THE EFFECT OF INTRAVENOUS INJECTION OF TRYPsin INHIBITOR ON THE COAGULATION OF BLOOD

By H. J. Tagonon, M.D., and J. P. Soulier, M.D.

The TRYPSIN inhibitor isolated from soya bean flour by Kunitz has been shown to have anticoagulant activity in vitro. The present communication deals with the effects of the intravenous injection of this material into experimental animals. The action on the clotting time, the prothrombin time, and the antitryptic activity of blood plasma and serum were studied.

METHODS

Trypsin inhibitor: A crude preparation was obtained by the method described previously. Dialysis of the final preparation for twenty-four hours against saline in the ice-box was found to be necessary for the removal of a toxic factor present in the undialyzed material. Without dialysis, small amounts (0.5-1 cc.) of the material regularly killed the 5-pound rabbits in a very short time (30 to 60 seconds) with cardiac standstill in diastole. It is possible that the toxicity was due to the presence of potassium ions in the preparation.

The final preparation was spun at 1500 rpm for 15 minutes in order to remove all insoluble material, and the pH was adjusted to 7.4 by the addition of NaOH N/10. Enough material was prepared in one batch so that all experiments except one (in which the crystalline inhibitor was used) were carried out with the same batch of material. It was kept frozen at -30°C and warmed up to 37°C shortly before the injection was given.

The preparation of inhibitor was assayed against crystallin trypsin (obtained from the Plaut Research Laboratory, Bloomfield, N. Y.) by the method of Anson. Twenty-five milligrams of this trypsin preparation produced 0.1137 mg. of tyrosin in 10 minutes at 35°C. The addition of 0.1 cc. of the inhibitor preparation used in this work to this quantity of trypsin reduced the production of tyrosin from 0.1137 mg. to 0.008 mg.

A small quantity of crystallin trypsin inhibitor, recrystallized 3 times, obtained by the method of Kunitz was used in one single experiment as indicated below.

Experimental animals: 2 mongrel dogs and 3 rabbits received injections of the inhibitor preparation. One additional rabbit was injected with the crystallin material. The animals were anesthetized by the intravenous injection of from 25 to 35 mg. of nembutal per kg. Injections were made into the jugular vein in the dogs and the ear vein in the rabbits. Blood samples were obtained by syringe and needle from the other jugular vein in the dogs and from the carotid arteries in the rabbits. The blood samples were taken simultaneously with and without anticoagulant. A mixture of 2 parts of potassium oxalate and 3 parts of ammonium oxalate in dry form was used as an anticoagulant. Ten milligrams of the mixture was used for every 5 cc. of blood. The plasma was removed immediately by centrifuging.

The clotting time was studied on blood taken without anticoagulant by a modification of the method of Lee and White.

The prothrombin time was measured on the plasma by the method of Quick.

The presence of an anticoagulant in the samples showing a prolongation of the clotting time was tested as described previously.

In the experiments on rabbits the samples of plasma were tested for their antitryptic activity before and after the injection of trypsin inhibitor preparation. One half cubic centimeter of oxalated plasma was mixed with one half cubic centimeter, of a solution of crystallin trypsin and the proteolytic activity of

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts; and the Sloan-Kettering Institute of the Memorial Hospital for the treatment of Cancer and Allied Diseases New York, New York.
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The mixture measured on a hemoglobin substrate by incubation at 37°C for 4 hours, according to the method of Anson. The crystallin trypsin was the same used for the assay of the inhibitor. 

In the experiments on dogs the samples of blood serum were tested before and after the injection of trypsin inhibitor for antiproteolytic activity against the blood plasma enzyme. As shown previously, the trypsin inhibitor from soya bean is also inhibitor towards the blood plasma enzyme. This was done by measuring the rate of dissolution of 0.1 cc. of fibrinogen solution by a chloroform plasma preparation (containing the active plasma proteolytic enzyme) in the presence of each sample of serum, as described previously.

Results

Tables 1 and 2 show the overall results obtained in the experiments on two dogs and four rabbits.

1. Effect on clotting time. There was an immediate prolongation of the clotting time following the injection of the soya bean preparation in dogs as well as in rabbits. This effect was transient, lasting from forty minutes to one hour, after the injection of respectively 8 cc. per Kg. and 5 cc. per Kg. into the 2 dogs, and from 30 to 60 minutes in the 3 rabbits that were followed longer than one hour.

Table 1.—The Effect of the Intravenous Injection of Trypsin Inhibitor on Clotting Time, Prothrombin Time and Fibrinolysis

<table>
<thead>
<tr>
<th>Dog #</th>
<th>Trypsin Inhibitor (cc.)</th>
<th>Clotting time at 37°C Intervals**</th>
<th>Prothrombin time Intervals**</th>
<th>Time of lysis of fibrinogen in presence of serum from blood taken at stated intervals*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>8 55 40 24 11</td>
<td>9 7 10 9.5 9.5 9.5 9.5 9.5</td>
<td>3 106 136 136 112 124 76</td>
</tr>
<tr>
<td>16 Kg.</td>
<td>80</td>
<td>10 52 13 11</td>
<td>14 16 16 16 16 16</td>
<td>11 180* 180* 120*</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 Kg.</td>
<td>80</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 0.1 cc. fibrinogen + 0.1 cc. serum + 0.4 cc. chloroform plasma preparation.

** 1: before injection; 2: from 5-10 minutes after injection; 3: from 10-30 minutes after injection; 4: from 30-40 min. after injection; 5: from 46-60 min. after injection; 6: from 60-90 min. after injection.

The injection of the small available quantity of crystallin inhibitor into rabbit # 4 produced a small prolongation of the clotting time (table 2, exp. 4).

2. Effect on prothrombin time. This was prolonged following the injection into dogs and rabbits and remained prolonged for a longer period than the clotting time (tables 1 and 2).

3. Effect on antiproteolytic activity of blood serum and blood plasma. In the 2 experiments on dogs a chloroform plasma preparation (containing the active plasma proteolytic enzyme) was mixed with serum from blood obtained before and after the intravenous injection of trypsin inhibitor, and the mixture was tested for fibrinolytic activity on a solution of fibrinogen. Table 1 shows that the time for complete fibrinolysis of the clot increased sharply in the presence of serum from blood obtained immediately after the injection of the inhibitor. The time of fibrinolysis was still considerably prolonged at the end of the 2 experiments (table 1). Figure 1 presents the results of experiment 1 in graph form.

In the 4 experiments on rabbits, the amount of tyrosin produced by trypsin in
The Effect of Intravenous Injection of Trypsin Inhibitor on Clotting Time Prothrombin Time and Antiprotolytic Activity of Plasma

<table>
<thead>
<tr>
<th>Rabbits</th>
<th>Type of preparation</th>
<th>Clotting time at 37°C, min.</th>
<th>Prothrombin time, sec.</th>
<th>Quantity of tyrosin produced by trypsin in pres. of plasma (mg X 10^-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Soya bean prepar., 17 cc.</td>
<td>10.25</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>1</td>
<td>Soya bean prepar., 30 cc.</td>
<td>1</td>
<td>0.24</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>Soya bean prepar., 30 cc.</td>
<td>11.17</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>Crystallin inhab. 45 mg. in 5 cc. saline</td>
<td>8.13</td>
<td>10</td>
<td>7</td>
</tr>
</tbody>
</table>

* 1: before injection; 2: 5 minutes after injection; 3: 15 minutes; 4: 30 min.; 5: 60 min.; 6: 2 hours; 7: 3 hours.

** 0.5 cc. oxalated plasma + 0.5 cc. solution of trypsin + 5 cc. hemoglobin substrate. Incubation for 1 hour at 37°C.

Solution of trypsin 30 mg. in 10 cc. of water in experiments 1 and 2.

2.0 " " 10 cc. " " 3 " 4.

**Fig. 1. The Effect of Intravenous Injection of Soya Bean Preparation on Antiprotolytic Activity of Blood Serum, Clotting Time and Prothrombin Time**

the presence of samples of plasma showed a sharp decrease with plasmas obtained after injection of the inhibitor (table 2). There was a gradual disappearance of the
increased antitryptic effect of plasma and normal or near normal values were obtained between 1 and 2 hours after the injection. Figure 2 shows the results of experiment #4 (table 2) in graph form.

**FIG. 2. THE EFFECT OF INTRAVENOUS INJECTION OF CRYSTALLIN TRYPsin INHIBITOR ON ANTI-TRYPTIC ACTIVITY OF BLOOD PLASMA, CLOTTING TIME AND PROTHROMBIN TIME**

**TABLE 3.—Protamine Titration of Plasma after Injection of Soya Bean Preparation**

One-tenth cc. of citrated plasma obtained from blood showing a prolonged clotting time following the injection of soya bean preparation was added to varying quantities of protamine. The plasma was recalcified and the clotting time noted.

<table>
<thead>
<tr>
<th>Protamine</th>
<th>Clotting time at 37 C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dog #2</td>
</tr>
<tr>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>0.01</td>
<td>30+</td>
</tr>
<tr>
<td>0.004</td>
<td>30</td>
</tr>
<tr>
<td>0.001</td>
<td>11</td>
</tr>
<tr>
<td>0.0005</td>
<td>11</td>
</tr>
<tr>
<td>0.0001</td>
<td>11</td>
</tr>
</tbody>
</table>

In order to rule out the presence of heparin in blood showing a prolonged clotting time following the intravenous injection of soya bean preparation, a protamine titration was carried out on the plasma from such blood in the 2 experiments on dogs. Table 3 gives a typical example of such a titration in dog #2. The results
show that there was no significant shortening of the clotting time of the recalcified plasma by the addition of various quantities of protamin.

**Discussion**

The data show that a soya bean preparation containing the trypsin inhibitor produced the following effects when injected intravenously into 2 dogs and 3 rabbits: it prolonged the clotting time of the blood, the prothrombin time of the blood plasma, and increased the antiproteolytic activity of the blood serum or plasma. The same effects were obtained in one experiment on a rabbit, in which a highly purified, three times recrystallized trypsin inhibitor preparation from soya bean was used.

There was some parallelism among the effects of the injection on the clotting time, the prothrombin time and the antiproteolytic activity of the plasma. This parallelism was most apparent in the experiments on rabbits because they were conducted for a longer time than those on dogs. The coincidence of the three actions appears clearly in figure 1.

The one single experiment carried out with the purified material gave results essentially similar with those obtained with the cruder material.

If one considers the fact that the trypsin inhibitor produced all these effects when added to blood in vitro, it seems that the data reported here might constitute evidence that the trypsin inhibitor prolonged the clotting time in vivo by the same mechanism by which it prolongs it in vitro, and not indirectly by provoking the organism to release an anticoagulant in the blood stream. This is further confirmed by the fact that the blood showing a prolonged clotting time following the injection of the trypsin inhibitor did not contain any heparin in the two experiments in which a protamin titration was carried out. It is well known that the prolongation of the clotting time following the intravenous injection of peptone or of antigen in sensitized animals is due to the appearance of heparin in the blood of such animals.

Three different trypsin inhibitors so far have been reported as having anticlotting properties: the trypsin inhibitor from pancreas, from blood serum and that from soya bean. These three substances differ chemically and their similar action on the blood coagulation mechanism parallels their similar action on trypsin. It is to be noted that the trypsin inhibitor from soya bean and from pancreas also inhibits the proteolytic enzyme of blood plasma. The exact role of the plasma enzyme in blood coagulation is unknown: a recent communication presents evidence that the enzyme has no clotting activity by the usual tests. It is nevertheless interesting to note that these substances which inhibit the proteolytic action of the enzyme also are anticoagulant agents.

The practical use of the trypsin inhibitor from soya bean for anticoagulant therapy must await further study. The material does not appear to be toxic and could conceivably be used, in purified form, for prolonging the clotting time in vivo when such action is desired. However it is quite possible that the inhibitor is antigenic and this point should be clarified before an attempt at practical application is made.
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Summary

1. The intravenous injection of a soya bean preparation containing a trypsin inhibitor into 2 dogs and 3 rabbits produced the following effects: prolongation of the clotting time and of the prothrombin time, and increase in the antiproteolytic activity of the blood plasma or serum.

2. Identical results were obtained in one experiment in which a crystalline trypsin inhibitor from soya bean was used.

3. The significance of these results is briefly discussed.

REFERENCES

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