THE ANTIPROTEOLYTIC ACTIVITY OF SERUM

II. Physiological Significance. The Influence of Purified Trypsin Inhibitor on the Coagulation of the Blood

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Morawitz (1904) originally suggested, and Eagle and Harris (1937) have presented considerable evidence that, in the course of physiological coagulation, calcium plus thromboplastic substance constitute an enzyme system, analogous to trypsin and to several other proetolytic enzymes. This enzyme system is believed to be responsible for the transformation of prothrombin to thrombin, which itself is thought to be a proteolytic enzyme that accelerates the transformation of fibrinogen to fibrin (as first suggested by Schmidt, 1892).

In view of the evidence for this mechanism, it was suspected that serum antiprotease might normally play some rôle in the process of coagulation. This was investigated by studying the effect on coagulation not only of purified serum antiprotease (prepared after the method of Schmitz (1938), which provided a yield of 40 mg. from 5 liters of pig's blood), but also of crystalline pancreatic trypsin inhibitor (prepared from beef pancreas after Northrop and Kunitz (1932–33)), which is believed by Schmitz to be almost identical with the serum compound, and which can be obtained in far greater amounts.

The effect of varying amounts of 0.5 per cent solutions of these preparations (at neutral pH) on the clotting time of oxalated rabbit's plasma, recalcified with an optimum amount of Ca^{++} (as determined in Table I), is recorded in Table II. The clotting time was taken at 37°C. as the time between the addition of the Ca^{++} and the formation of a clot firm enough not to move down standard Wassermann tubes (run in duplicate) when these were inverted. It is seen that some substance in these preparations is capable of markedly increasing the coagulation time. Control tubes showed that ammonium sulfate, from which the preparations could not be entirely freed, was not important in this respect.

Table III describes the effect of adding increasing amounts of crude trypsin to plasma to which serum antitrypsin or pancreatic trypsin inhibitor had been added, and the resulting solution allowed to stand for 15 minutes before the addition of Ca^{++}. (It was found that a time interval elapsed before the anti-

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coagulant action was complete.) It is observed that the effect of the trypsin was to progressively neutralize the effect of the trypsin inhibitor preparation.

TABLE I

Optimum Concentration of Ca^{++}

Oxalated plasma	1 per cent $CaCl_2$	0.5 per cent Pancreatic trypsin inhibitor	0.5 per cent Serum antitrypsin	0.1 per cent Crude trypsin	0.85 per cent NaCl	Coagulation time
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*min.*
0.5	0.2	—	—	—	0.8	∞
0.5	0.3	—	—	—	0.7	∞
0.5	0.4	—	—	—	0.6	5.2
0.5	0.5	—	—	—	0.5	4.0
0.5	0.6	—	—	—	0.4	6.8
0.5	0.7	—	—	—	0.3	9.1

TABLE II

Anticoagulant Action of Purified Antiprotease

Oxalated plasma	1 per cent $CaCl_2$	0.5 per cent Pancreatic trypsin inhibitor	0.5 per cent Serum antitrypsin	0.1 per cent Crude trypsin	0.85 per cent NaCl	Coagulation time
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*min.*
0.5	0.5	0.0	—	—	0.5	4.0
0.5	0.5	0.05	—	—	0.45	5.1
0.5	0.5	0.1	—	—	0.4	6.3
0.5	0.5	0.2	—	—	0.3	9.0
0.5	0.5	0.3	—	—	0.2	22
0.5	0.5	0.4	—	—	0.1	57
0.5	0.5	0.5	—	—	0.0	20 hrs.
0.3	0.3	—	0.0	—	0.4	4.5
0.3	0.3	—	0.1	—	0.3	6.0
0.3	0.3	—	0.2	—	0.2	8
0.3	0.3	—	0.3	—	0.1	11
0.3	0.3	—	0.35	—	0.05	20
0.3	0.3	—	0.4	—	0.0	120

Table IV serves as a control to show that the addition of trypsin (in the amounts employed) to oxalated plasma plus Ca^{++} of itself only slightly accelerates coagulation. Hence the effect of trypsin described above must be chiefly a specific neutralizing (or destructive?) effect on the anticoagulant.

The addition of trypsin to oxalated or citrated plasma is known to cause coagulation. In Table V is described the effect of adding increasing amounts of trypsin inhibitor to the oxalated plasma plus trypsin. This is seen to have

resulted in a progressive increase in the time required for the plasma to coagulate.

These experimental results strongly indicate that a sufficient concentration of serum antitrypsin or pancreatic trypsin inhibitor, prepared as indicated above, strongly inhibits the coagulation of plasma *in vitro*. This effect is largely prevented by the addition of trypsin. That an anticoagulant other

TABLE III

Neutralizing Action of Trypsin on the Anticoagulant

Oxalated plasma	1 per cent $CaCl_2$	0.5 per cent Pancreatic trypsin inhibitor	0.5 per cent Serum antitrypsin	0.1 per cent Crude trypsin	0.85 per cent NaCl	Coagulation time
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*min.*
0.5	0.5	0.3	—	0.0	0.3	24
0.5	0.5	0.3	—	0.05	0.25	18
0.5	0.5	0.3	—	0.1	0.2	14
0.5	0.5	0.3	—	0.2	0.1	12
0.5	0.5	0.3	—	0.3	0.0	10
0.3	0.3	—	0.3	0	0.3	11
0.3	0.3	—	0.3	0.1	0.2	11
0.3	0.3	—	0.3	0.2	0.1	9
0.3	0.3	—	0.3	0.3	0.0	5.5

TABLE IV

Non-Influence of 0.1 Per Cent Trypsin on Coagulation by Ca^{++}

Oxalated plasma	1 per cent $CaCl_2$	0.5 per cent Pancreatic trypsin inhibitor	0.5 per cent Serum antitrypsin	0.1 per cent Crude trypsin	0.85 per cent NaCl	Coagulation time
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*min.*
0.5	0.5	—	—	0.0	0.3	4
0.5	0.5	—	—	0.1	0.2	4
0.5	0.5	—	—	0.2	0.1	3.8
0.5	0.5	—	—	0.3	0.0	3.6

than a trypsin inhibitor was present in one or both of the preparations is of course possible, but is contraindicated by the neutralizing effect of trypsin.

In an attempt to determine the mode of action of the anticoagulant varying amounts of pancreatic trypsin inhibitor were added to thrombin (prepared by adding thromboplastin (after Mills and Guest, 1921) and Ca^{++} to prothrombin (after Mellanby, 1931)), a time interval of 15 minutes allowed to elapse, and fibrinogen (salted from plasma with sodium chloride) added. Table VI indicates the absence of any appreciable antithrombic effect by the trypsin inhibitor.

The "antiprothrombic" effect of pancreatic trypsin inhibitor was investigated by adding varying amounts to prothrombin plus thromboplastin, and adding calcium after 15 minutes. Then, after 2, 5, and 10 minutes a given

TABLE V

Neutralization of Tryptic Action by Purified Antiprotease

Oxalated plasma	1 per cent $CaCl_2$	0.5 per cent Pancreatic trypsin inhibitor	0.5 per cent Serum antitrypsin	0.1 per cent Crude trypsin	0.85 per cent NaCl	Coagulation time
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*min.*
0.5	—	0	—	0.4	0.4	9
0.5	—	0.1	—	0.4	0.3	10
0.5	—	0.2	—	0.4	0.2	12
0.5	—	0.3	—	0.4	0.1	14
0.5	—	0.4	—	0.4	0	17
0.3	—	—	0	0.4	0.4	8
0.3	—	—	0.1	0.4	0.3	8
0.3	—	—	0.2	0.4	0.2	9
0.3	—	—	0.3	0.4	0.1	10
0.3	—	—	0.4	0.4	0	13

TABLE VI

Lack of Antithrombic Activity of Trypsin Inhibitor

Fibrinogen (in 0.85 per cent NaCl)	Thrombin (in 0.85 per cent NaCl)	0.5 per cent Pancreatic trypsin inhibitor	0.85 per cent NaCl	Coagulation time
cc.	*cc.*	*cc.*	*cc.*	*min.*
0.5	0.4	—	0.3	2.6
0.5	0.3	—	0.4	3.1
0.5	0.2	—	0.5	5.0
0.5	0.1	—	0.6	11
0.5	0.05	—	0.65	30
0.5	0.4	0.3	0	3.0
0.5	0.3	0.3	0.1	3.5
0.5	0.2	0.3	0.2	5.4
0.5	0.1	0.3	0.3	10
0.5	0.05	0.3	0.35	32

amount of each mixture was tested for thrombic activity (Mellanby, 1917) by adding it to a solution of fibrinogen and determining the time that elapsed before coagulation took place. Table VII shows that the trypsin inhibitor markedly inhibited the conversion of prothrombin to thrombin by the action of thromboplastin plus Ca^{++}. Whether it did so by acting on the prothrombin, or

thromboplastin, or both, was not experimentally determined. But if the mechanism of coagulation described above is even partly correct, action on the thromboplastin is strongly indicated. That the inhibitor does not act by reducing the concentration of ionized calcium was easily proved by demonstrating that the presence of excess Ca^{++} would not neutralize the effect of the anticoagulant. In fact, it resulted in a further prolongation of the coagulation time.

Various workers have suggested that in addition to their probable rôle in the coagulation process proteolytic enzymes are responsible for thrombinolysis,

TABLE VII

Antiprothrombic Activity of Trypsin Inhibitor

	Prothrombin (in 0.85 per cent NaCl)	1 per cent $CaCl_2$	Thromboplastin (in 0.85 per cent NaCl)	0.5 per cent Pancreatic trypsin inhibitor	0.85 per cent NaCl	Coagulation time
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*min.*
A	0.5	0.1	0.1	0	0.3	—
B	0.5	0.1	0.1	0.3	0	—
Fibrinogen *cc.*						
0.5 + 0.1 cc. of A after 2 min. incubation						50
0.5 + 0.1 cc. of A after 5 min. incubation						24
0.5 + 0.1 cc. of A after 10 min. incubation						3
0.5 + 0.1 cc. of B after 2 min. of incubation						>24 hrs.
0.5 + 0.1 cc. of B after 5 min. of incubation						120 min.
0.5 + 0.1 cc. of B after 10 min. of incubation						10 min.

fibrinolysis, and syneresis. Inhibition by purified antiprotease would afford evidence that proteases are involved in these processes.

Hemophilia

Almost all the current theories on the etiology of hemophilia agree in attributing the prolonged coagulation time observed in this condition to a delay in the formation of thrombin. That a deficiency of active thromboplastic substance may be the cause of this is indicated by the fact that hemophilic blood can be made to coagulate in normal time by adding additional platelets (normal or hemophilic), or by adding trypsin (Tyson and West, 1937; Ferguson and Erickson, 1939). In view of these findings, and of the evidence of Addis (1911) and of Eagle (1935) that the platelets and prothrombin content of hemophilic blood are normal, the possibility that an inhibitor of thromboplastin is responsible for the delayed activation of prothrombin to thrombin must be considered.

Evidence that, if such a thromboplastin inhibitor is present in hemophilic

blood, it does *not* possess antiproteolytic activity, was supplied by examination of the plasma and serum of two hemophiliacs (one of whom was studied through the courtesy of Dr. W. H. Howell). In both of these the antiproteolytic activity was in the normal range, despite greatly prolonged coagulation time.

Heparin

If we assume serum antiprotease to be a circulating anticoagulant the possibility of its identity with one of the known circulating anticoagulants must be considered.

It has been found by independent investigators that anaphylactic shock or peptone injection results in an appreciable increase in the antithrombin content of the serum as well as in its antiproteolytic activity (Jobling, Petersen, and Eggstein, 1915). In addition, both antithrombic (Mellanby, 1908) and

TABLE VIII

	Dog 1		Dog 2	
	Coagulation time	Antiproteolytic activity	Coagulation time	Antiproteolytic activity
	min.	*S units/cc.*	*min.*	*S units/cc.*
Before heparinization	20	3.6	17	4.5
During heparinization	115	3.8	90	4.1
(Later) during heparinization	45	3.2	53	4.6

antiproteolytic activity (Fujimoto, 1918) are destroyed by heating plasma or serum at 75° for 10 minutes. However, that antiprotease and antithrombin are separate entities is indicated by the experiments of Mellanby and Pratt (1938), and by the evidence presented above that pancreas trypsin inhibitor has little or no antithrombic activity.

Recently Jaques and Waters (1940) have shown that heparin is also increased in peptone shock, while other investigators (Glazko and Ferguson, 1940, and Horwitt, 1940) have found that heparin, in sufficient concentration, inhibits the action of trypsin. However, some evidence against the identity of heparin and serum antiprotease is supplied by the finding that heparinization does not increase the antiproteolytic activity of the blood.

The blood of two heparinized dogs was followed in the Hunterian laboratory through the courtesy of Dr. H. B. Schumacker. Table VIII illustrates the lack of any relation found between the heparin content of the blood (as indicated by the clotting time) and the antiproteolytic activity of the serum. (The antiproteolytic activity of plasma and of serum are normally equal.) The serum

of the heparinized blood was allowed to remain in contact with the alkaline trypsin (crude) for 30 minutes before the casein was added, as suggested by Horwitt. This is a condition which is *not* ordinarily necessary for the antiproteolytic action of serum. Additional experiments showed that the inhibiting action of purified heparin on crude trypsin is much less than the action described by Horwitt on crystalline trypsin. Serum, on the other hand, is equally active against the crude and the crystalline enzyme. Furthermore, as Tyson and West (1937) have shown, trypsin does not accelerate the coagulation of heparinized blood, while it has been known to hasten the clotting of oxalated or citrated blood.

These considerations indicate that while a relatively high concentration of heparin may, *in vitro*, have some antitryptic activity, it is probably not responsible for an appreciable part of the antitryptic activity of the serum.

Horwitt has made the interesting observation that many basic dyes which are powerful anticoagulants are also antiproteolytic. As in the case of heparin this parallelism deserves investigation.

SUMMARY

1. Serum antitrypsin and pancreatic trypsin inhibitor inhibited the coagulation of plasma *in vitro*.

2. This could be largely prevented by trypsin.

3. The anticoagulant action of the trypsin inhibitor was apparently due to its antiprothrombic action. It had no appreciable antithrombic action.

4. Examination of the blood of two hemophiliacs indicated that the prolonged coagulation time of their blood is not due to an excess of trypsin inhibitor.

5. Examination of the blood of heparinized dogs indicated that heparin does not appreciably contribute to the antiproteolytic activity of the serum.

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