Stable Complex of Fibrinogen and Fibrin

Abstract. *Fibrin-stabilizing factor acts on monomeric fibrin combined with fibrinogen to form a stable complex that is soluble at physiologic* p*H and ionic strength. The complex has been isolated by chromatography on agarose gel and characterized by sedimentation rate near 24* $s_{w,20}$ *at both* p*H 5.3 and 7.4, by coagulability when treated with thrombin, and by content of fibrinopeptides. Formation of the complex could provide a pathway for solubilization of monomeric fibrin produced in circulating blood.*

Fibrinogen inhibits reversible polymerization of fibrin by interacting with fibrin monomers to block polymerization sites (*1*). In the absence of fibrinogen, reversible polymerization of fibrin rapidly and spontaneously forms a clot that can be redissolved by acidification. As contrasted with the reversible variety (*2*), irreversible polymerization yielding an acid-insoluble clot is catalyzed by a transamidase, an enzyme known as fibrin-stabilizing factor (factor XIII) (*3*). We have observed that fibrinogen blocks this polymerization as well, by combining with the fibrin. We now describe formation, purification, and some properties of a stable complex resulting from action of the stabilizing factor on fibrin combined with fibrinogen.

The materials used were human fibrinogen purified according to Finlayson and Mosesson (*4*) for preparation of chromatographic fraction I; monomeric fibrin as prepared by Donnelly *et al.* (*5*), but dissolved in a solution containing 0.27*M* tris-hydroxymethylaminomethane hydrochloride (tris-HCl) and 0.03*M* tris-acetate at *p*H 5.3; fibrin-stabilizing factor (1280 units per milligram) as fraction 5 described by Loewy *et al.* (*6*); and bovine thrombin purified by Rasmussen's method (*7*). Just prior to use, the stabilizing factor was activated by thrombin, and the thrombin was subsequently inhibited by addition of benzoylarginine methyl ester (*8*) in the manner prescribed by Lorand and Konishi (*9*). The monomeric fibrin used in our experiments was devoid of both fibrinopeptides A and B (*1*). Parallel experiments were performed with rabbit fibrinogen and fibrin and with preparations of fibrin devoid of fibrinopeptide A alone. No important differences were obtained in these auxiliary studies.

As already shown (*1*), limited amounts of monomeric fibrin could be added to solutions of fibrinogen without formation of a coagulum at *p*H 7.4. Instead of polymerizing into a coagulum, the monomers interacted with fibrinogen, forming a soluble complex. This complex sedimented at a rate near 24 $s_{w,20}$ relative to fibrinogen at 8 $s_{w,20}$ at *p*H 7.4, but it dissociated completely into monomeric fibrin and fibrinogen on acidification to *p*H 5.3 (Fig. 1*A*). It could be inferred that the acid-labile complex dissociates at *p*H 7.4 as well, but its dissociation is obscured by recombination of fibrinogen and fibrin (*1*).

When fibrin-stabilizing factor was added to solutions of fibrinogen and fibrin at *p*H 7.4, the acid-labile complex was converted to a stable congener still sedimenting near 24 $s_{w,20}$, but it was no longer broken by acidification (Fig. 1*B*). Nor was 5*M* urea effective in breaking the complex incubated with fibrin-stabilizing factor. Conversion of the labile to the stable complex was dependent upon both time

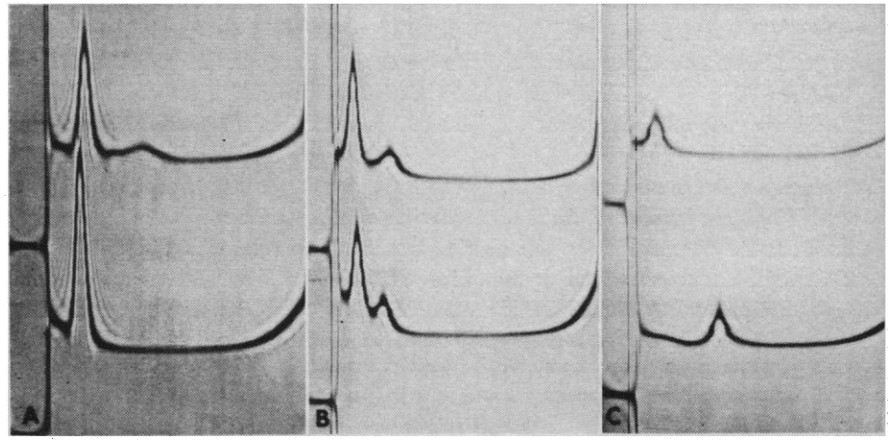

Fig. 1. Sedimentation of fibrinogen-fibrin complexes. *A*, The top curve shows fibrinogen and the acid-labile complex at *p*H 7.4. The acid-labile complex formed by interaction of monomeric fibrin (0.42 mg/ml) with fibrinogen (2.09 mg/ml) sedimented approximately three times faster than the bulk of the fibrinogen (8 $s_{w,20}$) not bound as a complex with the fibrin. The suspending medium contained 0.23*M* NaCl, 0.066*M* tris-HCl, and 0.003*M* tris-acetate. Picture at a schlieren element angle of 55°, 16 minutes after rotor reached operating speed of 52,640 rev/min. The bottom curve shows fibrinogen and monomeric fibrin at *p*H 5.3. After acidification, the complex dissociated completely into monomeric fibrin and fibrinogen which have identical sedimentation rate of 8 $s_{w,20}$. Acidification by addition of 0.166 ml of 0.3*M* sodium acetate (*p*H 4.78) and 0.334 ml of 0.3*M* tris-acetate (*p*H 5.3) to 0.6 ml of solution containing the complex at 11/6 of the concentration analyzed in the upper pattern. *B*, The top pattern shows fibrinogen and the acid-stable complex at *p*H 7.4. The acid-stable complex, formed by action of fibrin-stabilizing factor on fibrin in solution with fibrinogen, sedimented at the same rate relative to fibrinogen as did its predecessor, the acid-labile complex. Ultracentrifugation was performed after (i) addition of 0.5 ml of solution containing 82 units of fibrin-stabilizing factor to 1.44 ml of solution containing 10 mg of fibrinogen and 2 mg of monomeric fibrin, (ii) incubation for 30 minutes at 30°C, (iii) dilution with 5 ml of 0.3*M* NaCl, and (iv) subsequent addition of 0.1 ml of 0.04*M* EDTA. The solution of the stabilizing factor contained 0.028*M* $CaCl_2$, 0.006*M* cysteine, 0.03*M* tris-HCl (*p*H 7.4), 0.12*M* NaCl, 0.036*M* benzoylarginine methyl ester hydrochloride, and thrombin (1.4 units per milliliter). The solution of fibrinogen and fibrin contained 0.20*M* NaCl, 0.12*M* tris-HCl, and 0.006*M* tris-acetate at *p*H 7.4. Picture was taken at schlieren angle of 50° after 10 minutes at 52,640 rev/min. The bottom pattern shows fibrinogen and the acid-stable complex at *p*H 5.3. Protein from the reaction mixture prepared as described above was precipitated by addition of one-third volume of saturated ammonium sulfate, then washed with 1*M* glycine at −4°C to remove the ammonium sulfate (*1*), and dissolved in a solution of 0.24*M* tris-HCl and 0.06*M* tris-acetate at *p*H 5.3. A similar pattern was obtained when *p*H was adjusted by simply diluting with tris-acetate buffer at *p*H 5.3. *C*, The top curve shows fibrinogen (0.77 mg/ml) in solution containing 0.24*M* NaCl and 0.06*M* tris-HCl at *p*H 7.4. Picture was taken at schlieren angle of 45° after 20 minutes' centrifugation at 42,040 rev/min. The bottom curve shows purified acid-stable complex. The complex was separated by chromatography of reaction mixture prepared as described for the top pattern in *B*. Prior to centrifugation, the complex was dialyzed against saline for 4 hours at 25°C, precipitated by chilling to 0°C, and redissolved (0.9 mg/ml) in solution of 0.24*M* NaCl and 0.06*M* tris-HCl at *p*H 7.4.

and concentration of the stabilizing factor. Prolonging incubation for a period four times that needed for complete stabilization of the soluble complex did not change the ultracentrifuge pattern.

From the amount contained in the reaction mixture, we calculated that the stabilizing factor could not constitute more than 2 percent of the mass of the stable complex. The number of molecules of the stabilizing factor would be too small (*6*) to explain stabilization by nonenzymic means. Enzymic action presupposes that the stabilizing factor is not incorporated as a permanent constituent in product formation. Our studies show that a major portion of the stabilizing factor could be recovered from the reaction mixture by chromatography, and that the stable complex contained less than 1 unit/mg.

Reaction mixtures containing the stable complex were chromatographed on columns of granulated 3 percent agarose gel (*10*). The columns were equilibrated with buffer containing 0.285*M* tris-HCl, 0.015*M* benzoylarginine methyl ester hydrochloride, and 0.001*M* ethylenediaminetetraacetate (EDTA) at *p*H 7.4. The same buffer was used for elution.

Ultracentrifugation showed that the stable complex was eluted as the leading boundary of protein in the effluent solution. Recovery of the complex was approximately 70 percent. A second boundary contained fibrinogen and the stabilizing factor (Figs. 1*C* and 2). The latter's presence at concentrations exceeding 2 units per milliliter was determined by the method of Loewy *et al.* (*6*). Fibrinogen was measured by ultracentrifugation and by coagulation with thrombin.

The stable complex sedimented at a rate near 24 $s_{w,20}$ at both *p*H 7.4 and *p*H 5.3 as it did prior to chromatographic purification, but losses of about 30 percent were consistently incurred by denaturation on adjusting to *p*H 5.3.

No loss or change in the stable complex occurred during dialysis or storage for a period of 2 days, at concentrations of 0.5 mg/ml, in warm buffer (25° to 27°C) containing 0.12*M* NaCl, 0.03*M* tris-HCl, and 0.008*M* benzoylarginine methyl ester hydrochloride at *p*H 7.4; whereas it precipitated at concentrations above 0.05 mg/ml when stored in the cold. The precipitate redissolved in saline at 37°C.

The labile complex was also precipitable by cold, but it differed (*1*) in that its dissolution on warming was dependent on the concentration of fibrinogen in the solution. The labile complex separated into component fibrinogen and fibrin within minutes after it dissolved in warm saline, but it did not break down in solution containing fibrinogen in large excess over that in the complex. Repeated precipitation and solution of the stable complex in saline indicated that its stability at neutral *p*H was not dependent on fibrinogen concentration in the solvent.

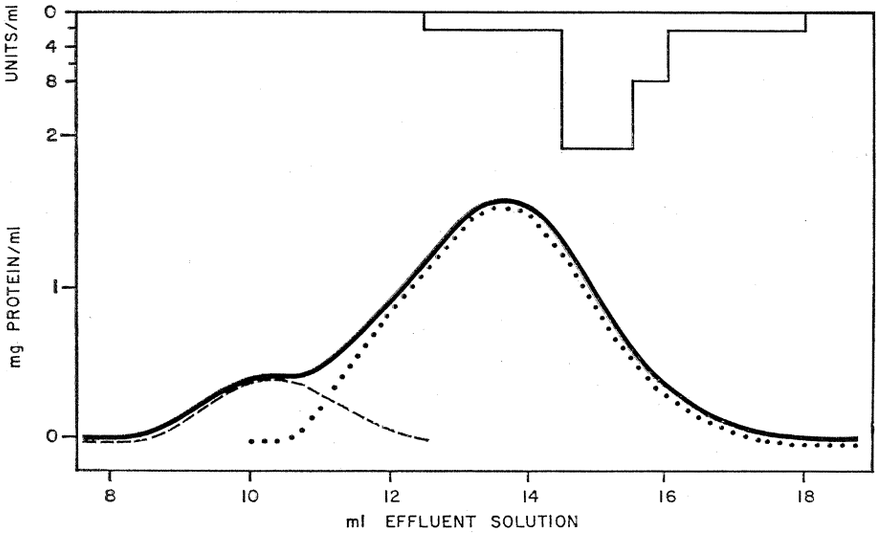

Fig. 2. Chromatographic separation of the acid-stable complex from fibrinogen and fibrin-stabilizing factor. The stable complex was prepared by incubating 82 units of fibrin-stabilizing factor with 2 mg of fibrin in solution with 10 mg of fibrinogen as described under Fig. 1*B*. The reaction mixture (1.9 ml) was applied to an 18.6-ml column of granulated agarose gel and washed through the column at a rate of 4 to 6 ml/hr. Effluent solution was collected in 0.5-ml fractions and analyzed for total protein (solid line), stable complex (broken line), fibrinogen (dotted line), and fibrin-stabilizing factor (histogram at top).

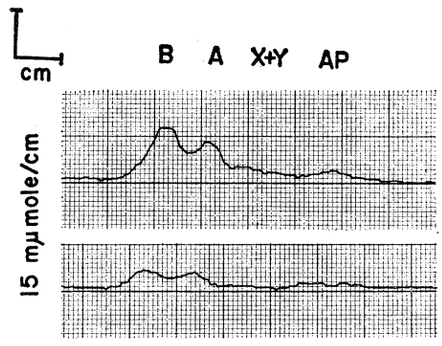

Fig. 3. Paper electrophoresis of fibrinopeptides liberated on treating 3.8 mg of fibrinogen (top tracing) and chromatographically isolated stable complex (bottom) with thrombin. NaOH (0.05*M*) buffered with boric acid to *p*H 8.3 was used as electrolyte. Separation and measurement (Sakaguchi reaction) were performed as described previously (*1*) except for the 400 volts applied across the 30-cm strips for 4.5 hours. Measurement by elution showed 18, 10.5, 3, and 4 mμmole of fibrinopeptides B, A, X+Y, and AP from 3.8 mg of fibrinogen, and 9, 6.5, 2, and 3.5 mμmole from 5.4 mg of the complex. The peptide designated as X has not been described previously, but it has been found in all preparations of human fibrinogen that we analyzed. The designation X is used only to distinguish the peptide from Y (*12*), from which it is separable by electrophoresis at *p*H 2.3.

The stable complex could be made to coagulate by treating it with thrombin. Complete coagulability was demonstrated by the fact that fluid extruded from the clot contained no protein precipitable with 5 percent trichloroacetic acid.

Coagulability with thrombin provided confirmatory evidence that the stable complex was formed in part from fibrinogen. The amount of fibrinogen incorporated into the complex was determinable from fibrinopeptides liberated by thrombin. The amount of fibrinopeptide A liberated from 5.4 mg of complex was equivalent to that in 2.4 mg of fibrinogen. Fibrinopeptide B provided a second estimate equivalent to 2.0 mg of fibrinogen (Fig. 3). The amount of fibrin incorporated into the complex was calculated from the difference between weight of the complex and its content of fibrinogen, as given by the fibrinopeptide measurements.

This calculation indicated that the proportion of fibrinogen to fibrin incorporated into the complex was between 0.6 and 0.8 mole of fibrinogen per mole of fibrin.

The question whether fibrin-stabilizing factor might act to form "hybrid polymers" of fibrinogen and fibrin has been raised (*11*). Our results provide an affirmative answer. However, the speculation (*11*) that cross-linking action of fibrin-stabilizing factor is responsible for formation of the cold-insoluble globulin that we specifically characterized as "cryoprofibrin" proved invalid. It has been shown (*1*) that cryoprofibrin separates into component fibrinogen and fibrin when dissolved in saline. Nonseparability of the stable complex that is formed with aid of fibrin-stabilizing factor reaffirms our identification of cryoprofibrin as a labile complex.

Our previous work (*1*) has shown the significance of formation of the labile complex in endotoxin-treated rabbits. Intravascular deposition of fibrin may begin when the labile complex is elevated to threshold concentrations. It is not as yet known whether the stable complex also occurs. Formation of the stable complex is dependent on fibrin-stabilizing factor which may exist in an inactive form (*9*) in blood. Our results now show that fibrin spent in formation of the stable complex would remain soluble until the complex is acted upon by thrombin.

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Replication of Chloroplast DNA of Tobacco

Abstract. *An experimental method has been designed for determining the relative rates of replication of the chloroplast and nuclear DNA's of* Nicotiana tabacum. *By this method chloroplast DNA in week-old seedlings is being replicated several times faster than nuclear DNA.*

The presence of DNA in chloroplasts and mitochondria is now well established (*1*). In the organisms so far examined, it has been possible to show that these "satellite" DNA's have a base composition different from those of the corresponding nuclear DNA's, and therefore they can be separated from them in CsCl gradients (*2*). The evidence for cytoplasmic inheritance of chloroplasts and mitochondria, the work on chloroplast mutations in *Euglena* (*3*), and the absence of chloroplast satellite DNA in aplastidic mutants of *Euglena* (*4*) suggest strongly that the DNA of these organelles does carry genetic information which is vital to the existence and development of the organelles. In addition, there is some evidence for DNA-directed RNA synthesis in chloroplasts (*5*) and mitochondria (*6*) and for protein synthesis in chloroplasts (*7*).

It is generally believed (*8*) that the number of chloroplasts per cell in a higher plant increases as the cell matures, up to a relatively late stage of development. If chloroplast DNA is a stable carrier of essential genetic information, it should replicate when the chloroplasts or proplastids divide. It therefore should be replicating faster than the nuclear DNA. If young seedlings in which the primary leaves are still developing are pulse-labeled with P^{32} for a long enough time so that pools of nucleotide will have a chance to equilibrate, but a short enough time so that the nuclei will have gone through, on the average, less than one division, the relative specific activities of the chloroplast and nuclear DNA's at the end of this period should be equal to their relative rates of synthesis.

There are two satellite DNA's in *Nicotiana tabacum* (*9*). One of these, which forms bands at 1.706 g/cm^3 in CsCl as compared to 1.696 g/cm^3 for the nuclear DNA, is associated with the chloroplasts. The other satellite, which forms bands at 1.709 to 1.712 g/cm^3, has not been localized in the cell, but may be mitochondrial. The existence of chloroplast DNA in tobacco has been confirmed in essence by Shipp *et al.* (*10*), although their figures for its buoyant densities in CsCl do not agree with ours, nor with Marmur's value for nuclear DNA (*11*).

Since the nuclear and chloroplast DNA's of tobacco form bands close together, their densities being only 0.010 g/cm^3 apart, the two DNA's cannot be resolved in a preparative CsCl gradient unless very small amounts of DNA (~1 μg) are used, although it is possible to obtain a fraction enriched in satellite DNA by careful selection of fractions. Therefore, it is difficult to determine the absolute specific activities of the two DNA's; for example, when 1 μg of DNA is used there is not enough material in either peak to determine the optical density, and thence the weight of DNA in the test samples. It is, however, possible to determine the relative specific activities of the two DNA's by the following three-step experiment.

1) Seeds of *Nicotiana tabacum* var. Maryland were sterilized by soaking in saturated bromine water for 10 to 15 minutes (*12*); the seeds were then placed in petri plates on sterile glucose-agar (1 percent glucose, 0.4 percent agar) in the dark. After 6 to 7 days, the seedlings were 1.5 to 2 cm long and had two small, yellow, unfolded primary leaves. One millicurie of sterile, carrier-free P^{32}, in the form of the neutralized inorganic phosphate, was added to each plate of 100 to 200 seedlings. Half the plates were placed under light (1650 lu/m^2) for the 12- to 14-hour labeling period, and the other half were kept in the dark. At the end of the labeling period, the seedlings were washed off, and DNA was extracted from them by Ray and