AN HEPATIC INHIBITOR OF ACTIVATED CLOTTING FACTOR X (Stuart)

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We have recently reported (1) that the perfused liver rapidly removes activated blood clotting factor X (Stuart) from the perfusing medium, but that precursor or inactive factor X was unaffected by hepatic perfusion. In the present study we have examined the ability of cell-free preparations of liver to attenuate activated factor X.

Materials and Methods.

Fresh bovine livers were obtained at a slaughter house and were immediately chilled and promptly homogenized with a loose-fitting teflon pestle in veronal buffer, pH 7.4 (2.5 ml/gm tissue). Cell debris, nuclei, and mitochondria were removed by centrifugation at 10,000 x g for 30 minutes. Microsomes were sedimented by centrifugation for 60 minutes at 104,000 x g. Proteins were fractionally precipitated from the supernatant (S 104,000) by addition of powdered $(NH_4)_2SO_4$ at melting-ice temperature. Factors X and II (prothrombin) were prepared from bovine plasma by the method of Nemerson (2). Bovine factor V (proaccelerin) was prepared from barium sulfate adsorbed plasma (100 mg/ml) by collecting the fraction which precipitated between 33 and 50% saturation with ammonium sulfate. Clotting factor activity was assayed as described previously (1). The total factor X and factor V activities in 1 ml of normal bovine plasma were assigned the value of 100 units. Crude vegetable phospholipids (inositihin, Lakeside Associates), bovine fibrinogen (Armour), and Russell's viper venom ("Stypven", Burroughs-Wellcome) were obtained commercially.

Results and Discussion.

In our first studies we examined the effects of liver fractions on the clotting of normal plasma (Table 1). We utilized three clotting assays: the partial thromboplastin time (a measure of the intrinsic pathway), the one-stage prothrombin time (a measure of the thromboplastin activated extrinsic pathway), and the thrombin time (a measure of the thrombin-fibrinogen interaction). The supernatant from the 104,000 x g centrifugation grossly prolonged the partial thromboplastin time and the prothrombin time, and slightly prolonged the thrombin time. The anti-coagulant activity in the soluble cell sap was further concentrated in a narrow ammonium sulfate fraction (Table 1). Further isolation and initial purification of the clot-retarding principle in liver was achieved by gel filtration of the active ammonium sulfate fraction on a 100 cm Sephadex G-100 column (Figure 1). The active fraction isolated from the column

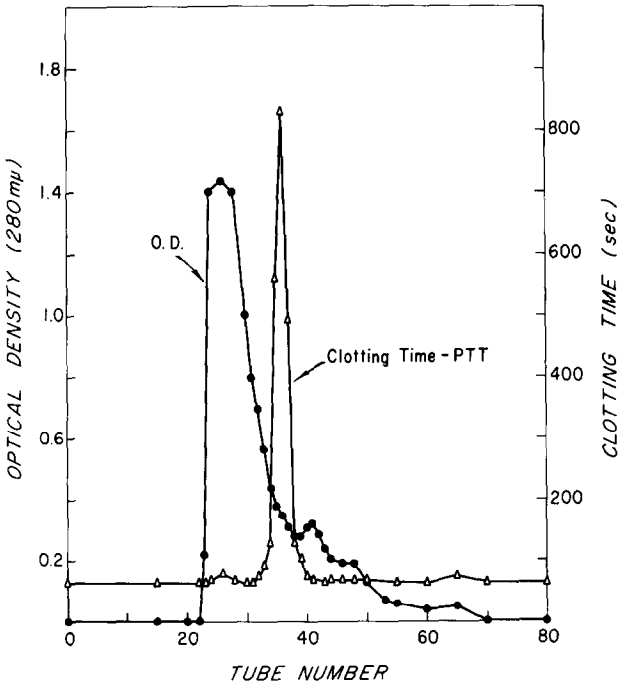

Fig. 1. Separation of anticoagulant activity on Sephadex G-100. Fraction volume: 3.5 ml.

Table 1. Effect of liver fractions on coagulation assays

	Protein (mg/ml)	Partial thromboplastin time (seconds)	Prothrombin time (seconds)	Thrombin time (seconds)
Buffer control*		68	19	17
S-104,000	30	1,450	59	24
$(NH_4)_2SO_4$ Fx				
0 - 23%	40	185	-	-
23% - 37%	100	2,400	180	21
37% - 100%	4.4	135	-	-
Tube 36	1.6	870	89	18

Reaction mixtures: partial thromboplastin time - 0.1 normal oxalated plasma, 0.1 ml phospholipid emulsion, 0.1 ml liver fraction, 0.1 ml 0.02 M $CaCl_2$; prothrombin time - 0.1 normal oxalated plasma, 0.1 ml brain thromboplastin, 0.1 ml liver fraction, 0.1 ml 0.02 M $CaCl_2$; thrombin time - 0.1 normal oxalated plasma; 0.1 ml bovine thrombin (5 NIH u/ml); 0.1 ml bovine fibrinogen (10 mg/ml).

* 0.1 ml buffer replaces liver fraction.

prolonged both the activated partial thromboplastin time and the prothrombin time but it did not affect the thrombin-fibrinogen reaction (Table 1). These data distinguish the anticoagulant from heparin. The anticoagulant activity was completely inactivated by heating to 56° C for 10 minutes. Its effect was immediate; it disappeared on serial dilution of the liver fractions; and it was non-progressive on incubation at 37° C. The high speed supernatant fractions from kidney, heart, and intestine were inert in all clotting systems. Similar fractions from spleen and lung prolonged the partial thromboplastin time but also greatly prolonged the thrombin time, presumably reflecting heparin activity. These data indicate that the anticoagulant activity in liver reflected the presence of a specific inhibitor which either blocked the activation of factor X or prevented the conversion of prothrombin to thrombin by active factor X in the presence of factor V, calcium, and phospholipid.

To identify further the site of action of the inhibitor, we used a three-stage clotting sequence. In the first stage purified bovine factor X was activated with Russell's viper venom (5 ug/ml). In the second stage the activated factor X was diluted 25 to 500-fold and incubated with calcium, phospholipid, and factor V. Prothrombin was then added and serial samples of the incubation mixture were added to bovine fibrinogen in the third stage to measure the rate of thrombin formation. Addition of the inhibitor in the first stage of the assay did not retard thrombin formation, indicating that the inhibitor did not prevent the activation of factor X. Addition of the inhibitor in the third stage was also ineffective, confirming that it was not an antithrombin. However, addition of the inhibitor in the second stage strikingly reduced the rate of thrombin evolution (Figure 2). In the absence of any single component of the first two stages of the assay system, no thrombin

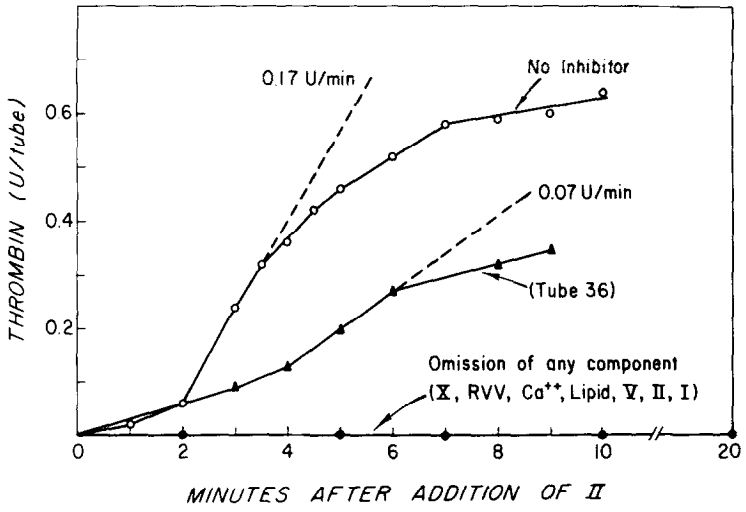

Fig. 2. Effect of hepatic inhibitor on thrombin evolution. Second stage reaction mixture: activated factor X, 2 units (1 ml normal bovine plasma = 100 units); factor V, 2.5 units (1 ml normal bovine plasma = 100 units); hepatic inhibitor (tube 36, 0.2 mg) or buffer; crude phospholipid, 100 ug; $CaCl_2$ (.04 M 0.1 ml; prothrombin 10 NIH units, all in a final volume of 6.0 ml. Serial samples of 0.6 ml added to 0.1 ml of bovine fibrinogen (10 mg/ml) in third stage of assay.

evolved. In the absence of added fibrinogen, no clot developed.

Increasing the concentration of activated factor X increased the rate of thrombin evolution in the presence or absence of inhibitor (Figure 3a). When the data were plotted in reciprocal units (Figure 3b), the curves formed straight lines which intercepted at the ordinate, indicating competitive inhibition between active factor X and the hepatic inhibitor.

In previous studies (3) we have shown that in the presence of circulating activated procoagulants red thrombi form in areas of stasis, but not in the general circulation. We have also shown that in the intact animal (3) or as an isolated perfused organ (1) the liver removes activated procoagulants. We have suggested that thrombosis in areas of stasis may in

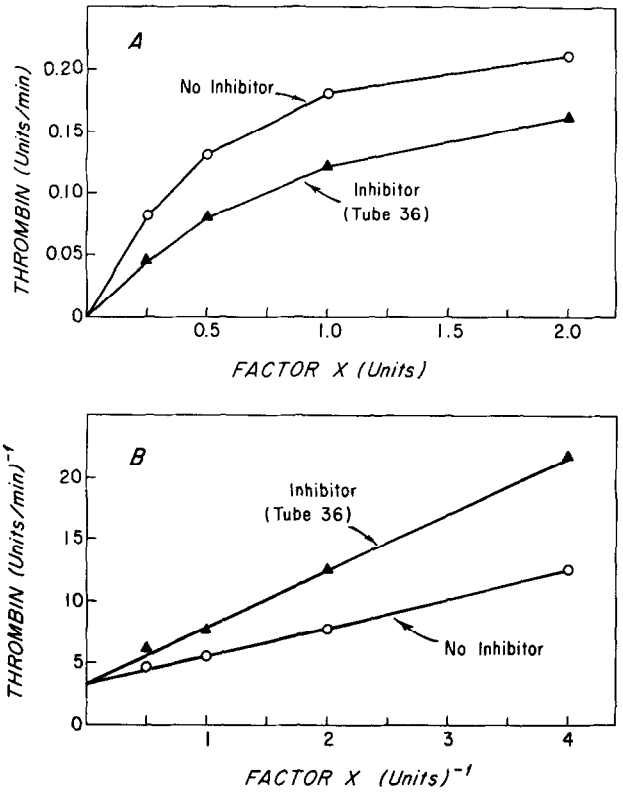

Fig. 3. Effect of increasing factor X concentration on rate of thrombin evolution. Conditions as in Figure 2 with varying factor X concentration.

part be due to local persistence of activated procoagulants that are removed from the flowing circulation by hepatic clearance mechanisms. In the present study, we have isolated from the soluble fraction of liver homogenates a potent inhibitor that competitively blocks activated factor X. Our present data suggest, therefore, that at least one mechanism by which activated factor X is removed from the circulation is intrahepatic binding of the procoagulant.

Summary.

A potent heat-labile inhibitor of blood clotting was isolated from the soluble fraction of bovine liver. It prolongs both the prothrombin

time and partial thromboplastin assays, but it does not affect the thrombin-fibrinogen interaction. The inhibitor was isolated by salt fractionation and gel filtration. In assay systems using purified coagulation factors, the inhibitor retards the reaction between activated factor X, factor V (proaccelerin), and phospholipid by competitive inhibition of activated factor X. Our present data suggest that at least one component of the hepatic attenuation of factor X may be intrahepatic binding of the activated procoagulant.

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