively small amounts of antithrombin concentrate significantly prolongs the clotting time of dog blood.

REFERENCES

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Circ Res. 1955;3:397-402
doi: 10.1161/01.RES.3.4.397

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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