# Failure of Fibrinogen Degradation Products to Increase Plasma Fibrinogen in Rabbits[1] (37540)

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The agents controlling fibrinogen synthesis are poorly understood. Two groups of investigators have described increased fibrinogen production in dogs after infusion of exogenously prepared fibrinogen degradation products (1, 2). However, a third group of investigators were unable to demonstrate elevated plasma fibrinogen levels in rabbits following infusion of exogenously prepared fibrinogen degradation products in rabbits (3). The experiments reported herein were designed to test further the hypothesis that fibrinogen degradation products function as major regulatory agents of fibrinogen synthesis in the rabbit.

*Materials and Methods.* Male New Zealand rabbits, 1.8 to 2.8 kg in weight, were housed in separate cages for 14 days before use and allowed free access to Purina rabbit chow and water.

*Fibrinolysin (human) streptokinase-activated (Thrombolysin, Merck, Sharp, and Dohme).* The contents of 10 vials of Thrombolysin (40 casein units/vial) were dissolved in 40 ml sterile distilled water and stored in 2 ml aliquots at −70°. Fourteen hundredths (0.14) casein units of this material completely digested 1 mg of rabbit fibrinogen to fragments D and E within 2 hr.

*Epsilon aminocaproic acid (EACA).* A 39.3 g% (w/v) solution was made in distilled water and sterilized by autoclaving.

*Rabbit fibrinogen.* Nine volumes of rabbit blood, obtained by cardiac puncture, were added to 1 vol of an anticoagulant solution containing 2% $Na_2EDTA$ and 0.26% EACA. Ammonium sulfate was added to the plasma to 25% saturation. The resultant precipitate was dissolved in 0.005 *M* sodium citrate. Precipitation at 25% ammonium sulfate saturation was repeated two more times. The final precipitate was dissolved in 0.005 *M* sodium citrate, dialyzed for 24 hr at 4° against large volumes of sodium citrate, and concentrated by vacuum suction at 4° to a fibrinogen concentration of 9–15 mg/ml. Clottability of all preparations exceeded 97%. Preparations were kept at 4° and used within 24 hr.

*Soybean trypsin inhibitor (SBTI), Type I-S* (Sigma Chemical Co.) was dissolved in sterile distilled water in a concentration of 10 mg/ml.

*Preparation of infusion mixtures containing fibrinogen degradation products (FDP) and of control infusion mixtures.* Between 78 and 115 mg of fibrinogen, an amount equivalent to 33% of the test animal's plasma fibrinogen pool as estimated from body weight and assumed values of 3 mg/ml for fibrinogen and 40 ml/kg for plasma volume, was digested at 37° with 0.14 casein units of Thrombolysin/mg of fibrinogen (9–16 casein units). After either 5 min or 2 hr, EACA solution was added to stop further fibrinolysis (133 mg EACA/casein unit) (4). A 0.2 ml portion was removed from each preparation for acrylamide gel electrophoresis (5, 6). The 5 min incubation mixtures consistently contained primarily fragments X and Y. The 2 hr mixtures contained only fragments D and E (see Fig. 1). Control preparations were made by substituting 0.005 *M* sodium citrate

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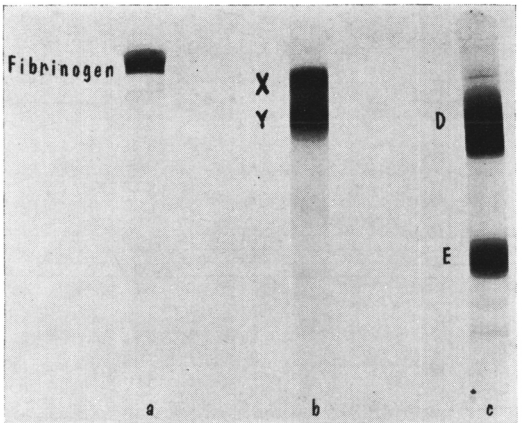


FIG. 1. Acrylamide gel electrophoresis of fibrinogen degradation products: (a) control with undigested fibrinogen, (b) 5 min incubation mixture containing fragments X, Y and D, (c) 2 hr incubation mixture containing fragments D and E.

solution for the fibrinogen solution.

In a preliminary experiment, two control preparations containing 18 and 19 casein units were neutralized with 36 and 38 mg of SBTI instead of EACA. Two milligrams of SBTI fully inhibited the fibrinolytic activity of 1 casein unit of Thrombolysin as shown by the persistence of fragments X and Y in mixtures of fibrinogen and Thrombolysin which were incubated for 5 min, then treated with SBTI, and allowed to stand for 24 hr before acrylamide gel electrophoresis.

The following preparations were made: 4 preparations containing fragments X and Y, 14 preparations containing fragments D and E, and 7 control preparations. Sterile technique was used throughout, including the isolation of the fibrinogen, in preparing eight of the fragment D and E preparations and all five control preparations which were neutralized with EACA. Bacterial cultures taken immediately before injection were negative. Sterile technique was not used in making the four fragment X and Y preparations, six of the fragment D and E preparations, and the two control preparations which were neutralized with SBTI. However, each preparation was passed through a Millipore filter (0.45 $\mu$m) prior to injection. Cultures after Millipore filtration were negative except for 3 of the fragment D and E preparations, which contained small numbers of *Staphylococcus albus*. One of the latter preparations also contained small numbers of diphtheroids.

*Preparation and injection of labeled degradation products.* A rabbit, given 80 IU of ACTH (Corticotropin Injection, Armour) and 0.5 ml of 1/1000 adrenalin (Epinephrine Injection, Parke, Davis) intravenously to stimulate fibrinogen synthesis, was injected intravenously 4 hr later with 350 $\mu$Ci of $^{75}$selenomethionine (Sethotope, Abbott). The animal was exsanguinated by cardiac puncture after 18 hr, and its fibrinogen was isolated as described above. The isolated fibrinogen preparation contained endogenously labeled $^{75}$selenomethionine-fibrinogen ($^{75}$SeM-fibrinogen) with a specific activity of 4308 cpm/mg. Seventy milligrams of this fibrinogen was placed in each of two tubes containing 9.7 casein units of Thrombolysin. Fibrinolysis was stopped in the first tube after 5 min and in the second tube after 2 hr by adding 4.4 ml of EACA solution (1.7 g). One-tenth milliliter of each preparation was added to 2.9 ml of saline solution and counted to obtain a value for total radioactivity. The first preparation contained a total of 264,604 cpm in 15 ml total volume and the second preparation contained a total of 245,437 cpm in 14 ml total volume. One-tenth milliliter of each preparation was also subjected to acrylamide gel electrophoresis to measure radioactivity in isolated degradation products. Each stainable band was cut out, added to a counting tube containing 2 ml of saline solution, and counted. The first preparation contained the following distribution of radioactivity: fragment X, 44%; fragment Y, 35%; and fragment D, 16% of the total radioactivity. The second preparation contained 58% of the total radioactivity in fragment D and 35% in fragment E. Each preparation was passed through a Millipore filter and injected into a 2.5 kg rabbit. Twelve blood samples of 1.8 ml were drawn over the next 24 hr for counting whole plasma radioactivity. The radioactivity of the first sample, drawn at 5 min, was called 100% and subsequent values were expressed as a percentage of this value.

*Protocol for evaluation of fibrinogen syn-*

*thesis.* Control preparations or preparations containing degradation products (approximately 7 ml/kg body weight) were infused over 5 min into the marginal vein of the left ear. Four hours later, 1 ml of $^{75}$SeM solution (containing 35 to 40 $\mu$Ci) was injected into a vein of the same ear by a technique described earlier (7) which ensured exactness of the volume injected. Blood samples (1.8 ml) were withdrawn for measurement of plasma fibrinogen level just before infusion of the test material and just before injection of the $^{75}$SeM. Blood samples (3.6 ml) were withdrawn from the opposite ear for measurement of both fibrinogen level and incorporation of $^{75}$SeM into fibrinogen at 3, 6, 18, 24, 48, 72, and 96 hr after the injection of the $^{75}$SeM. All blood samples were obtained by allowing the blood to flow directly from the hub of the needle into marked plastic tubes containing 1/9 vol of the above-described anticoagulant solution.

*Determination of plasma fibrinogen level.* Plasma fibrinogen concentration was measured by clotting diluted plasma, dissolving the washed clot in alkaline urea solution, and measuring optical density (8). Values were corrected for dilution of plasma with anticoagulant and for change in hematocrit during the experiment (7).

*Measurement of plasma $^{75}$SeM-fibrinogen.* $^{75}$SeM-fibrinogen was separated and counted as thrombin-clottable radioactivity. Counts were corrected for dilution of plasma with anticoagulant, changes in hematocrit, and loss of radioactivity due to prior sampling and then expressed as percentage of injected dose incorporated into the fibrinogen of 1.5 ml of plasma. The procedure has been described in detail elsewhere (7). Sufficient counts were accumulated to give a counting error of less than 3%.

*Results. Disappearance of labeled degradation products.* Figure 2 is a plot of the plasma radioactivity curves obtained following the injection into one rabbit of the preparation containing primarily $^{75}$SeM-labeled fragments X and Y and the injection into a second rabbit of the preparation containing primarily $^{75}$SeM-labeled fragments D and E. These radioactivity curves were fitted to double exponential equations by use of the SAAM program (9) on a digital computer (see legend to Fig. 2). The equations did not differ significantly from each other and the following $t_{1/2}$ values were calculated from their exponential rate constants: for the preparation containing fragments X and Y, initial slope, 0.7 hr, terminal slope, 7.7 hr; for the preparation containing fragments D and E, initial slope, 0.7 hr, terminal slope, 9.9 hr.

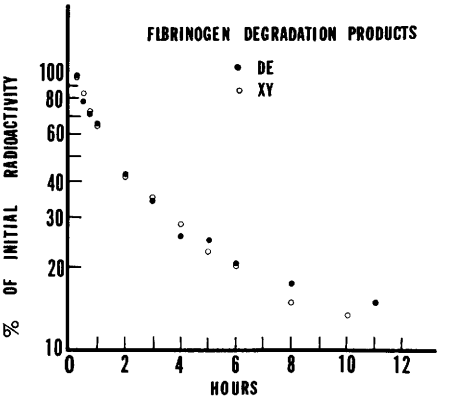


FIG. 2. Plasma fibrinogen radioactivity following the infusion of a 5 min incubation mixture containing primarily labeled fragments X and Y (○) and following the infusion of a 2 hr incubation mixture containing primarily labeled fragments D and E (●). The points for the disappearance of fragments X and Y can be fitted to a curve given by the equation: $f(t) = 0.77e^{-1.02t} + 0.23e^{-0.09t}$. The points for the disappearance of fragments D and E can be fitted to a curve given by the equation: $f(t) = 0.77e^{-1.03t} + 0.23e^{-0.07t}$.

*Effect of control preparations upon fibrinogen concentration and upon incorporation of $^{75}$SeM into fibrinogen.* Plasma fibrinogen levels rose strikingly in two rabbits infused with control preparations containing citrate solution, Thrombolysin and SBTI (see Fig. 3). This rise was associated with a markedly increased incorporation of $^{75}$SeM into fibrinogen (see Fig. 4). In contrast, plasma fibrinogen levels did not rise in rabbits infused with control preparations containing citrate solution, Thrombolysin, and EACA, and these animals did not exhibit increased incorporation of $^{75}$SeM into fibrinogen (Fig. 4). Their mean maximum value for percent-

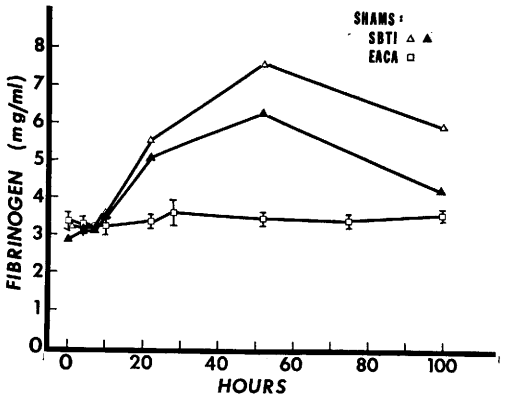


FIG. 3. Fibrinogen levels of rabbits infused with control preparations (sham mixtures). (△ ▲) The levels in two rabbits infused with a citrate–Thrombolysin–SBTI mixture; (□) the mean fibrinogen levels of five rabbits infused with a citrate–Thrombolysin–EACA mixture. Brackets delineate 1 SE. Control preparations were infused immediately after drawing the initial sample at 0 hr.

age incorporation of $^{75}$SeM into fibrinogen was 0.0100 ± 0.0008 (SE), which did not differ significantly from the mean maximum value of 0.0098 ± 0.0013 obtained for 17 control rabbits injected subcutaneously with 2 ml of saline in an earlier study from this laboratory (7). Thus, SBTI appeared to be the stimulatory agent in control preparations containing this material and it was not used in preparing degradation products.

*Effect of preparations of degradation products upon fibrinogen concentration and incorporation of $^{75}$SeM into fibrinogen.* Mean fibrinogen levels for rabbits infused with degradation product preparations are contrasted with mean fibrinogen levels for rabbits infused with citrate–Thrombolysin–EACA control preparations in Fig. 5. As shown, fibrinogen levels did not rise appreciably after infusion of either fragment X and Y preparations or fragment D and E preparations. For each group, the highest mean values observed after infusion did not differ significantly from the mean value prior to infusion. The mean maximum values for percentage incorporation of $^{75}$SeM into fibrinogen were as follows: fragment X and Y preparations, 0.0083 ± 0.0007; fragment D and E preparations, 0.0094 ± 0.0005. Thus, as indicated by these values and the radioactivity curves illustrated in Fig. 4, infusion of degradation products did not increase the incorporation into fibrinogen of $^{75}$SeM injected 4 hr after the degradation products.

*Discussion.* The data presented above indicate that the infusion of large amounts of either early (fragments X and Y) or late (fragments D and E) rabbit fibrinogen deg-

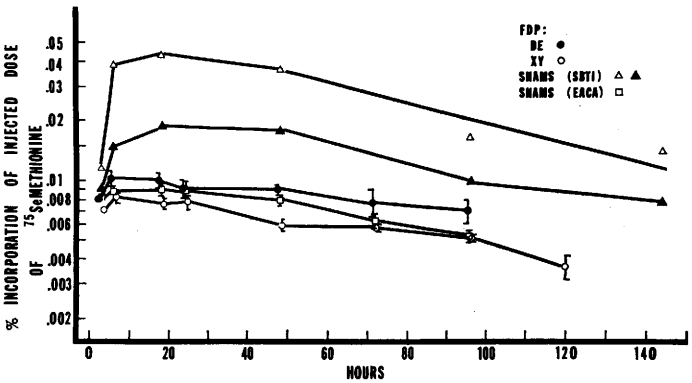


FIG. 4. Mean $^{75}$SeM-fibrinogen radioactivity curves in 5 control rabbits infused with sham mixtures containing citrate–Thrombolysin–EACA (□), 4 rabbits infused with FDP preparations containing fragments X and Y (○), and 14 rabbits infused with FDP preparations containing fragments D and E (●). Brackets delineate 1 SE. The $^{75}$SeM-fibrinogen radioactivity curves in the two rabbits given sham mixtures containing citrate–Thrombolysin–SBTI are also plotted (▲ △) to illustrate the degree of increase in $^{75}$SeM incorporation observed when fibrinogen synthesis is stimulated. 0 hr represents the time of injection of $^{75}$SeM, which was 4 hr after infusion of the test material.

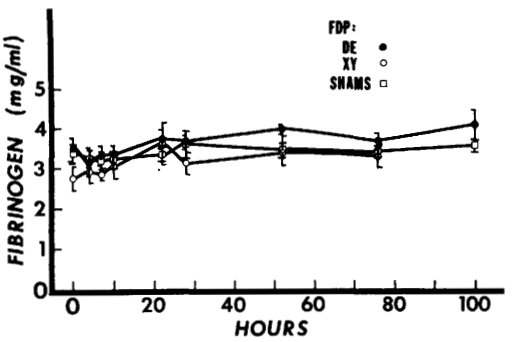


FIG. 5. Mean fibrinogen levels for control rabbits infused with sham mixtures containing citrate–Thrombolysin–EACA (□), for rabbits infused with FDP preparations containing fragments X and Y (○), and for rabbits infused with FDP preparations containing fragments D and E (●). Numbers of animals as listed in legend to Fig. 4. Brackets are 1 SE. Test materials were infused immediately after drawing the initial sample at 0 hr.

radation products fails to stimulate fibrinogen synthesis in the rabbit. Thus, serial fibrinogen levels failed to rise appreciably over 96 hr (Fig. 3) in animals infused with a quantity of fibrinogen degradation products calculated to equal the quantity that could have formed from one-third of the plasma fibrinogen pool. These observations fit with an earlier report of Kropatkin and Izak (3) that fibrinogen levels failed to rise in four rabbits infused with material prepared by adding streptokinase and thrombin to bovine fibrinogen.

Moreover, the injection of $^{75}$SeM 4 hr after infusion of degradation products did not yield evidence of increased synthesis of labeled plasma fibrinogen (Fig. 4). A 4 hr interval was selected because Barnhart and coworkers (1) had reported that dogs infused with fibrinogen degradation products had peak uptake of these products in liver reticuloendothelial cells at 4 hr and maximum evidence of hepatocyte fibrinogen synthesis at 5 hr. In rabbits, purified fragment D has been shown to disappear rapidly from plasma (10) and in our preliminary experiments approximately 75% of both a labeled fragment X and Y preparation and a labeled fragment D and E preparation disappeared from the circulation within 4 hr (see Fig. 2). Thus, our failure to find increased incorporation into fibrinogen of $^{75}$SeM given 4 hr after infusion of degradation products appears to us as strong evidence against even a transient stimulation by degradation products of fibrinogen synthesis in the rabbit.

The inertness of degradation products was made even more apparent by the unexpected finding that a control citrate–Thrombolysin–SBTI mixture markedly increased fibrinogen levels and incorporation of $^{75}$SeM into fibrinogen. This stimulatory effect of SBTI, albeit known for pancreatic proteins (11), has not been reported previously to our knowledge for fibrinogen, and the mechanism by which it stimulates fibrinogen production is unknown.

On the basis of increased hepatocyte fluorescence with anti-fibrinogen antisera and increased release of fibrinogen into thoracic duct lymph following infusion of fibrinogen degradation products into dogs, Barnhart and co-workers (1) have suggested that such agents may serve as physiologic stimuli for fibrinogen production. Our experiments do not rule out the possibility that small amounts of fibrinogen degradation products may function as physiologic, steady-state regulators of synthesis, since conceivably all receptor sites for such regulators could normally be saturated and unable to respond to the infusion of excess amounts. Our data strongly suggest, however, that in the rabbit, fibrinogen degradation products do not mediate the acute increases in fibrinogen synthesis that follow the infusion of coagulation or fibrinolytic agents (12, 13) or that occur in a variety of infectious, inflammatory and traumatic states.

*Summary.* Infusion of large quantities of early or late fibrinogen degradation products into rabbits failed to increase plasma fibrinogen levels or the incorporation of $^{75}$SeM into newly synthesized fibrinogen. Thus, no evidence could be obtained for a key role of fibrinogen degradation products in mediating the increased fibrinogen synthesis associated with pathologic states in this species.

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