suppression of background activity, if any exists, so that the area above the tuning curve may be called the excitatory or inhibitory response area. Here, the excitatory response area is simply called the excitatory area.
7. The Q value is the best frequency divided by the band width of an excitatory area at 10 db above the minimum threshold.
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Coagulation Inhibitor Elicited by Thrombin

Abstract. *A protein fraction present in bovine prothrombin complex, or in prothrombin-free preparations obtained from serum, is converted by thrombin into an anticoagulant that strongly interferes with the intrinsic pathway of blood coagulation and with thrombin formation, initiated also by the extrinsic mechanism. If one assumes that a similar phenomenon takes place in vivo, the association between thrombosis and hemorrhage may be better understood.*

Thrombin, the basic procoagulant and the unique clotting factor that accounts for fibrin formation, when infused or continuously released into circulation brings about a reaction in which bleeding rather than thrombosis is a predominant symptom (*1*, *2*). The marked hemorrhagic tendency is often manifested by a prolongation of clotting time to the limit of uncoagulability, for which the accepted knowledge of hemostasis does not provide a sufficient explanation. The decrease of platelets and coagulation factors is not great enough to delay the clot formation that extensively, and the presence of heparin in blood was never convincingly demonstrated. Fibrinolysis with formation of breakdown products does not occur regularly and, at least in some experimental thrombinemias, was ruled out as a cause of impaired clotting (*2*). The present study, although made in vitro, may clarify certain mechanisms underlying abnormalities in coagulation under circumstances in which thrombin is present in the blood.

An earlier observation (*3*) indicated that a coagulation inhibitor can be isolated by chromatography from preparations of bovine prothrombin complex treated with a small amount of thrombin or converted into thrombin in strong salt solutions. Since in both cases prothrombin undergoes functional and structural alterations (*4*, *5*), the anticoagulant that became evident at this stage was considered to be a prothrombin derivative that inhibits prothrombin conversion on a competitive basis. I have now obtained evidence that a protein fraction normally present in bovine blood, and independent of prothrombin, is converted by thrombin into a clot-retarding factor of great potency. The precursor (proinhibitor) absorbs together with the vitamin K–dependent coagulation factors from plasma and serum and is therefore present in preparations of prothrombin complex.

For study, preparations were obtained according to the method described previously (*5*), with the use of barium carbonate or barium sulfate for the initial absorption of proteins. The starting material was either oxalated bovine plasma or normal bovine serum. From the chromatograms shown in Fig. 1, it is evident that inhibitor activity was elicited by thrombin in regular prothrombin preparations absorbed from plasma, as well as in prothrombin-free preparations obtained in a similar manner from serum. Thrombin as a procoagulant was eluted early from the column, and then a spectacular clot-retarding activity became evident. It is not represented by a distinct protein peak in chromatograms; therefore, it may be assumed that the corresponding protein fraction was rather small. I was also able to separate first the proinhibitor from nonactivated preparations as a fraction trailing the prothrombin peak (fraction 2) and subsequently activate it with thrombin. The prothrombin portion from the same ion exchange procedure (fraction 1) generated no anticoagulant activity upon contact with thrombin. Nonactivated serum preparations contained a weak and preformed clot-retarding activity, probably elicited by thrombin during blood clotting. Additional contact with thrombin brought about a severalfold potentiation of inhibitor. Prolongation of partial thromboplastin time in bovine plasma with inhibitor-containing fractions was often over 20 minutes. In many assays only a partial clot appeared first, and a solid one formed several minutes later. In those samples, the prothrombin consumption was greatly impaired. One hour after coagulation the two-stage determination indicated 75 to 100 units of residual prothrombin per milliliter (less than 10 units in the control).

Table 1. Influence of inhibitor on coagulation tests in bovine plasma. TPLN, thromboplastin.

Reagents used in the test	Clotting time (sec) of bovine plasma (0.1 ml)	
	Plus saline (0.1 ml)	Plus inhibitor* (0.1 ml)
Calcium	74	190
Partial thromboplastin†	58	720
Partial TPLN + kaolin	51	380
TPLN (bovine brain)†	18	28
Stypven†	24	27
Stypven + phospholipid†	13	48
Factor Xa†	31	40
Factor Xa + phospholipid†	17	83
Thrombin (purified)	15	15

* Fraction from chromatography of serum product activated with thrombin. † Calcium also present.

Table 2. Bovine inhibitor in coagulation of plasmas from different species.

Species	Partial thromboplastin time (sec) of species plasma (0.1 ml)	
	Plus saline (0.1 ml)	Plus inhibitor* (0.1 ml)
Cow	57	> 600
Man	61	50
Dog	21	42
Horse	85	61
Sheep	50	> 600
Goat	48	420

*Fraction from activated proinhibitor.

The new anticoagulant also interferes with other coagulation tests in bovine plasma (see Table 1). In all the assays, only the thrombin time remained undisturbed. Of interest is the almost paradoxical fact that the clotting mechanism was much more inhibited in the presence of lipids than in their absence. There was also a striking inhibition of the thromboplastin generation test composed of bovine reagents. Certain fractions of activated proinhibitor (Fig. 1), when used as a diluent for one of the four components, gave less than 1 percent of the original activity in the test. Human plasma was used as a substrate to eliminate a possibility of a secondary inhibition, since, as presented in Table 2, the same inhibitor that renders bovine plasma almost unclottable has little or

no influence on human, dog, or horse plasma. Clotting of plasma from species that are more closely related, such as goat or sheep, is effectively retarded. In view of those results, the species specificity of the new anticoagulant is very probable. For final proof, however, a substance having similar activity must first be isolated from blood of another species.

Studies were initiated to elucidate the disturbance in coagulation mechanism more precisely, with the use of prothrombin and factor X in purified form (*4, 5*). Preliminary observation indicated that inhibitor interferes with the coagulation mechanism at two different stages. In the early stage, it inhibits activation of factor X in a reaction that involves factors VIII and IX, phospholipids, and calcium ions. When this stage is omitted by substituting either tissue thromboplastin or Stypven for intrinsic activators, factor Xa forms, undisturbed by the inhibitor. However, the next step, namely, conversion of prothrombin to thrombin, is now impaired. This explains why, of all coagulation tests in bovine plasma, only thrombin time remained unchanged by the presence of the anticoagulant.

Finally, an effort has been made to determine more closely the nature of the protein associated with clot-retarding activity. Fractions with this activity, obtained on a diethylaminoethyl (DEAE) cellulose column from the thrombin-treated prothrombin complex, were concentrated by precipitation with ammonium sulfate (60 percent saturation) and filtered through Sephadex G-200. A considerable amount of inert material left the column with the void volume. A second flat peak obtained in elution corresponded to a protein associated with the anticoagulant and also with factor VII activity. There was no detectable prothrombin, factor X, or factor IX activity. The volume in which the clot-retarding substance passed through Sephadex, when applied to the equation of Determann and Michel (*6*), indicated that it may consist of particles with a molecular weight in the neighborhood of 80,000. The ability to retard coagulation and to inhibit the thromboplastin generation test was preserved at −30°C for at least 2 months and only partly lost after 30 minutes at 56°C. This ability was totally lost after the substance was heated at 85°C for 20 minutes.

In contrast to this considerable stability of the isolated form, a progressive loss of anticoagulant activity took place rather rapidly after admixing it with native bovine plasma or serum. This mechanism remains to be explained and may represent a major difficulty in demonstrating a substance similar to the one elicited by thrombin in vitro, in association with either experimental or clinical intravascular coagulation.

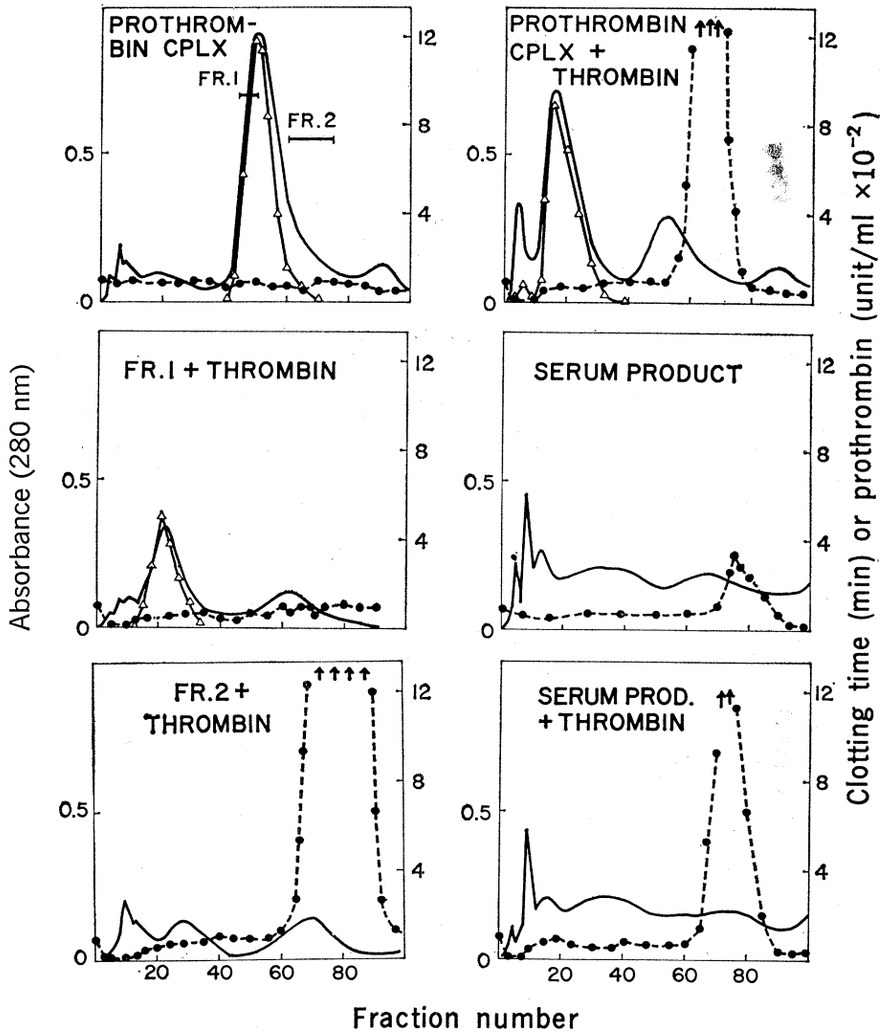

Fig. 1. Chromatography on DEAE cellulose. Columns (1.5 by 20 cm) eluted by linear gradient. Starting concentration of NaCl was 0.08*M* in 0.04*M* tris, *p*H 7.8, and slope of the gradient was 2 m*M* NaCl per fraction (4 ml in volume). The prothrombin complex (*CPLX*) for single chromatography was isolated from approximately 1200 ml of plasma and the serum product from 750 ml of serum. For activation, 900 to 1200 Iowa units of thrombin, purified on IRC-50 resin (*7*), was added to the sample 90 minutes prior to chromatography. For clot-moderating activity, 0.1 ml of chromatographed fraction was mixed with 0.1 ml of bovine plasma, 0.2 ml of phospholipid, and 0.2 ml of 0.025*M* $CaCl_2$, and the clotting time was recorded. Protein, solid line; clotting time, dotted line; prothrombin, triangles. Alterations resulting from the contact with thrombin and concerning thrombin zymogen are as described previously (*4*).

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