SIGNIFICANCE OF CRYOPROFIBRIN IN FIBRINOGEN-FIBRIN CONVERSION*,‡

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A cold precipitable fibrin-precursor, designated cryoprofibrin, was separated from the plasma of endotoxin-treated rabbits, and shown on the basis of its peptide composition to correspond to a product of limited action of thrombin on fibrinogen (1). The present communication is concerned with the stability and the mechanism of formation of cryoprofibrin. The studies were undertaken principally to evaluate the proposed utility of cryoprofibrin in determining intravascular deposition of fibrin.

Two moles of each of the peptide residues called cofibrins A and B or fibrinopeptides A and B are liberated from the fibrinogen molecule by action of thrombin (2–4), and the altered fibrinogen polymerizes to form fibrin. Fibrinopeptide B is liberated at considerably slower rate than A (1, 3–7). Liberation of B does not appear necessary for production of fibrin, but may contribute to side by side aggregation of fibrin strands (3). Cryoprofibrin contains one-half as much fibrinopeptide A but as much B as does fibrinogen. Since fibrin can be produced by liberation of fibrinopeptide A alone, cryoprofibrin differs only in content of fibrinopeptide A and solubility from fibrin that would be produced by limited action of thrombin on fibrinogen.

In dilute solution fibrinogen tends to dissociate into subunits that are approximately one-third the size usually found to be characteristic of the fibrinogen molecule (8). Cryoprofibrin might consist of a complex between subunits of both native fibrinogen containing fibrinopeptide A and altered fibrinogen lacking it. Our studies indicate that in the presence of low concentrations of fibrinogen cryoprofibrin decomposes to form fibrinogen and fibrin in equimolecular proportions. The fibrin separating from cryoprofibrin seems to result from polymerization of altered fibrinogen lacking fibrinopeptide A, while the native fibrinogen remains in solution. This separation is reversible, the fibrin being depolymerized and solubilized in presence of excess fibrinogen to form cryoprofibrin.

The formation of cryoprofibrin was studied in relation to both the production of fibrin and the thrombin-catalyzed liberation of fibrinopeptides. Results of

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these studies conformed with two alternate possibilities for the mechanism by which native and altered fibrinogen combine to form cryoprofibrin, and suggested that intravascular deposition of fibrin may be determined by the relative concentrations of cryoprofibrin and fibrinogen in plasma.

Materials and Methods

Rabbit fibrinogen was freshly prepared as follows: It was (*a*) precipitated as Cohn fraction I from fresh citrated plasma (9), (*b*) washed with glycine solution as specifically prepared by Blombäck and Blombäck (10), (*c*) dissolved at concentrations ranging from 2 to 6 mg/ml in a 0.136 M ammonium acetate and 0.015 M tosyl-L-arginine methyl ester hydrochloride (TAME)[1] solution, (*d*) stored at 0°C for 5 to 18 hours to remove cryoprofibrin, (*e*) precipitated by adding one-third volume of saturated ammonium sulfate, (*f*) washed with the glycine solution to remove ammonium sulfate and TAME, and (*g*) dissolved in either 0.15 M ammonium acetate at pH 6.8 or in 0.15 M saline containing one-tenth volume of 0.15 M sodium barbital at pH 7.4. The preparation formed a single sharp boundary in the analytical ultracentrifuge, and contained less than 4 per cent non-coagulable protein precipitable with 5 per cent trichloracetic acid. The fibrinogen was converted to fibrin for assay as previously described (1).

Bovine thrombin was derived from the Parke, Davis & Company product, and was purified ten times further by the method of Rasmussen (11). There was a marked tendency for substantial amounts of the purified thrombin to become absorbed from dilute solutions on to glass surfaces, as described by Rasmussen. It was not possible to designate the absolute thrombin concentration used in various experiments, because the amount adsorbed was dependent on the manner in which the stock solution was diluted. The amount of thrombin used is designated in terms of arbitrary units calculated on the basis of its original concentration in NIH units and its dilution. Experimental error in comparative serial experiments was minimized by using the same stock of diluted thrombin solution and pipette in each reaction.

Tosyl-L-arginine methyl ester hydrochloride at 0.015 M concentration was used to stop reactions between thrombin and fibrinogen by competitive inhibition. Use of TAME as a competitive inhibitor of thrombin was described by Sherry and Troll (12), and by Ehrenpreis, Laskowski, Donnelly, and Scheraga (13). It was estimated from the data of Sherry and Troll that at least 95 per cent inhibition of action of thrombin on fibrinogen was affected by the 0.015 M TAME. The degree of inhibition was found to be sufficient, largely because inhibited reaction mixtures were kept at 0°C except for a relatively short 10 minute period used for centrifugation. Decreased recovery of fibrin clot from calibrated solutions of bovine fibrin was observed by Ehrenpreis *et al.* (13) to result from use of 0.04 M TAME. We found that 0.015 M TAME did not inhibit polymerization of radioiodized rabbit fibrin in solutions containing 0.135 M ammonium acetate and 0.05 M sodium bromide.

Fibrin was separated at 37°C as insoluble coagulum from reaction mixtures, and was washed with the 0.136 M ammonium acetate and 0.015 M TAME solution. Separation of fibrin was facilitated by centrifuging reaction mixtures for 8 minutes at 4500 RPM in thermally insulated centrifuge tubes at temperatures between 35° and 37°C. Quantitative recovery of fibrin was assumed because fibrin is insoluble in the solvent system used. The supernatant solutions remained clear when kept at 37°C. Intermittent samples of coagulum were characterized chemically on the basis of the amount of peptides that could be liberated when the samples were allowed to react further with thrombin. The following procedure was used to liberate fibrinopeptides from the fibrin: (*a*) the fibrin was dissolved in 0.5 ml of 1 M sodium

[1] TAME, tosyl-L-arginine methyl ester hydrochloride.

bromide at pH 5.4, (*b*) mixed with 10 units of thrombin, (*c*) diluted with 10 ml of a solution containing 0.05 M sodium barbital and 0.05 M ammonium acetate at pH 7.4, and (*d*) incubated at 37°C for 4 hours, after which the coagulum was removed and the mother-liquor was analyzed for fibrinopeptides. Small amounts of fibrinopeptide A contained in the coagulum were assumed to be derived from occluded fibrinogen. The amount of fibrin was calculated from the weight of the coagulum minus the weight of occluded fibrinogen. Clots that were estimated to be greater than 5 mg were dried at 110°C for 12 to 16 hours, and weighed to within 0.02 mg. Smaller clots were dissolved in 1 M NaOH by heating at 50°C for 15 minutes, and were compared with calibrated solutions of fibrin by means of Folin-Ciocalteu reagent (14).

Cryoprofibrin was separated by precipitating it at 0°C from reaction mixtures contained in 0.136 M ammonium acetate and 0.015 M TAME. After reactions in ammonium acetate solutions were inhibited with TAME, the solutions were rapidly chilled. The solutions were then subjected to continuous and gentle agitation for 5 hours during the precipitation of protein. To eliminate contamination by fibrinogen, the precipitates were: (*a*) washed twice at 0°C with ammonium acetate and TAME solution, and (*b*) washed once at 0°C with 0.15 M ammonium acetate. Complete removal of contaminating fibrinogen from the precipitate was indicated by absence of protein in the fluid from the second and third washings. Fibrinogen was not washed from cryoprofibrin precipitates in our previous study (1); in this instance we were primarily concerned with measurement of fibrinopeptides liberated from fibrinogen.

The following procedure was used to separate cryoprofibrin from reaction mixtures in saline and plasma: (*a*) cryoprofibrin and fibrinogen were precipitated as Cohn fraction I after inhibiting reactions with TAME, (*b*) transferred to 0.36 M ammonium acetate and 0.04 M TAME solution at 37°C, (*c*) stored at 0°C for about one-half hour until precipitation of cryoprofibrin subsided, and (*d*) diluted with 1.7 volumes of cold water to complete the precipitation of cryoprofibrin.

Nearly complete recovery of cryoprofibrin from inhibited reaction mixtures was assumed for the following reasons: (*a*) The supernatant fibrinogen solution contained no dimers within limits detectable by analytical ultracentrifugation, whereas a large amount of dimers were found when cryoprofibrin was not removed. (*b*) Good correspondence was observed between thrombin-catalyzed liberation of fibrinopeptides and the amounts of cryoprofibrin separated from reaction mixtures. And (*c*) by virtue of the procedure used in preparing the fibrinogen, the solutions would have been saturated with respect to the amount of cryoprofibrin that tended to remain dissolved at 0°C. Consequently, newly formed cryoprofibrin would be precipitable.

Fibrinopeptides were separated and measured as follows: (*a*) Protein was removed by precipitation with 5 per cent trichloracetic acid and 1 per cent acetic acid. (*b*) The acids were extracted from solution with 2:1 ligroine-ether mixture. (*c*) The solution was concentrated in conical tubes by evaporation using a rotating evaporator. (*d*) A minimal amount of dimethylformamide was added to dissolve any crystalline tosylarginine derived from hydrolysis of TAME during deproteinization. (*e*) Volume was adjusted to 1.5 ml with water. (*f*) The solution was transferred to a 1 cm diameter column containing 3 gm of sephadex (grade G-25) which was suspended in 0.05 M pyridine and packed into 12.2 ml. (*g*) When large amounts of crystalline tosylarginine had been dissolved in the concentrated sample, 0.5 ml of a 50 per cent solution of dimethylformamide in 0.05 M pyridine was added to the column both before and after application of the sample. (*h*) The first 7.0 ml of effluent following application of the 1.5 ml sample was collected from the column. (*i*) The effluent was evaporated to 0.2 ml, spotted along with a rinse on to Schleicher and Schüll paper strips, and subjected to electrophoresis at 7.8 volts/cm in 0.05 M sodium acetate at pH 4.0. (*j*) Strips were dried at 105°C in an oven with uniform draft. (*k*) Peptides were then located, eluted with 1 or 2 ml of 1 per cent potassium hydroxide, and measured to within ½ millimicromole per ml by means of a Sakaguchi reaction as described previously (1).

RESULTS

Cryoprofibrin and Fibrin Produced by Limited Action of Thrombin.—

Fibrinogen in 0.15 M ammonium acetate at pH 6.8 was gently stirred and allowed to react with bovine thrombin at concentrations below 0.01 NIH units per mg of fibrinogen at 37°C,

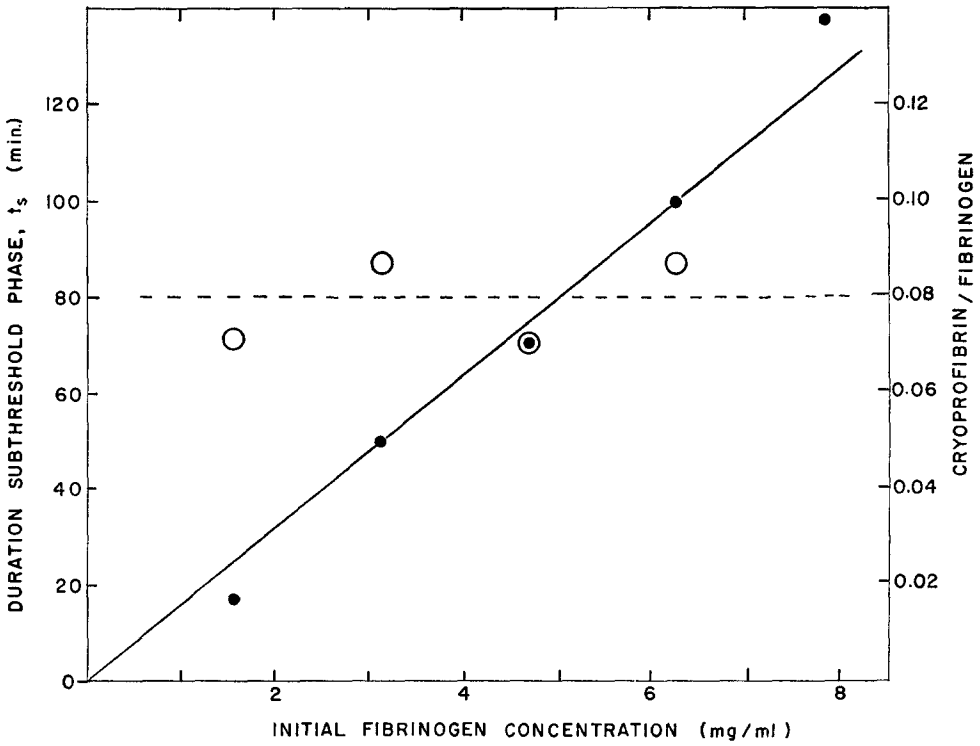

FIG. 1. Duration of subthreshold phase (●) and relative concentrations of cryoprofibrin and fibrinogen (○) at end of subthreshold phase as functions of initial concentration of fibrinogen. Solvent: 0.15 M ammonium acetate at pH 6.8. Thrombin concentration: 9.5×10^{-4} units/ml. Volume: 4.2 ml. Temperature: 37°C. Cryoprofibrin was measured after inhibiting reaction by adding one-tenth volume of 0.16 M TAME at pH 6.8.

and the reaction was inhibited after intervals by adding one-tenth volume of 0.16 M TAME. During an initial subthreshold phase turbidity changes were not detectable to within a 0.01 increment in optical density at 340 mμ as measured in 1 cm cuvettes with a Beckman DU spectrophotometer. Fibrin strands were not detectable during this phase. The end of the subthreshold phase was marked by the abrupt appearance of an opalescent sheen in the reaction mixture. The duration of the subthreshold phase was directly proportional to the concentration of fibrinogen (Fig. 1).

When TAME was added during the subthreshold phase, and the mixture brought to 0°C, protein having composition of cryoprofibrin separated as a flocculent precipitate (Table I, *d*).

The cryoprofibrin redissolved without difficulty on adjusting the temperature of the solution back to 37°C.

A thin film of protein deposited on stirring rods in the region of the triple interface between the rod, solution, and air. The protein appeared to be surface-denatured fibrinogen rather than a product of reaction between thrombin and fibrinogen, because it deposited from fibrinogen solutions as well as reaction mixtures, and deposition did not increase measurably during the subthreshold phase of the reaction (Fig. 2).

TABLE I

Content of Fibronopeptides in Fibrinogen, Cryoprofibrin, and Fibrin

Material	Source	No. of specimens analyzed	Fibrinopeptide A per mg coagulable protein, mμmoles/mg ± SEM	Ratio of fibrinopeptides (A/B) ± SEM
(a) Fibrinogen	Separated from plasma	7	5.20 ± 0.25	1.10 ± 0.017
(*b*) Fibrinogen	Separated from cryoprofibrin	3	4.64 ± 0.46	0.96 ± 0.031
(*c*) Cryoprofibrin	Separated from plasma	4	2.45 ± 0.09	0.56 ± 0.045
(*d*) Cryoprofibrin	Limited action of thrombin on fibrinogen, $t <$ duration subthreshold phase t_s	5	2.86 ± 0.26	0.52 ± 0.041
(*e*) Cryoprofibrin	Limited action of thrombin on fibrinogen, $t = 2t_s$	2	2.76 ± 0.16	0.56 ± 0.002
(*f*) Cryoprofibrin	Separated from mixture of fibrinogen and fibrin	1	2.55	0.57
(*g*) Fibrin	Limited action of thrombin on fibrinogen, $t_s < t < 4t_s$	5	0.39 ± 0.04	0.086 ± 0.006
(*h*) Fibrin	Separated from cryoprofibrin	3	0.48 ± 0.10	0.110 ± 0.020

As the reaction proceeded beyond the subthreshold phase, fibrin separated from solution as web-like strands that were incorporated into a coagulum. On adding TAME and removing fibrin, the supernatant solution remained clear when kept at 37°C. The washed coagulum had solubility characteristics of fibrin; *i.e.*, insoluble in ammonium acetate and TAME solution, soluble in 1 M sodium bromide at pH 5.4 and 37°C, and again polymerized into a coagulum when solutions in sodium bromide were diluted twentyfold with ammonium acetate and TAME at pH 6.8. As indicated by a small content of fibrinopeptide A in the coagulum (Table I, *g*), native fibrinogen equal to 7.5 ± 1.1 (SD) per cent of the mass of the coagulum was occluded in the fibrin. The fibrin that separated from partially coagulated reaction mixtures contained nearly as much fibrinopeptide B (Table I, *g*) as contained in fibrinogen (Table I, *a*). The high content of fibrinopeptide B was consistent with previous observations that it is liberated at much slower rates than fibrinopeptide A (1), and its liberation is not necessary for production of fibrin (4).

The following test provided further evidence of stability of the protein

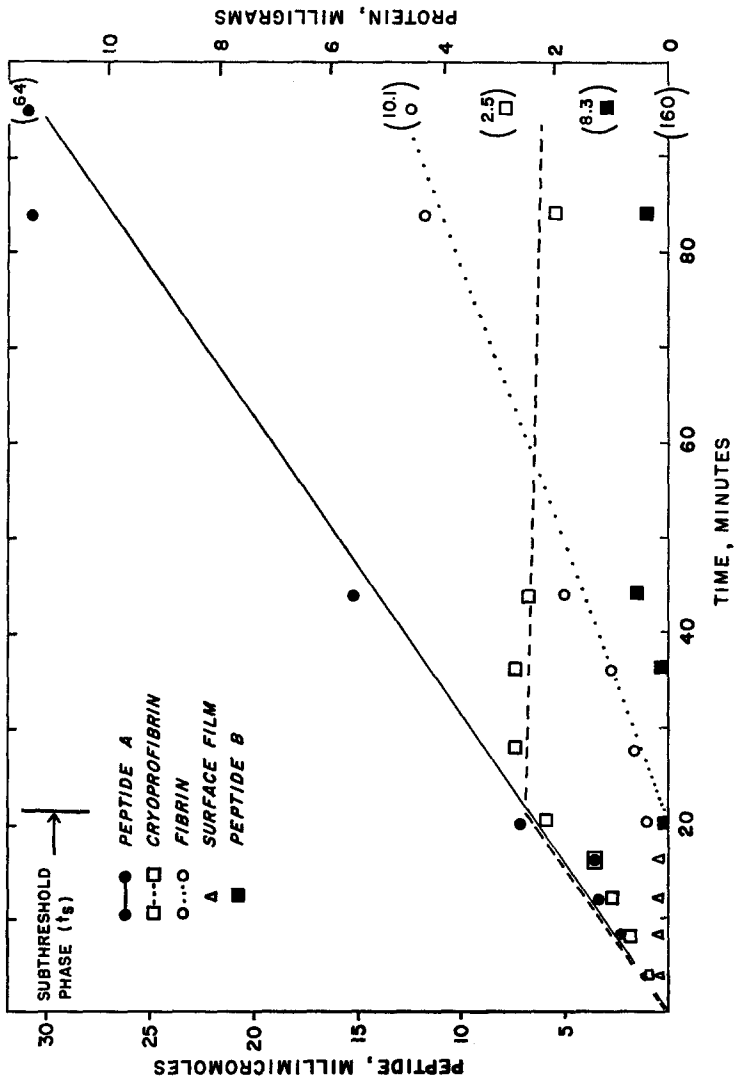

FIG. 2. Liberation of fibrinopeptides and production of cryoprofibrin and fibrin as functions of time. Solvent: 0.15 M ammonium acetate at pH 6.8. Initial amount of fibrinogen: 34.6 mg. Volume: 8.2 ml. Thrombin: 0.02 units. Temperature: 37°C. Measurements were performed after inhibiting reaction by adding one-tenth volume of 0.16 M TAME at pH 6.8. The plotted lines were drawn in accordance with Equations 1 to 3 *a*, using the average values 0.38 mg/millimicromole, 0.32 millimicromole/minute, and 1/0.082 for W, V, and K, respectively. Points in parentheses, representing measurements for reaction period of 160 minutes, were located in accordance with Equations 1 to 3 *a* to correspond to the change in abscissa; they were not used in calculating values for V and K.

solution remaining after TAME was added and fibrin removed: (a) fibrinogen and protein reaction products were precipitated with ammonium sulfate and washed with glycine as used in preparing fibrinogen, and (*b*) were rapidly redissolved in the ammonium acetate and TAME solution at 37°C. The protein redissolved without difficulty, and the solution remained clear for a period exceeding 4 hours.

Protein having composition of cryoprofibrin (Table I, *e*) precipitated when the clear solutions remaining after removal of fibrin were brought to 0°C, and the cryoprofibrin redissolved in the supernatant fibrinogen solution at 37°C. The reversible precipitability of the cryoprofibrin provided further evidence that fibrin did not tend to separate from the clear solutions.

Composition and Properties of Cryoprofibrin.—The experiments described below demonstrate that cryoprofibrin can be separated into fibrinogen and fibrin without liberating fibrinopeptides, and that fibrin can, in turn, combine with fibrinogen to form cryoprofibrin.

When four samples of cryoprofibrin ranging from 6 to 8 mg were dissolved in 3 ml of 0.36 M ammonium acetate containing 0.04 M TAME and 0.0002 M versene, and were diluted with 5 ml water, they became increasingly turbid, and approximately half, 48 ± 9 (SD) per cent, of the protein separated as insoluble coagulum. Two of the samples of cryoprofibrin were derived from plasma, and two from fibrinogen that was subjected to limited action of thrombin. Based on content of fibrinopeptides (Table I, *h*), the coagulum separating from cryoprofibrin was indistinguishable from fibrin (Table I, *g*). Liberated fibrinopeptides were not found in pooled portions of the supernatant solution remaining after separation of fibrin from cryoprofibrin, and would have been found if as little as 0.5 millimicromole per mg of protein had been liberated. Since fibrinopeptides were not liberated the fibrin was not produced by action of thrombin on cryoprofibrin, but was contained in an unpolymerized form within the cryoprofibrin. The soluble protein, remaining after separation of fibrin from cryoprofibrin, had nearly the same content of fibrinopeptides (Table I, *b*) as found in native fibrinogen (Table I, *a*). As based on its content of fibrinopeptides and its coagulability when subjected to action of thrombin, the soluble protein consisted predominately of fibrinogen and, possibly, a small amount of undecomposed cryoprofibrin.

Since cryoprofibrin was separable by physical means alone into fibrinogen and fibrin, it must have been a complex formed by combination of the two substances. Apparently, in the presence of high concentrations of fibrinogen, the altered fibrinogen-lacking fibrinopeptide A did not polymerize into fibrin, but instead combined with native fibrinogen to form a soluble complex which was precipitable as cryoprofibrin in the cold. When the cryoprofibrin was separated from excess fibrinogen and was redissolved, the concentration of native fibrinogen was no longer sufficient to prevent the altered fibrinogen from polymerizing into fibrin. The cryoprofibrin then separated into fibrinogen and fibrin.

Laskowski *et al.* (15) have shown that adsorption of fibrinopeptide inhibits polymerization. The possibility, therefore, existed that cryoprofibrin was

produced by adsorption of fibrinopeptide A on to unpolymerized fibrin. That adsorption was not involved in the formation of cryoprofibrin was indicated by an observation that the fibrinopeptides contained in cryoprofibrin could not be displaced by 5 per cent trichloracetic acid or 0.5 M ammonium hydroxide, but by thrombin. We calculated that adsorption of peptide could have contributed maximally to formation of only 2 per cent of the cryoprofibrin which was separated from TAME-inhibited reaction mixtures. This conclusion arose from an observation that 1 per cent additional fibrin was recovered in presence or absence of TAME when fibrinopeptides were dialyzed from solutions extruded from clots that formed in 0.15 M ammonium acetate. Further evidence that adsorption was not involved in formation of cryoprofibrin was obtained by demonstrating that the separation of fibrin from cryoprofibrin was reversible in the absence of liberated peptide, as described below.

When solutions of fibrin in 1 M sodium bromide at pH 5.4 were diluted with twenty volumes of 0.15 M ammonium acetate adjusted to pH 7.4 with sodium phosphate, the fibrin coagulated to within 2 per cent. However, when it was mixed with an amount of fibrinogen 20 times greater than the fibrin, the fibrin remained dissolved.[2] These observations were made both with fibrin produced by limited action of thrombin and containing fibrinopeptide B, and with fibrin that had lost B in addition to A by extensive action of thrombin. The amount of fibrin converted to cryoprofibrin was not measured, because the presence of bromide prevented complete precipitation of cryoprofibrin when solutions were brought to 0°C. Conversion of fibrin to cryoprofibrin was measured in one case, as described below.

A 5.1 mg sample of fibrin containing fibrinopeptide B but not A was suspended along with 74 mg of fibrinogen in 5 ml of 0.4 M sodium bromide and 0.04 M TAME, and was diluted tenfold with 0.135 M ammonium acetate and 0.015 M TAME to form a slightly turbid solution. The protein was precipitated with ammonium sulfate and subsequently washed with glycine solution as in preparation of fibrinogen. All but 1.4 mg of the protein redissolved in ammonium acetate and TAME solution. The fibrin that remained soluble had combined with fibrinogen to form cryoprofibrin, as evidenced by the content of fibrinopeptides in 7.1 mg of protein precipitating at 0°C (Table I, *f*). The amount of cryoprofibrin produced was twice the amount of fibrin that redissolved in the fibrinogen solution, and was equal to 10.0 per cent of the amount of fibrinogen in the supernatant solution remaining after precipitation of cryoprofibrin.

As described in the following section, there is a point of equilibrium wherein decomposition of cryoprofibrin into fibrinogen and fibrin is balanced by recombination of fibrinogen and fibrin. Kinetic data indicate that the formation

[2] As indicated in a personal communication, Dr. S. Ehrenpreis, Georgetown School of Medicine, Washington, D. C., has observed a similar effect of high concentrations of fibrinogen on fibrin.

of cryoprofibrin is in equilibrium with the formation of fibrin in ammonium acetate solution when the concentration of cryoprofibrin equals 8.2 per cent of the fibrinogen. The amount of cryoprofibrin formed in the present experiment is in fair agreement with that expected from kinetic data.

Fibrin clots, in the form of dense insoluble coagula, dissolved partially when allowed to react with fibrinogen; however, we have not as yet succeeded in dissolving within a 15 minute period more than 50 per cent of the fibrin from a clot. We attributed at least part of the difficulty to stabilization of the clots, because the portions that did not dissolve easily were insoluble in 1 M sodium bromide at pH 5.4. Formation of cryoprofibrin accompanied dissolution of the clots.

Quantitative Aspects of Fibrinogen-Fibrin Conversion in Ammonium Acetate Solution.—As shown in the previous section, altered fibrinogen lacking fibrinopeptide A combines with native fibrinogen containing fibrinopeptide A to form cryoprofibrin. High concentrations of native fibrinogen relative to altered fibrinogen appear necessary to prevent the altered fibrinogen from polymerizing into fibrin. When dissolved in solutions containing no fibrinogen, cryoprofibrin breaks down into fibrinogen and fibrin. Altered fibrinogen tends to remain dissolved during the subthreshold phase of reaction between thrombin and fibrinogen, as indicated by the absence of fibrin and the stability of the solution after adding TAME to inhibit thrombin. Reasonably, the altered fibrinogen would not tend to polymerize into fibrin until conditions for equilibrium between both its incorporation into cryoprofibrin and its separation from cryoprofibrin are satisfied. The tendency for altered fibrinogen to be incorporated into cryoprofibrin would be dependent on the concentration of fibrinogen. Accordingly, the duration of the subthreshold phase would correspond to the the time needed for catalytic action of thrombin to bring the concentration of cryoprofibrin into equilibrium with the concentration of fibrinogen.

To determine whether the quantitative aspects of fibrinogen-fibrin conversion conform with this viewpoint, the following was done: (*a*) The formation of cryoprofibrin and thrombin-catalyzed liberation of fibrinopeptides were studied as functions of time to establish a kinetic relationship between the production of altered fibrinogen and formation of cryoprofibrin. (*b*) Concentrations of cryoprofibrin appearing necessary for formation (precipitation) of fibrin in the presence of various concentrations of fibrinogen were determined to establish that conditions for equilibrium in formation of cryoprofibrin and fibrin can be expressed in terms of a mass action relationship between cryoprofibrin and fibrinogen. (*c*) The formation of fibrin was studied as a function of time to establish that it could be expressed as a function of the production of altered fibrinogen in excess of the amount tending to be incorporated into cryoprofibrin in accordance with the mass action relationship.

Peptide A was liberated at a constant velocity of 0.32 millimicromole per minute (Fig. 2) both during and after the subthreshold phase.[3] The liberation of fibrinopeptide A at constant velocity indicated fibrinogen was in large excess relative to thrombin. Liberation of peptide B was negligibly small in comparison with A, and did not contribute substantially to production of cryoprofibrin or fibrin over the periods of reaction that were studied.

The amounts of cryoprofibrin formed during the subthreshold phase corresponded to 0.36 mg per millimicromole of liberated peptide A (average of three points between $t = 8$ and $t = 16$ minutes in Fig. 2). On the assumption that cryoprofibrin contains one-half as much fibrinopeptide A as does fibrinogen, we calculated that 0.38 mg cryoprofibrin would be formed per millimicromole of liberated fibrinopeptide A. Since the observed yield agreed with that expected, we concluded that the fibrinogen altered by thrombin-catalyzed liberation of fibrinopeptide A is partitioned predominantly into cryoprofibrin during the subthreshold phase of the reaction. The quantitative relationships between reactants and products of the subthreshold phase can, accordingly, be expressed as in Equation 1.

$$-\frac{dx}{dt} = \frac{dy}{dt} = \frac{WdA}{dt} \equiv WV, \text{ and } \frac{dz}{dt} = 0 \text{ when } t < t_s \tag{1}$$

in which x = milligrams fibrinogen (coagulable protein)
y = milligrams cryoprofibrin (coagulable protein)
z = milligrams fibrin
A = millimicromole peptide A
W = milligrams cryoprofibrin forming per millimicromole of peptide A
t = period of reaction
t_s = duration of subthreshold phase of reaction.

The concentration of cryoprofibrin reached a level equal to 8.2 ± 0.42 (SEM) per cent of the fibrinogen, and remained at this level as reactions proceeded beyond the subthreshold phase. The same relative proportions of cryoprofibrin and fibrinogen were found at the end of the subthreshold phase

[3] *Experimental Error:* Variations in reaction velocity appear to be greater than variations due to errors in measuring protein or peptide in the experiment of Fig. 2. This is indicated by the larger size of coefficients of variation for kinetic relationships as compared with stoichiometric relationships between variables. Pearson's coefficient of variation (V) is the per cent relative size of standard deviation and mean. For the purpose of statistical inference, ratios of squared coefficients of variation in the present case would be distributed as is Fisher's variance ratio, because the data have common origin. The variance ($V^2 = 0.012$) for equivalent weights of cryoprofibrin and fibrin found per mole fibrinopeptide A was significantly smaller than the variance ($V^2 = 0.033$) in rate of liberation of peptide A or rate of formation of cryoprofibrin and fibrin ($V^2 = 0.045$).

of reactions involving a wide range of fibrinogen concentrations (Fig. 1). Incorporation of altered fibrinogen into cryoprofibrin and fibrin, therefore, appear to be in equilibrium when the concentration of cryoprofibrin equals 8.2 per cent of the concentration of fibrinogen. As found in the previous section, an amount of cryoprofibrin equal to 10 per cent of the fibrinogen was formed when fibrin was allowed to depolymerize in the presence of fibrinogen. The conditions for equilibrium in formation of cryoprofibrin, therefore, appear to be the same whether approached from a forward directon by liberation of fibrinopeptide A or from a backward direction by reaction between fibrinogen and fibrin. When interpreted in terms of the laws of mass action, the conditions for equilibrium in formation of cryoprofibrin conform with those for the reversible reaction represented by Equations 2 and 2 *a*, in which $K = 1/0.082$ is the apparent mass equilibrium constant for the reaction in the ammonium acetate solvent.

$$\text{cryoprofibrin} \overset{K}{\rightleftarrows} \text{fibrinogen} + \text{fibrin (insoluble)} \tag{2}$$

$$K = \frac{\text{fibrinogen}}{\text{cryoprofibrin}} = \frac{x}{y} \tag{2 a}$$

Since cryoprofibrin separates into fibrinogen and fibrin in equal proportions (in terms of weight of coagulable protein), the amount of fibrin formed should equal one-half the cryoprofibrin decomposed. The formation of fibrin would, accordingly, be given by Equations 3 and 3 *a*, in which decomposed cryoprofibrin is equated with the difference between the total amount produced in accordance with Equation 1 and the amount remaining undecomposed in accordance with Equation 2 *a*.

$$z = \frac{1}{2}\left(WVt - \frac{x}{K}\right), \quad \text{in which} \quad t > t_s \tag{3}$$

$$z = \frac{(K+1)}{(2K+1)} WV(t - t_s), \quad \text{in which} \quad t_s = x_0/WV(1+K) < t \tag{3 a}$$

The amount of fibrin found during the reaction between thrombin and fibrinogen (Fig. 3) after periods of reaction ranging between 20 and 84 minutes are described by the empirical relationship ($z = bt - a$), in which $b = 0.0648 \pm$ $= 0.0648 \pm 0.0042$ (SD) mg/minute and $a = -1.122$ mg. The regression coefficient (b) of the empirical relationship is the same within experimental error as the rate (0.0631 mg/minute) of formation of fibrin beyond the subthreshold phase as predicted by substituting experimental values for WV and K in Equation 3. The observed duration of the subthreshold phase ($t = 17.6 \pm 2.4$ ($S_{t \cdot z}$) minutes, when $z = 0$) and that predicted ($t_s = 21.2$ minutes) from

Equation 3 were also the same. The linear dependence of duration of the subthreshold phase on the initial concentration of fibrinogen as observed in Fig. 1 is also predicted by Equation 3 *a*. We calculate that the slope of Fig. 1 is 20 per cent smaller than that predicted from data of Fig. 2; however, the thrombin used in obtaining data of Fig. 1 was very dilute and some may have become adsorbed on to the container. We conclude that the course of fibrinogen-fibrin conversion can be expressed as a function of the velocity of production of altered fibrinogen by thrombin-catalyzed liberation of fibrinopeptide A, and the mass action relationship describing conditions for equilibrium between incorporation of altered fibrinogen into cryoprofibrin and fibrin.

Quantitative Aspects of Fibrinogen-Fibrin Conversion under Physiological Conditions.—Physiologic saline, 0.15 ionic strength and pH = 7.4, was used to simulate the ionic composition of plasma, because study of the reaction between thrombin and fibrinogen in plasma was made difficult by the presence of antithrombin activity and by high initial turbidity. Study of the reaction between thrombin and fibrinogen in saline revealed two aspects that were not indicated by studies with the ammonium acetate solvent. One aspect implicated thrombin as an inhibitor in the process of polymerization of fibrin. The other demonstrated that ionic composition of the medium has a large influence on the equilibrium between formation of cryoprofibrin and its separation into fibrinogen and fibrin.

Although TAME appeared to have little effect on polymerization in the ammonium acetate solvent, it had a substantial effect in promoting polymerization in saline. Addition of TAME resulted in sudden separation of fibrin from solutions wherein the reaction between thrombin and fibrinogen had progressed for only one-half, or more, of the time required for fibrin to separate in the absence of TAME. Small changes in pH, ionic strength, or dielectric constant, as affected by glycine or arginine, did not have a comparable effect; and studies by Ehrenpreis *et al.* (13) indicate TAME would tend to retard rather than accelerate separation of fibrin. Since effects resulting from alteration of the medium or interaction of TAME with fibrin were not indicated, the action of TAME appeared to be related to its inhibitory properties towards thrombin or, possibly, another enzyme present in the preparation. Conceivably, adsorption of thrombin on to altered fibrinogen could have blocked binding sites involved in polymerization. The TAME might, then, have functioned in unmasking the binding sites by displacing the altered fibrinogen from its combination with thrombin. The 0.016 M TAME used to inhibit the reaction between thrombin and fibrinogen was sufficient to eliminate the effect on polymerization, because doubling the TAME did not bring about additional separation of fibrin from TAME-inhibited reaction mixtures. Quantitative aspects of formation and decomposition of cryoprofibrin could, therefore, be

determined after inhibiting thrombin with TAME as was done with the ammonium acetate solvent.

Measurements of fibrinogen, cryoprofibrin, fibrin, and liberated fibrinopeptides in the TAME-inhibited reaction mixtures are plotted in Fig. 3.

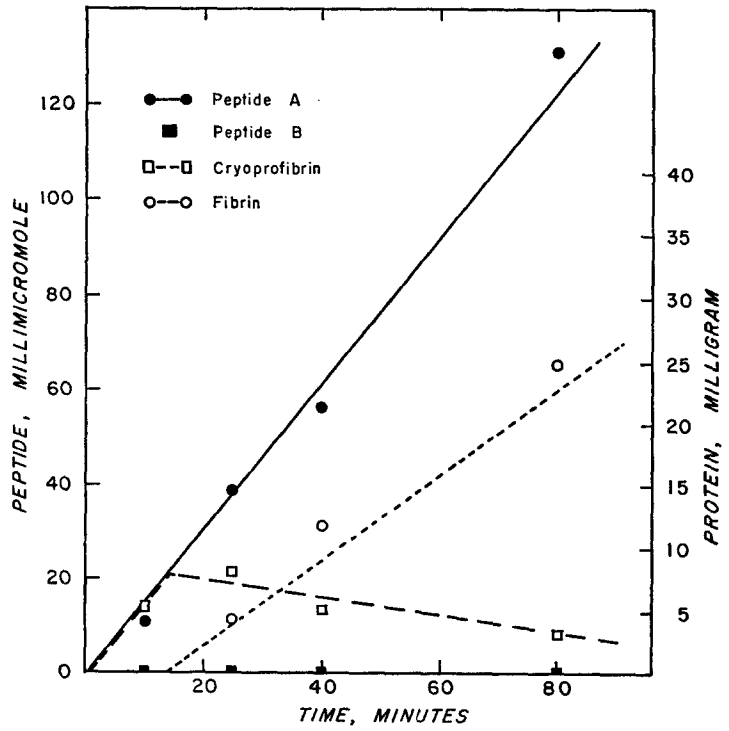

FIG. 3. Liberation of fibrinopeptides and production of cryoprofibrin and fibrin as functions of time. Solvent: 0.136 M sodium chloride and 0.014 M sodium barbital at pH 7.4. Initial amount of fibrinogen: 38.7 mg. Volume: 15 ml. Thrombin: 0.03 units. Temperature: 37°C. The plotted lines were drawn in accordance with Equations 1 to 3 *a*, using average values 0.38 mg/millimicromole, 1.48 millimicromole/minute, and 1/0.26 for W, V, and K, respectively.

Equilibration of cryoprofibrin with respect to the concentration of fibrinogen accompanied the formation of fibrin in reaction mixtures where up to 65 per cent of the fibrinogen was converted to fibrin, as evidenced by the concentration of cryoprofibrin remaining equal to 26 ± approximately 4 per cent of concentration of fibrinogen. Equilibration appeared to be necessary to predispose incorporation of altered fibrinogen into fibrin, because fibrin was not formed when the concentration of cryoprofibrin was equal to 16 per cent of

fibrinogen. The production of cryoprofibrin paralleled the liberation of fibrinopeptide A, since the amount of cryoprofibrin remaining in solution or separating into fibrinogen and fibrin was equivalent to 0.459 ± 0.018 (SEM) mg per millimicromole of fibrinopeptide A, which is in satisfactory agreement with the equivalent weight of cryoprofibrin as measured by direct analysis (Table I).

The rate of liberation of fibrinopeptide B was immeasurably small.[4]

As described in Fig. 3, the kinetic and quantitative aspects of formation of cryoprofibrin as a consequence of limited action of thrombin under the simulated physiologic conditions conforms with Equations 1 to 3; however, the constant (K = fibrinogen/cryoprofibrin = 1/0.26) representing conditions for equilibrium in the formation of cryoprofibrin and fibrin is one-third that circumscribing equilibrium in the ammonium acetate solvent.

The conditions for equilibrium in formation of cryoprofibrin in plasma appeared to be the same as in the saline solution, because the concentration of cryoprofibrin found in four partially coagulated samples of plasma ranged from 25 to 30 per cent of the concentration of fibrinogen after thrombin was inhibited by TAME. Therefore, plasma proteins other than those affected by TAME did not influence the equilibrium.

We conclude that under physiologic conditions formation of threshold quantities of cryoprofibrin (equal to approximately 26 per cent of fibrinogen) is a necessary condition for the formation of fibrin, but even this may not be sufficient because thrombin, or a similar enzyme, may inhibit polymerization.

DISCUSSION

Thrombin acts on fibrinogen to liberate fibrinopeptide A. The fibrinogen is altered so as to polymerize into fibrin. The altered fibrinogen may also combine with native fibrinogen to form a cold-precipitable complex called cryoprofibrin, and the polymerization of altered fibrinogen into fibrin is restrained by its incorporation into cryoprofibrin.

Waugh and Livingstone (16) and Ehrenpreis *et al.* (13) measured the production of fibrin alone, and demonstrated that the underlying process involved first order kinetics. Blombäck and Laurent (5) observed that fibrin was not produced during an early phase of the reaction between thrombin and fibrinogen despite the liberation of fibrinopeptide A. Our results indicate that during a subthreshold phase, when the concentration of fibrinogen is high, cryoprofibrin rather than fibrin is formed in proportion to liberation of fibrinopeptide. The concentration of cryoprofibrin is limited by the concentration of fibrinogen present, and when the maximum amount of cryoprofibrin has been formed the altered fibrinogen begins to separate from solution as insoluble fibrin strands.

[4] We have observed that fibrinopeptide B is liberated at measurable rate when the reaction between thrombin and fibrinogen is allowed to proceed without stirring, and fibrin is allowed to form a gel rather than an insoluble coagulum.

Some Aspects of the Mechanism of Formation of Cryoprofibrin.—As based on *N*-terminal amino acids and on a molecular weight of 360,000 for fibrinogen at physiologic concentrations, two residues of fibrinopeptide A are contained in the fibrinogen molecule (4). Physicochemical data of Caspary and Kekwick (8) indicate the fibrinogen molecule is dissociable into at least three subunits. The two residues of A might be located on one, or possibly two separate subunits within the fibrinogen molecule. However, if the two residues of A are located on a single subunit they must be liberated concurrently by action of thrombin, since subunits capable of forming fibrin containing no A are produced in stoichiometric proportion to liberated A.

If it is supposed that two residues of fibrinopeptide A are liberated concurrently from one subunit of the fibrinogen molecule, the altered fibrinogen containing no fibrinopeptide A would have the peptide composition of fibrin, and would correspond to a fibrin monomer. Accordingly, cryoprofibrin, in being separable into fibrinogen and fibrin in equimolecular proportions, would correspond to a fibrinogen molecule combined with fibrin monomer, the two possibly combining in the manner described below.

Polymerization involves interaction of tyrosyl and histidyl residues (17). Liberation of fibrinopeptides unmasks previously unavailable binding sites (3). Donnelly *et al.* (18) suggested that the histidyl residues may not be masked on native fibrinogen, and only the tyrosyl residues may be exposed by liberation of fibrinopeptides. If only the tyrosyl groups are unmasked, the histidyl groups on both fibrinogen molecules and fibrin monomers would compete for the exposed tyrosyl groups on fibrin monomers. The fibrinogen molecules lacking exposed tyrosyl groups would on combining with fibrin monomers block further polymerization, and in the presence of high concentrations of fibrinogen the fibrin monomers would be prevented from polymerizing into fibrin.

The formation of cryoprofibrin and its separation into fibrinogen and fibrin can be explained also in terms of the alternate assumption that each of the two residues of fibrinopeptide A are located on separate subunits within the fibrinogen molecule. Liberation of fibrinopeptide A from one of the two subunits would produce a molecule having the fibrinopeptide content of cryoprofibrin. The altered molecule, which we tentatively call "cryoprofibrin monomer," would contain both a native fibrinogen subunit, containing A, and an altered fibrinogen subunit, lacking A. Dissociation of these subunits from cryoprofibrin, and recombination of the subunits in randomized pairs would not only reconstitute some of the cryoprofibrin monomers, but would also result in formation of fibrinogen molecules and fibrin monomers. Fibrinogen molecules would form when two native subunits combine. Fibrin monomers, called this because they have the molecular weight of fibrinogen but contain no fibrinopeptide A, would result from combination of two altered subunits. The shuttling of subunits between fibrinogen molecules and cryoprofibrin and fibrin monomers

would provide a dynamic basis for the equilibrium involved in separation of cryoprofibrin into fibrinogen and fibrin.

The histidyl and tyrosyl residues involved in polymerization may both be unmasked by liberation of fibrinopeptide A. If so, cryoprofibrin monomers in possessing one altered subunit containing a set of unmasked sites could combine to form dimers. The fibrin monomers by possessing two altered subunits could form polymers, and would tend to polymerize into an insoluble coagulum under conditions in which fibrin itself is insoluble. It can be shown that when the ratio of fibrinogen to cryoprofibrin dimer is greater than the value of the equilibrium constant K (Equation 3 *a*) fibrin monomers do not polymerize into an insoluble coagulum, but combine with cryoprofibrin monomers to a limited extent to form soluble intermediary polymers with content of fibrinopeptides in between cryoprofibrin and fibrin.

When the formation of cryoprofibrin is interpreted in terms of the first suggestion that it is a complex of a fibrinogen molecule in combination with fibrin monomer, the intermediary polymers would correspond to a molecule of fibrinogen combined with a polymer of fibrin. Since the values of equilibrium constants for conversion of "(n)-mers" to "($n + 1$)-mers" increase only slightly with degree of polymerization "n" at low values of n, as shown by Donnelly *et al.* (18), it can be calculated in accordance with laws of mass action that for either viewpoint of the structure of intermediary polymers their concentration would be very small in comparison with cryoprofibrin under conditions of the present study in which the value of the mass equilibrium constant K (Equation 3 *a*) is much greater than 1.

Soluble complexes between native fibrinogen and fibrinogen altered by liberation of fibrinopeptides have not been studied previously, and available data do not enable us to determine whether cryoprofibrin consists predominantly of fibrinogen molecules combined with fibrin monomers, or of dimers of molecules differing from fibrinogen only in lacking one residue of fibrinopeptide A.

Cryoprofibrin as an Indicator of Intravascular Fibrin Deposition.—Histopathologic evidence suggests intravascular deposition of fibrin in cardiovascular disease. Methods have not been available for evaluation of quantitative aspects of intravascular conversion of fibrinogen to fibrin. Cryoprofibrin represents a soluble product of the action of thrombin, it can be measured in blood, and its *in vivo* origin was previously demonstrated (1). Results of the present study indicate that in plasma or saline at pH 7.4, simulating physiologic conditions, altered fibrinogen does not tend to polymerize into fibrin until the concentration of cryoprofibrin is at a threshold near or equal to 26 per cent of the concentration of fibrinogen. Deposition of fibrin on blood vessels would similarly be precluded at relatively low levels of cryoprofibrin in blood, but may accompany the action of thrombin in presence of threshold concentrations of cryoprofibrin.

During the reaction between thrombin and fibrinogen in saline or plasma, greater amounts of altered fibrinogen remained soluble than would be solubilized in forming cryoprofibrin alone. But when TAME was added to inhibit thrombin, the altered fibrinogen that was not incorporated into cryoprofibrin rapidly polymerized into fibrin. The TAME appeared to be displacing adsorbed thrombin that was blocking binding sites involved in polymerization. Waugh and Livingstone (16) concluded from their kinetic data that adsorption of thrombin on to fibrin inhibits production of fibrin. While Ehrenpreis and Scheraga (19) observed that thrombin is not adsorbed on to fibrin, the fibrin that is produced by limited action of thrombin contains fibrinopeptide B and differs from that used in their studies. That thrombin may reversibly inhibit polymerization warrants further study, because administration of an inhibitor of thrombin to counter coagulation in a patient having threshold concentration of cryoprofibrin might provoke, rather than prevent, formation of a thrombus. Since thrombin, or possibly a similar enzyme, may inhibit polymerization of fibrin, the concentration of cryoprofibrin in plasma can be used at present only to determine whether minimal conditions for deposition of fibrin exist within the blood vessels.

Measurements of cryoprofibrin in normal and endotoxin-treated rabbits support the conclusion that levels near 26 per cent of the concentration of fibrinogen are necessary for deposition of fibrin. Plasma from endotoxin-treated rabbits contains 0.2 $\pm$ 0.1 (SD) mg cryoprofibrin per ml, and normal plasma contains 0.03 $\pm$ 0.04 mg per ml (1). Studies by Thomas and Good (20) indicate the concentration of coagulable protein remains at a level near 2 mg per ml for a 4 hour period following treatment with endotoxin. It can be inferred that a small percentage of the endotoxin-treated rabbits had a level of cryoprofibrin near the threshold for polymerization of altered fibrinogen into fibrin. Brunson, Gamble, and Thomas (21) found fibrinoid deposits in 10 per cent of rabbits subjected to single doses of varying amounts of endotoxin, but not in normal rabbits. Their finding of fibrinoid in only a small percentage of treated rabbits appears consistent with our prediction based on levels of cryoprofibrin.

The level of cryoprofibrin in plasma from normal rabbits is far below the threshold for incorporation of altered fibrinogen into fibrin. It can tentatively be concluded that fibrin is not deposited on blood vessels of normal rabbits. This conclusion is consistent with Gitlin's observations that fibrin, as demonstrated by fluorescent antibodies, does not appear to be a component of normal tissues (22).

The presence of a small amount of cryoprofibrin in normal blood indicates there is at least a low level of thrombin activity. Since the concentration of cryoprofibrin remains low, fibrinogen altered by liberation of fibrinopeptide A must be removed from circulation by means other than coagulation. The reticuloendothelial cells function in the turnover of plasma protein. Small doses of

endotoxin impair phagocytosis (23). The increase in cryoprofibrin following treatment with endotoxin might have resulted from disturbance of the reticuloendothelial cells rather than increased thrombin activity.

Continuous fibrin deposition at low levels has been viewed as being necessary for preservation of normal characteristics of capillaries. Since high levels of cryoprofibrin are needed for deposition of fibrin, continual deposition does not appear to be a function of the coagulation system. Effects of deposition within capillaries might be limited to pathologic circumstances and healing processes. This implication would be consistent with observations on congenitally afibrinogenemic patients (24).

In absence of continuous deposition of fibrin the fibrinolytic enzyme, plasmin, would be needed only under abnormal circumstances. Sherry *et al.* (25), in demonstrating strong inhibitory power of plasma toward plasmin, suggested that circulating plasmin may not be effective in removal of fibrin. Astrup's (26) observation that only small concentrations of plasminogen activator exist in the intimal lining of arterial walls provides circumstantial evidence in favor of our conclusion that little would normally be needed.

Conceivably, localized deposition of fibrin might occur while cryoprofibrin in other regions of the body is below the threshold concentration. If localized deposition were to occur, the fibrin might subsequently be dissolved by combining with fibrinogen from inflowing blood to form additional cryoprofibrin. However, interrupted flow of blood would allow the fibrin to remain for a period sufficient for stabilization by the enzyme known as "fibrin-stabilizing factor" (27) or "fibrinase" (28) making the fibrin refractory to solubilization by fibrinogen. It appears that a fibrinolytic mechanism would be necessary for removal of thrombi.

Some evidence (1) suggests the heparin-precipitable fibrinogen described by Thomas and Good (20) consists largely of cryoprofibrin. Smith (30) measured a heparin-precipitable fraction of human plasma, and found that its level was increased in rheumatoid arthritis, acute bacterial infections, and pregnancy.

The apparent applicability of cryoprofibrin to determining fibrin deposition in endotoxin-treated rabbits provides some assurance that its measurement is useful in determining whether fibrin deposition precedes atherosclerosis as stressed by Duguid (29). Inability of the coagulation system to maintain the concentration of cryoprofibrin at low levels may provide the circumstance for this deposition.

SUMMARY

Fibrinogen altered by thrombin-catalyzed liberation of fibrinopeptide A was found to combine with native fibrinogen to form a cold-precipitable complex we have called "cryoprofibrin." The altered fibrinogen lacking fibrinopeptide A polymerized into fibrin, but not until conditions for equilibrium

between its incorporation into both cryoprofibrin and fibrin were satisfied. At equilibrium, the concentration of cryoprofibrin was maintained at a threshold proportional to the concentration of fibrinogen. When the concentration of cryoprofibrin was below threshold, fibrin could be depolymerized and solubilized by fibrinogen with resultant formation of cryoprofibrin. Since threshold concentrations of cryoprofibrin appear necessary for precipitation of fibrin, the concentration of cryoprofibrin in plasma provides a basis for determining intravascular deposition of fibrin. Intravascular deposition of fibrin does not appear to occur normally in rabbits, because the concentration of cryoprofibrin in plasma from normal rabbits is far below the threshold for precipitation of fibrin. The applicability of cryoprofibrin as an indicator of fibrin deposition is demonstrated by the occurrence of levels of cryoprofibrin approaching the threshold for precipitation of fibrin in plasma from endotoxin-treated rabbits.

The current concept that the fibrinogen molecule can dissociate into subunits can be used to explain the conversion of fibrinogen to cryoprofibrin. As one possibility, the two residues of fibrinopeptide A contained in fibrinogen may be located on two separate subunits of the molecule; cryoprofibrin is produced when one of these subunits is replaced by a subunit altered by loss of fibrinopeptide A. Recombination of native subunits with subunits altered by loss of A would counter dissociation of cryoprofibrin and inhibit polymerization of subunits lacking fibrinopeptide A. As an alternate mechanism, two residues of A may be liberated concurrently from a single subunit. Cryoprofibrin would then correspond to a fibrinogen molecule, containing a subunit with two residues of A, in combination with an altered molecule containing a subunit lacking two residues of A.

Liberation of fibrinopeptide B did not contribute measurably to production of fibrin resulting from limited action of thrombin on rabbit fibrinogen. Both fibrin containing B but not A, and fibrin containing neither B nor A, as is produced by extensive action of thrombin, could be solubilized by fibrinogen.

Thrombin, or another enzyme utilizing tosyl-L-arginine methyl ester as substrate, appeared reversibly to inhibit polymerization of fibrin containing fibrinopeptide B. This enzyme and fibrinogen were the only proteins appearing to inhibit polymerization in plasma from normal rabbits.

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