SOLUBLE FIBRIN COMPLEXES

Nils U. Bang, M.D., and Marge L. Chang, M.D.

A large volume of literature appearing over the last three decades has documented the existence of soluble fibrin-like macromolecules occurring *in vitro*, as well as *in vivo*. At the same time considerable confusion in terminology has arisen. Without attempting to contribute further to this semantic confusion we present in Table 1 the terminology and abbreviations utilized in this review, together with our definitions of these individual entities.

Historically, soluble fibrin complexes (SFC) first received intensive interest during the '50's and the early '60's, and investigations in many laboratories focusing on the physicochemical properties of these macromolecules contributed significantly to our understanding of the mechanisms involved in normal fibrin formation.

During these same years and even earlier there appeared scattered clinical reports, as well as reports from animal experimental studies, suggesting that SFC existed *in vivo* in a variety of thrombotic states [4, 15, 30, 63, 74, 82, 100, 114, 140].

In 1960 Shainoff and Page [126], using a precise biochemical approach, confirmed previous reports that the plasma of endotoxin-treated rabbits contained substantial quantities of soluble cold-precipitable thrombin-clottable high molecular weight SFC.

In the 1960's and in the 1970's macromolecular SFC were demonstrated with increasing frequency in patients and in experimental animals with intravascular coagulation. The demonstration of SFC became possible with the introduction of rapid screening technics (e.g.: the protamine sulfate gelation test [33, 81, 108] and the ethanol gelation test [18, 53]), which were shown to produce fibrin strands or gels in plasmas containing SFC.

Fletcher et al. [3, 41-48] have provided substantial preliminary evidence suggesting that the determination of SFC content in plasma samples from patients through gel exclusion chromatography represents a sensitive and specific assay capable of detecting ongoing subclinical intravascular thrombosis.

From the clinical data available it, therefore, appears that circulating SFC may represent an important molecular marker for intravascular fibrin formation and that the detection of SFC in patients may have considerable therapeutic potential.

It is the purpose of this review first to summarize available biochemical and biophysical data on SFC. Secondly, we wish to discuss the information available on

Lilly Laboratory for Clinical Research and Veterans Administration Hospital, Indiana University School of Medicine, Indianapolis, Ind.

TABLE 1.

Entity	*Description*	*Abbreviation*
Soluble Fibrin Complexes	Any polymeric material originating from fibrinogen or fibrin possessing molecular weight greater than fibrinogen. Maintained in solution under artificial or physiological conditions.	SFC
Fibrinogen	Soluble plasma protein; dimer of sets of Aα Bβ and γ chains; contains intact fibrinopeptides A and B.	
Fibrinopeptides A & B	Peptide material liberated from fibrinogen and certain fibrinogen fragments at the N-terminals of the α and β chains.	FP A & B
Fibrin Monomer	Fibrinogen devoid of *either* fibrinopeptides A or fibrinopeptides A and B; can be produced from thrombin treatment of fibrinogen in the presence of certain inhibitors or from noncrosslinked fibrin by agents capable of breaking hydrogen bonds.	FM
Fibrinogen Fragments X, Y, D & E	Hydrolysis products of fibrinogen incubated with plasmin characterized by acrylamide gel electrophoresis and immunoelectrophoresis according to Nussenzweig *et al.* (111) and Marder and Shulman (93); Fibrinogen X, Y and possibly E possess fibrinopeptides.	Fg X, Fg Y, Fg D, Fg E
Fibrin Fragments X, Y, D & E	Plasmin hydrolysis products of noncrosslinked fibrin; by electrophoretical and other bio-physical criteria nearly identical to fibrinogen fragments X, Y, D and E; however, fibrin X, Y and E contain no fibrinopeptides.	Fb X, Fb Y, Fb D, Fb E

the physiologic and possible pathophysiologic role of these macromolecules; and, thirdly, we wish to evaluate the clinical applications of different technics proposed for the detection of SFC *in vivo*.

An alternative approach to the detection of intravascular fibrin formation, the detection and quantification of degradation products of fibrinogen and fibrin in serum arising as a consequence of plasmin proteolysis, will not be included in this review; since the subject has been discussed extensively in recent reviews by Marder et al. [95] and Mosesson [105].

PHYSICOCHEMICAL CHARACTERISTICS OF SFC

In this section we will consider the different mechanisms proposed for the formation of SFC: (1) SFC formed through the direct action of thrombin on fibrinogen, (2) SFC formed through the interaction between fibrin monomer (FM) and fibrinogen, (3) SFC formed through the interactions between FM and Fg X, Y, D and E, (4) SFC arising as the direct consequence of plasmin proteolysis of fibrinogen, (5) SFC formed from components of lysing fibrin (Fb X, Y, D and E), and (6) SFC arising from crosslinking mediated by fibrin-stabilizing factor (factor XIII) of FM and fibrinogen or alternatively through crosslinking of native fibrinogen not exposed to thrombin.

(1) SFC Formed through Thrombin Action on Fibrinogen

The classic reaction scheme of the fibrinogen-fibrin conversion formulated by Laskowski et al. [84], using the abbreviations of this review, can be written as follows:

Step 1 – Proteolysis: $\text{Fibrinogen} \xrightarrow{\text{Thrombin}} \text{FM} + \text{FP A \& B}$

Step 2 – Polymerization: $\text{nFM} \rightleftharpoons \text{SFC}$

Step 3 – Clotting: $\text{mSFC} \rightleftharpoons \text{fibrin}$

where "n" is a variable number and "m" is some large number.

The existence of SFC occurring prior to the appearance of the fibrin gel was obtained through several different lines of investigation.

Shulman and Ferry [132], having made the observation that neutral alcohols inhibit fibrin formation, prepared SFC in mixtures of thrombin, fibrinogen and hexamethylene glycol. In their studies intrinsic viscosity, as well as analytical ultracentrifugal data, indicated that such inhibited clotting mixtures contained components of molecular weights appreciably higher than fibrinogen or fibrin monomer.

The existence of SFC in thrombin-fibrinogen mixtures or in solubilized noncrosslinked fibrin was rapidly confirmed by several other investigators using inhibitors other than hexamethylene glycol, such as 0.5 M lithium bromide, 1 M sodium bromide and 1 M urea [35, 36, 134]. At sufficiently low thrombin concentrations it was also possible to demonstrate SFC in clotting mixtures containing no inhibitor at all [7, 17, 133, 136, 139].

Figure 1 depicts an experiment conducted by Dr. G. F. Smith in our laboratory [36]. Gel exclusion chromatography on Bio Gel® A-5m, an agarose medium with an exclusion limit of 5,000,000 daltons, was utilized to illustrate the gradual conversion of fibrinogen into high molecular weight SFC.

A solution of purified human fibrinogen in 0.3 M NaCl was reacted with low concentrations of thrombin at room temperature. At Time ∅, at 1½ hours and at 5½ hours, at which time visible gel formation had not occurred in the mixture, aliquots were chromatographed on Bio Gel A-5m. Comparison of the chromatograms obtained at Time ∅ and after 5½ hours' incubation with thrombin

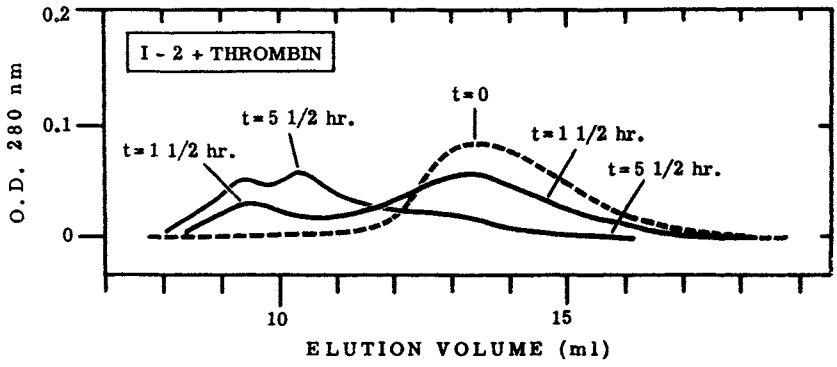

FIG. 1. Agarose gel chromatograms of purified fibrinogen (stippled line) and fibrinogen incubated with thrombin for varying time periods (solid lines). A 3 mg/ml solution of human fibrinogen fraction I_2 in 0.3 M NaCl was reacted with 0.01 units of thrombin/ml. 0.2 ml aliquots were removed at the times indicated and chromatographed on Bio Gel A-5m.

demonstrates that it is possible to convert virtually all fibrinogen in a coagulation mixture into SFC before gelation takes place.

Several investigators demonstrated SFC arising in the course of fibrin polymerization to be rod-like particles with a general size range of 300 to 600 nm by means of measurements by flow birefringence, light scattering and non-Newtonian viscosity [23, 37, 38, 51, 122, 139]. There is considerable evidence that the formation of SFC in polymerizing fibrin arises by a staggered overlapping of monomeric fibrin units to produce molecules of twice the width of fibrin monomer; this conclusion was derived from light scattering studies and from comparisons of length obtained from flow birefringence measurements and sedimentation constants [22, 23, 36]. Rod-shaped SFC have been directly visualized in the electron microscope [10, 61, 135].

Figure 2 shows an electron micrograph of a few fibrinogen molecules and a micrograph of an early string-shaped SFC [10]. Since these two preparations were reproduced to almost exactly the same magnification and were shadow-cast simultaneously at identical angles and with the same quantity of Pt-Pd alloy, the height of the fibrinogen molecules could be compared with the height of intermediate polymers through measurements of shadow length. The height of the SFC is almost twice the height of the fibrinogen molecule, thus confirming through direct visual observations the concept that SFC arise through staggered overlapping of monomeric fibrin units.

Thus, there has been ample evidence for a number of years that macromolecular SFC can be produced *in vitro* in highly purified systems, concisely defined in biochemical and in biophysical terms.

It has been demonstrated by several technics that SFC can be produced in the physiologic milieu of normal blood plasma through the addition of small amounts of thrombin. Gel formation can be observed upon addition of either protamine sulfate or ethanol to thrombin-treated plasma [18, 53, 108, 125]. Similarly, incorporation of ^{14}C-labeled glycine ethyl ester into fibrin monomer and SFC following treatment with fibrin stabilizing factor has been used to demonstrate the presence of SFC in thrombin-treated plasma [79].

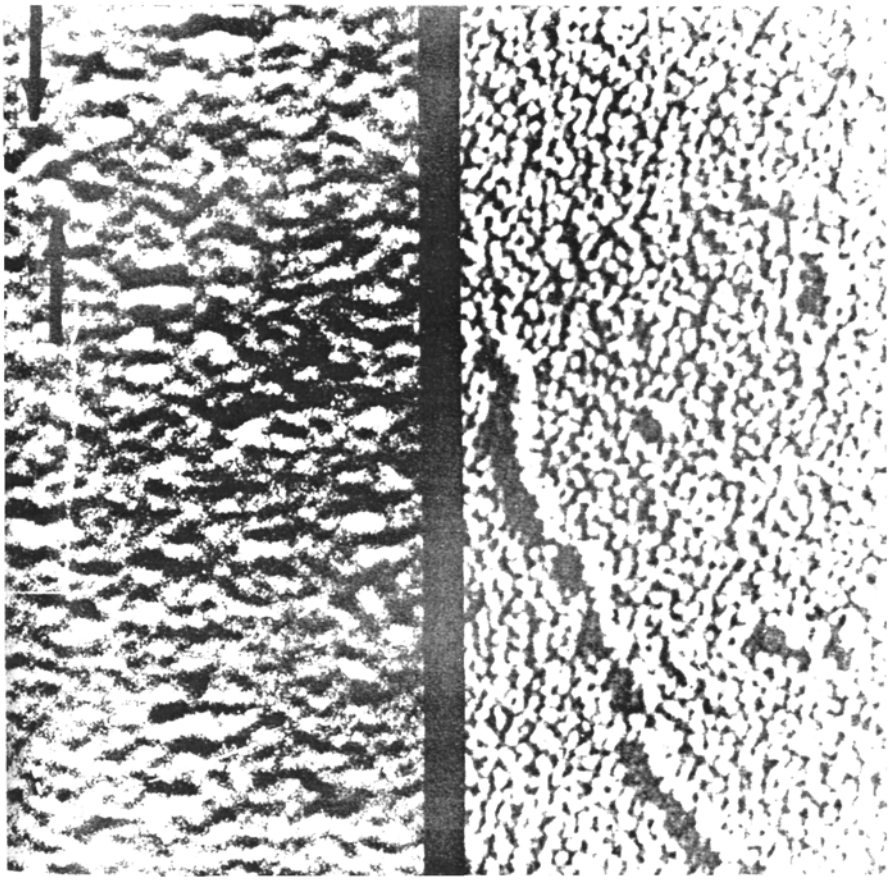

FIG. 2. Left Panel: a few fibrinogen molecules. Right Panel: an early string-shaped SFC. (for details, see text).

We have ample evidence from our own laboratory indicating that thrombin treatment of native plasma can give rise to SFC *in vitro*, readily demonstrable by gel exclusion chromatography [11, 64].

Kierulf and Abildgaard [76] observed that the clottable protein isolated from thrombin-treated plasma always contained N-terminal glycine, characteristic of fibrin monomer, indicating the presence of SFC.

Recently we have found evidence in our laboratory [12] suggesting that one nonfibrinogen protein, namely cold-insoluble globulin, participates in the formation of SFC from normal plasma. This protein has previously been shown by Mosesson and Umfleet [104] to be distinct and separate from either fibrinogen or fibrin monomer. Upon Bio Gel A-5m chromatography of normal plasma, cold-insoluble globulin elutes in a sharply defined symmetrical peak, superimposable on the fibrinogen peak.

As seen in Figure 3, the treatment of normal plasma with low levels of thrombin followed by hirudin results in the emergence of an early SFC peak, eluting close to the void volume. These manipulations also produce a new cold-insoluble globulin peak, eluting with the SFC peak. These findings, in

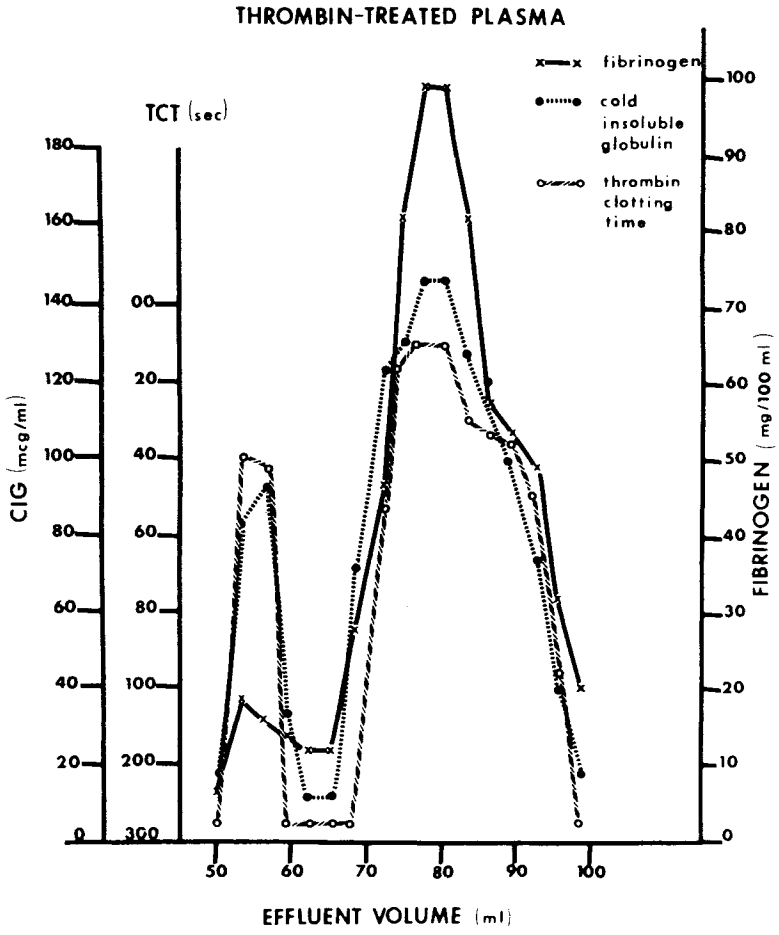

FIG. 3. Gel filtration behavior of fibrinogen and cold insoluble globulin in thrombin-treated plasma. Normal plasma was incubated at 0.04 units of thrombin for 5 minutes. 0.4 units of hirudin were added to block further thrombin action, and 5 ml of the thrombin-treated plasma subjected to Bio Gel A-5m chromatography. Immunoassayable fibrinogen (curve points represented by crosses) shows a two-peak pattern with a small SFC peak eluting close to the void volume. This early peak is readily identified by a thrombin clotting time assay (open circles). Cold-insoluble globulin (closed circles) also elutes in a two-peak pattern closely following the fibrinogen pattern.

combination with earlier reports that cold-insoluble globulin is unaffected by thrombin [104], suggest copolymerization between cold-insoluble globulin and SFC. The function of cold-insoluble globulin in SFC (e.g., maintenance of the complex in its soluble state) remains to be established.

(2) SFC Formed through the Interaction between Fibrin Monomer and Intact Fibrinogen

Shainoff and Page [126] suggested that the cryoprecipitable high molecular weight SFC found in endotoxin-treated rabbits was derived from fibrin monomer

with fibrinopeptide A removed by thrombin. In a subsequent communication they proposed an alternative mechanism [127].

On the basis of elegant kinetic analyses of interactions of solutions containing fibrinogen and FM, they proposed that fibrinogen altered by thrombin-catalyzed liberation of fibrinopeptide A could combine with native fibrinogen to form the cold-precipitable SFC. This suggestion seemed justified, in view of other work [35, 123], indicating that polymerization involves interaction between tyrosyl and histidyl residues. Liberation of fibrinopeptides unmasks previously unavailable polymerization sites.

It was suggested that histidyl residues are unmasked in native fibrinogen, and only the tyrosyl residues may be exposed by the liberation of fibrinopeptides. If only the tyrosyl groups are unmasked, histidyl groups on both fibrinogen and FM would compete for the exposed tyrosyl residues on FM. Fibrinogen molecules lacking exposed tyrosyl groups, when combining with FM, could block further polymerization; and, in the presence of high concentrations of fibrinogen, FM could be prevented from forming fibrin. This theory was further supported by later work from the same laboratory [121]. When purified FM was interacted with excess amounts of fibrinogen, a complex formed which in the analytical ultracentrifuge sedimented at a rate of 24S relative to fibrinogen at 8S at pH 7.4. The complex dissociated completely into monomeric fibrin and fibrinogen upon acidification to pH 5.3. When fibrin-stabilizing factor was added to solutions of FM and fibrinogen, the acid-labile SFC was converted to a stable complex which still sedimented at 24S but which no longer dissociated by either acidification or exposure to 5 M urea.

Subsequent work in other laboratories has provided further support for the existence of SFC composed of FM-fibrinogen. Thus, Jacobsen et al. [73] using gel exclusion chromatography, demonstrated complexing between ^{125}I-labeled fibrinogen and cold FM in the absence of thrombin, resulting in high molecular weight SFC.

Sherman et al. in a preliminary report [131], isolated complexes containing ^{125}I-labeled FM and ^{131}I-labeled fibrinogen. When this double-labeled material was injected into rabbits, parallel rapid multiphasic disappearance curves resulted for both ^{125}I FM and ^{131}I fibrinogen with mear residual plasma radioactivities at 24 hours of 5% and 8%, respectively. These results strongly suggest that FM-fibrinogen complexes can exist as SFC *in vivo* and may provide a mechanism for clearance of FM from the blood.

In contrast, Smith and Bang [136], using preliminary N-terminal analyses of mixtures of fibrinogen and SFC produced by incubating fibrinogen with trace amounts of thrombin, suggested that intact fibrinogen did not incorporate into SFC fractions.

In subsequent work Smith and Craft [137] failed to demonstrate incorporation of fibrinogen into SFC through gel exclusion chromatography of mixtures containing either ^{125}I-labeled SFC and "cold" fibrinogen or ^{125}I-labeled fibrinogen and "cold" SFC.

Thus, although the bulk of evidence favors the concept of SFC composed of FM-fibrinogen, the few published experiments which failed to demonstrate these entities suggest that further work is needed to completely clarify this issue.

(3) SFC formed through Interaction between FM and Fg X, Y, D and E

In 1962 Bang et al. [8] demonstrated that fibrin clots formed in the presence of purified Fg D or formed from the plasma of patients with pathological fibrinolysis possessed an abnormal ultrastructure. To account for these findings it was proposed that Fg D and FM form abnormal polymers during the early phases of coagulation. Residual free FM, although capable of forming normal fibrin, according to this theory, was so depleted that a complete and organized fibrin network could not be established.

Figure 4 [9] illustrates the sequence of events in "defective fibrin polymerization," as visualized in the electron microscope. When mixtures of fibrinogen, Fg D and thrombin were incubated, one can appreciate during the earliest phases of polymerization the appearance of globular structures with a diameter of approximately 50-80 nm. These SFC contrasted sharply to those produced by incubation of fibrinogen with thrombin, as illustrated in Figure 2. The right upper panel of Figure 4 depicts the later growth and aggregation of nonfibrous elements concomitantly with the formation of fibrin, and the right lower panel shows the final picture of fibrous aggregates built into the fibrin network as an integral part of the polymer.

Subsequently, the concept that SFC could arise through the combination of FM with a variety of plasmic derivatives of fibrinogen was investigated and seemingly confirmed by several investigators.

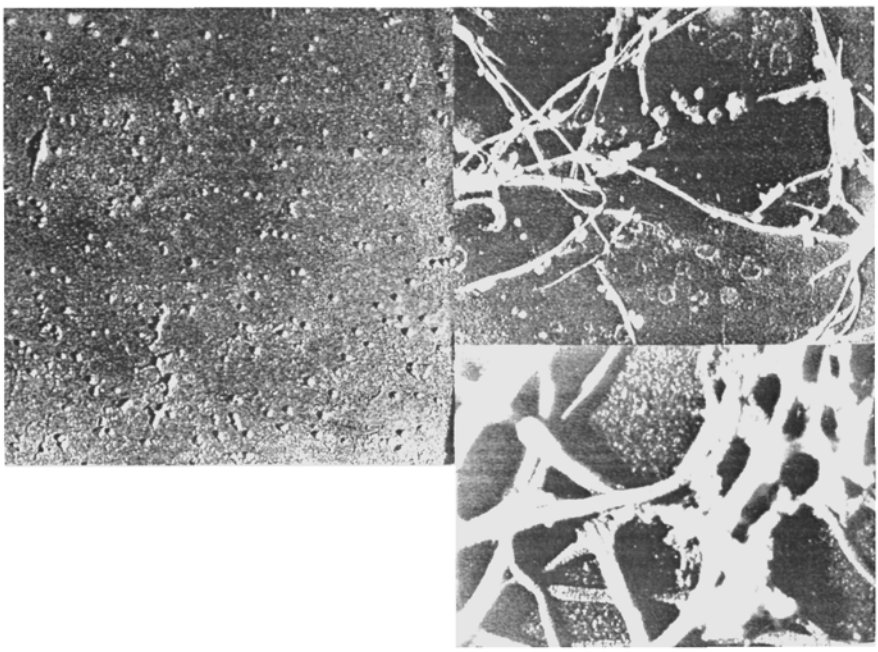

FIG. 4. The electronmicroscopic appearance of globular aggregates during the early phases of polymerization of fibrinogen and thrombin in the presence of Fg D (left panel). Later the growth of nonfibrous elements concomitantly with the formation of fibrin is observed (right upper panel). Finally, nonfibrous aggregates are built into the fibrin network as an integral part of the structure (right lower panel).

Lipinski and co-workers [90] conducted studies in which plasma containing ^{131}I-labeled fibrinogen was clotted in the presence of increasing quantities of either "early" or "late" fibrinogen degradation products, the former containing predominantly Fg X and Y and the latter containing mostly Fg D and E. As the concentration of fibrinogen degradation products was increased, the amount of nonclottable ^{131}I-labeled fibrinogen also rose, leaving the authors to hypothesize that the nonclottable fibrinogen existed in the form of a complex with nonclottable fibrinogen degradation products. However, direct confirmation of the existence of such complexes was not provided.

Similarly, Wegrzynowicz et al. [144] incubated purified ^{131}I-labeled FM with increasing concentrations of either "early" fibrinogen degradation products, i.e., Fg X and Y, or "late" fibrinogen degradation products, i.e., Fg D and E, and examined the supernatant for radioactivity after clotting was completed. Again, it appeared that increasing quantities of either early or late fibrinogen degradation products resulted in increasing quantities of radioactivity not incorporated in the clot. This suggested to the authors the formation of nonclottable SFC between fibrin monomers and components of the fibrinogen degradation products. But again no direct evidence for such complex formation was presented.

In analytical ultracentrifugal studies Marder and Shulman [94] noted the appearance of peaks of high sedimentation velocity in mixtures of fibrinogen, thrombin and Fg D or Y. The suggestion was made on the basis of these findings that the rapidly sedimenting peak consisted of FM complexed with either Fg D or with Fg Y; however, a direct demonstration of the presence of either Fg Y or Fg D in these rapidly sedimenting peaks was not provided.

Kudryck et al. [83] showed that approximately 50% of Fg D was capable of complexing with FM immobilized on cyanogen bromide-activated sepharose. Fg E, in contrast, did not complex with immobilized FM.

Mathias et al. [97] later demonstrated that Fg Y and X, as well as native fibrinogen, exhibited even greater affinity for immobilized FM than did Fg D. On the basis of these observations, both groups of investigators postulated that similar interactions may occur between FM and fibrinogen breakdown products in solution.

Recent work in our laboratory [11, 12, 86, 98, 136] does not support the concept of SFC formation through interaction between FM and fibrinogen degradation products.

In Figure 5 is shown an experiment [136] in which a coagulation mixture of "cold" fibrinogen (Blombäck fraction I_2) and a ^{125}I trace-labeled mixture of Fg D and E and lower molecular weight degradation products of fibrinogen were incubated with trace amounts of thrombin and the reaction allowed to proceed for 20 hours at room temperature. The reaction mixture was then chromatographed on Bio Gel A-5m and eluted fractions analyzed for radioactivity along with continuous OD-280 monitoring. Normal fibrinogen elutes here at a peak volume at 13.5 ml; but, as indicated by the stippled line representing OD-280 readings, SFC eluting earlier than fibrinogen are present in significant quantities. On the other hand, it appears that all of the radioactivity remains strictly associated with Fg D and E eluting later than fibrinogen, as indicated by the solid line. Thus, under the

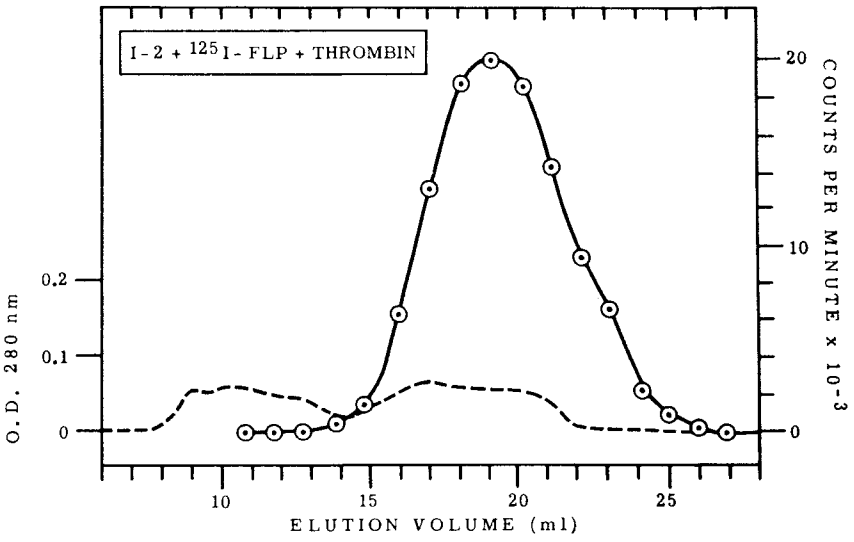

FIG. 5. Agarose gel chromatogram of a mixture of ^{125}I-labeled Fg D and E, "cold" fibrinogen and thrombin. 1.5 mg/ml of "cold" fibrinogen fraction I_2 and 1.3 mg/ml of ^{125}I-labeled end stage plasmin digest of fibrinogen were incubated with 0.0025 units/ml of thrombin for 20 hours at room temperature.

Solid line: ^{125}I CPM. Stippled line: OD-280 readings. Normal fibrinogen in this system elutes around a peak of 13.5 ml.

conditions of these experiments we failed to demonstrate incorporation of either Fg D or E into soluble fibrin polymers.

We subsequently examined interactions between FM and early fibrinogen digests largely composed of Fg X and Y [86, 98]. In the experiment depicted in Figure 6, ^{125}I-labeled "early" fibrinogen degradation products and ^{131}I-labeled fibrinogen were incubated with thrombin, followed by hirudin to block further thrombin activity. The mixture was chromatographed on Bio Gel and individual fractions assayed for both ^{131}I and ^{125}I. The effluent profile depicted in Figure 6 demonstrates a large peak eluting close to the void volume of the column and exhibiting both the ^{131}I and ^{125}I-label, indicating the participation in polymer formation of fibrin monomer, as well as components from the early fibrinogen digests. To elucidate which of the components from an early fibrinogen digest partakes in SFC formation, we conducted experiments with purified Fg Y.

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In the experiment depicted in Figure 7 we incubated ^{131}I-labeled fibrinogen, thrombin and ^{125}I-labeled Fg Y containing trace quantities of Fg D but no Fg X. Upon chromatography of this mixture fibrinogen labeled with ^{131}I eluted in a two-peak pattern with a very large SFC peak; however, in this area of effluent, no ^{125}I activity was demonstrable, clearly indicating that the Fg Y used in these

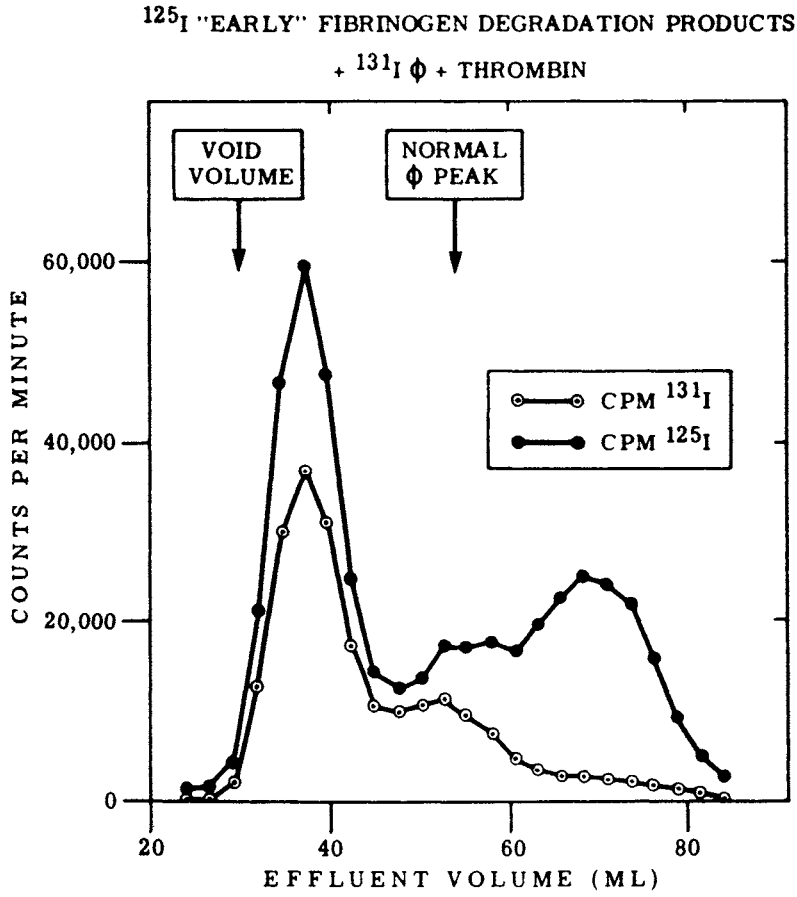

FIG. 6. Agarose gel chromatogram of a mixture of ^{125}I-labeled early fibrinogen degradation products and ^{131}I-labeled fibrinogen incubated with thrombin. ^{125}I-labeled early fibrin degradation products (2.5 mg) and 1.25 mg of ^{131}I-labeled fibrinogen were incubated with 1 unit of thrombin for 18 minutes at 37°C, followed by 10 units of hirudin.

experiments failed to incorporate into SFC. We conclude from our experiments that only clottable fibrinogen-related molecules, i.e., fibrinogen itself and Fg X, incorporate into SFC. Nonclottable Fg Y, D and E do not. Thus, these recent observations made in our laboratory directly contradict the suggestion made by ourselves 12 years ago [8] and also conflict with the conclusions of several other investigators.

We suggest at this point that nonclottable Fg Y, D and E in some unexplainable fashion may contribute toward maintaining SFC composed solely of FM and/or Fg X monomer in their soluble state. Admittedly, our recent observations do not completely exclude previously postulated theories of complex formation between FM and nonclottable Fg Y, D and E. The relatively high ionic strength used routinely in all our agarose gel chromatographic procedures [11, 136], as well as the exposure of the protein mixtures to agarose gels which are not ionically inert, could conceivably cause dissociation of very weakly bonded complexes.

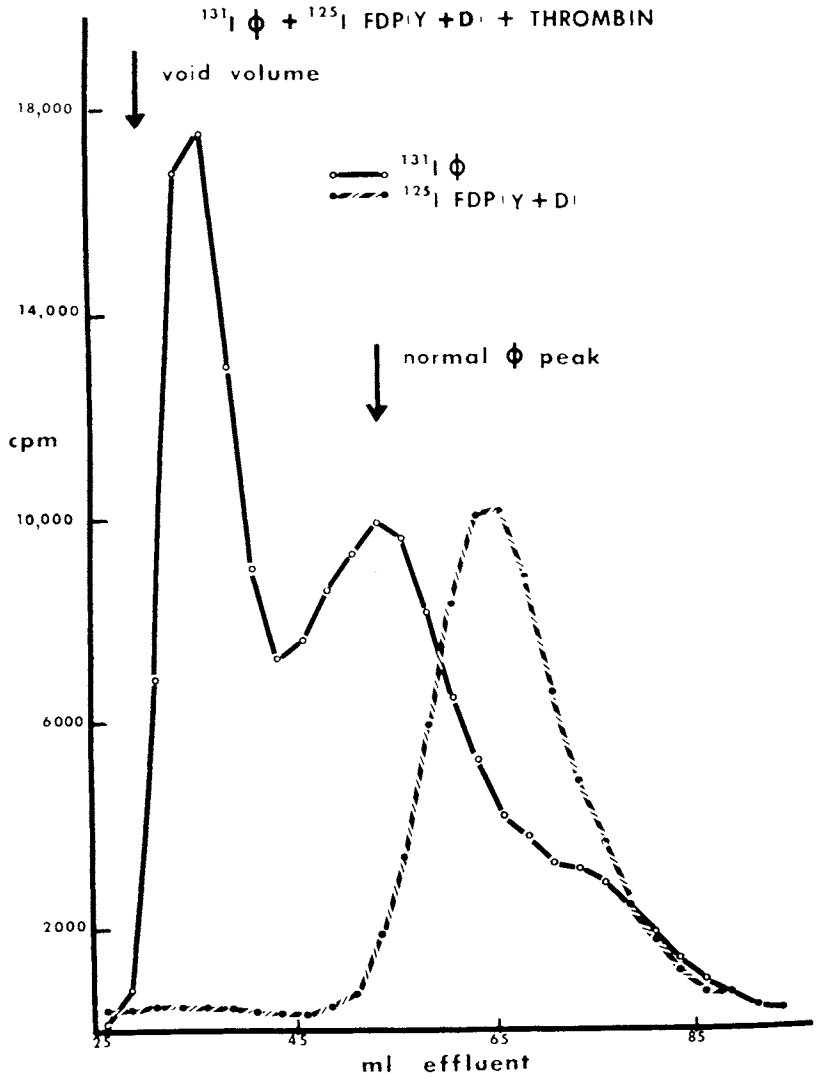

FIG. 7. Agarose gel chromatogram of incubation mixture of ^{131}I-labeled fibrinogen, ^{125}I-labeled partially purified Fg Y and thrombin. Fg Y (3.5 mg/ml) and fibrinogen (0.62 mg/ml) were incubated with 0.12 units of thrombin for 10 minutes, followed by 1.2 units of hirudin.

(4) SFC Arising from Plasmin Proteolysis of Fibrinogen

Fletcher et al. [40] systematically investigated the degradation products arising from plasmin hydrolysis of bovine and human fibrinogen in the analytical ultracentrifuge.

In the course of these investigations they noted that degradation of human but not bovine fibrinogen by plasmin resulted in significant polymer formation. Polymer formation occurred as early as 5 minutes after the start of incubation of

human fibrinogen with plasmin and increased and persisted until completion of the final proteolysis steps. The polymers displayed $S_{20}w$ values ranging from 8 to 14S. The amount of polymer in proportion to total protein ranged from 2% after 5 minutes to as high as 35% after 60 minutes of incubation of fibrinogen with plasmin and thereafter fell to zero during the final proteolytic stages. On this basis it was suggested plasmin might cleave arginine-glycine bonds (those susceptible to thrombin) in a random fashion releasing fibrinopeptides A and B. This hypothesis, which does represent an intriguing alternate mechanism for SFC formation, has not been confirmed in subsequent *in vitro* or *in vivo* experiments; and it remains speculative at this date.

(5) SFC Formed from Components of Lysing Fibrin (Fb X, Fb Y, Fb D, Fb E)

It has been known for a long time that the products of plasmin proteolysis of noncrosslinked fibrin under suitable experimental conditions may reaggregate to form gels and ordered polymers. Derechin [31-33] first reported that protamine sulfate causes nonenzymatic gelation of fibrin lysed by plasmin, and he referred to this phenomenon as the paracoagulation reaction. Other substances, including toluidine blue, a wide variety of inorganic salts (sulfates, mercuric chloride, uranium acetate), organic compounds, including aniline, ninhydrin, o-, m- and p-nitrophenols and tyramine, may produce gel formation in mixtures of fibrin degradation products. In addition, lowering of the ionic strength to less than 0.15 M, cooling or acidification to pH 5.5 may also induce the paracoagulation reaction in lysed fibrin [33, 81].

Horn et al. [70] and Niewiarowski et al. [107] independently observed that the gel produced by the addition of protamine sulfate to fibrin degradation products exhibit the highly organized structure and periodicity of a normal fibrin clot.

It was suggested by Niewiarowski et al. [107], that the paracoagulation reaction of plasmic degradation products of fibrin is mediated through Fb X. The earliest catabolic intermediate of fibrinogen, Fg X, is clottable by thrombin, since it retains fibrinopeptides A. In contrast, Fb X, arising from a plasmin proteolysis of already polymerized fibrin, is devoid of fibrinopeptides. In the hypothesis forwarded by Niewiarowski et al. it is assumed that Fb X under physiologic conditions is prevented from polymerizing because it undergoes SFC formation with nonclottable degradation products (Fg and Fb Y, D and E). The addition of protamine sulfate or other manipulations is supposed to cause dissociation of these complexes, liberating Fb X, which then, in turn, polymerizes to form ordered fibrin structures.

Basing our work on these earlier investigations, we have recently conducted a series of experiments in our laboratory exploring the possibility that molecular fragments arising from plasmin proteolysis of fibrin, rather than fibrinogen, may reaggregate or copolymerize under physiologic circumstances to form SFC.

The capability of fibrin fragments D and E (Fb D and Fb E) to participate in SFC formation was investigated by gel exclusion chromatography [26]. Noncrosslinked fibrin extensively digested by plasmin and purified Fb D and Fb E did not form higher molecular weight aggregates upon incubation with thrombin and upon

incubation with fibrinogen in the absence of thrombin. On the other hand, Fb D-E mixtures, as well as purified Fb D and purified Fb E upon incubation with fibrinogen and thrombin displayed moderate peak shifts toward a higher molecular weight range by agarose gel chromatography, suggesting aggregate or complex formation. Fb D and Fb E differ in this respect from fibrinogen fragments D and E (Fg D and Fg E), which fail to incorporate into SFC [136]. These experiments suggest that Fb D and Fb E retain a limited number of polymerization active sites.

In subsequent experiments [11] we turned our attention to "early" plasmin digests of fibrin containing no FM but large quantities of Fb X, as estimated by SDS gel electrophoresis. When an ^{125}I-labeled early fibrin digest was subjected to agarose gel chromatography (Figure 8), a very large early peak emerged, eluting

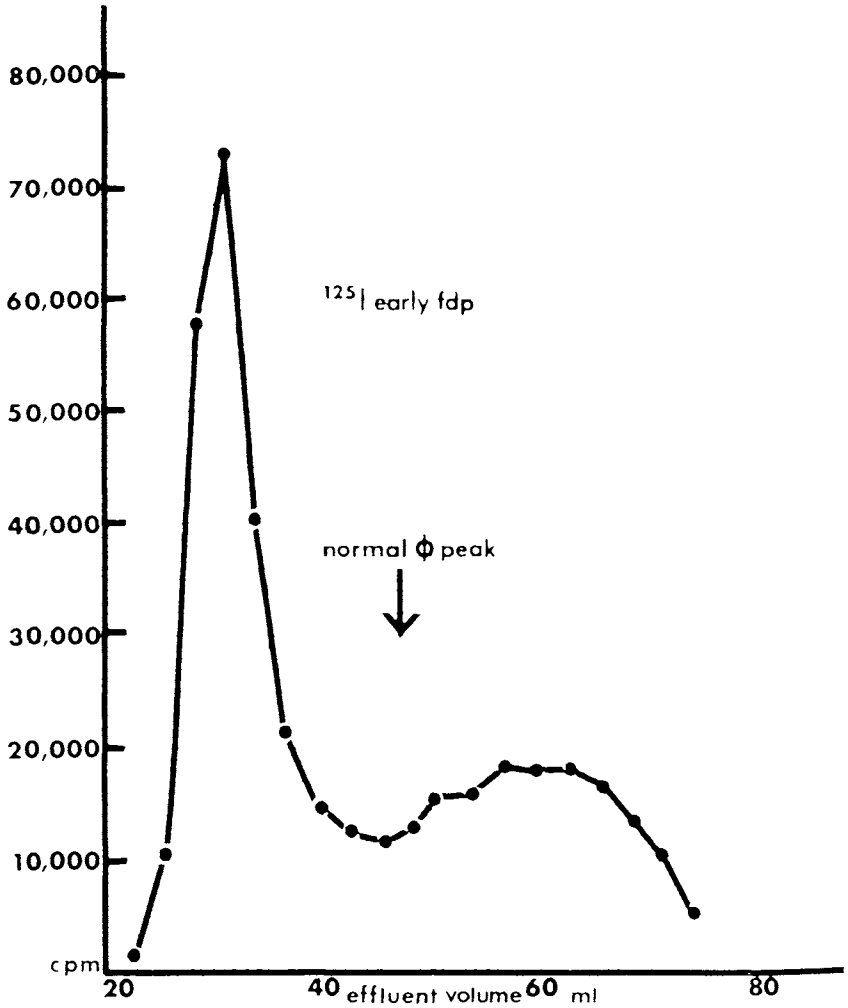

FIG. 8. Agarose gel chromatography of ^{125}I-labeled "early fibrin digest." Plasmin lysate of noncrosslinked fibrin was produced by the addition of 10 units of thrombin to 20 mg of ^{125}I trace-labeled fibrinogen followed by 2 units of plasmin at gel point. The reaction was stopped at the time of complete clot lysis after 5 minutes of incubation at 37 C by the addition of 2,000 units of Trasylol.

with the void volume and representing approximately 70% of the total radioactivity. This indicates that a large portion of early fibrin digests exist in macromolecular form. These early fibrin digests are strongly protamine sulfate positive and will occasionally gel upon simple dilution and lowering the ionic strength from 0.2 M to 0.15 M. In additional experiments [27] we have shown by SDS polyacrylamide gel electrophoresis that this high molecular weight peak material upon treatment with SDS and urea readily dissociates into Fb X.

We next produced an ^{125}I-labeled fibrin digest containing approximately 30% Fb X, 30% Fb Y and 40% Fb fragments of molecular weights less than Y [11].

Figure 9 (upper panel) shows the gel filtration behavior of an aliquot of this early fibrin digest eluting in a broad peak with radioactivity appearing both before and after the normal peak position for native fibrinogen indicated by the arrow. An aliquot of this ^{125}I-labeled digest was treated with Trasylol® and hirudin to block plasmin and thrombin activity, respectively, and then admixed with ^{131}I-labeled fibrinogen. The lower diagram in Figure 9 depicts the gel filtration behavior of the digest following incubation with fibrinogen. The solid tracing shows the distribution of ^{125}I counts denoting the elution profile for fibrin digest components, and the stippled line shows the elution profile of ^{131}I counts denoting the elution profile for fibrinogen under these circumstances. It can be seen that the two proteins now elute in superimposable peak formation. The two peaks occur earlier than the fibrinogen peak for the column, and the ^{125}I peak representing fibrin digest components is now shifted appreciably to the left, compared to its position in the control run illustrated in the upper panel of Figure 9.

This peak also elutes considerably later and, therefore, represents material of considerable lower molecular weight than that demonstrated in our previous experiments (Figs. 1 and 3), in which fibrinogen or plasma were treated with thrombin. The position of the polymeric peak noted in this experiment is consistent with SFC in the molecular weight range of 500,000 to 700,000, which could be a dimer between fibrinogen and Fb X. The appearance of SFC in the dimeric-size range is the most frequently noted abnormality in fibrinogen gel chromatograms from patients with thrombotic disorders [41, 65], suggesting that such clinical abnormalities arise as a consequence of fibrinolysis rather than thrombin generation.

(6) SFC Arising from Crosslinking

It has long been known [for review, see reference 39] that fibrin is converted from a loosely hydrogen-bonded aggregate into a "vulcanized" insoluble gel by an enzyme variably known as fibrin-stabilizing factor (FSF), fibrinase, fibrinoligase or factor XIII in the presence of calcium. This enzyme circulates in the blood as an inactive zymogen but is activated by thrombin in the presence of calcium. It produces the insolubilization of the fibrin clot through the formation of ϵ-(γ-glutamyl) lysine crosslinks.

As previously mentioned, Sasaki et al. [121] first noted that SFC formed through incubation of FM with excess fibrinogen could be stabilized or crosslinked by activated FSF.

In a preliminary report by Kanaide et al. [75], an intriguing explanation for the formation of SFC was forwarded. Having noted SFC with the characteristics of a

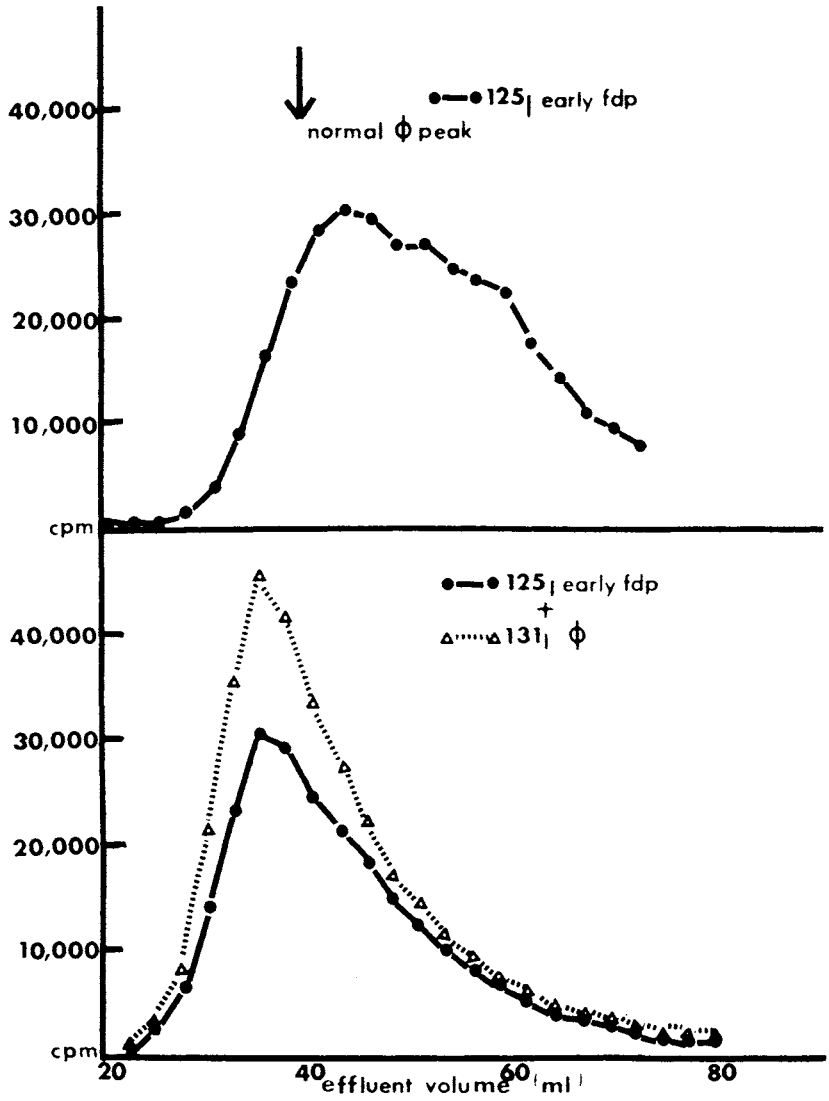

FIG. 9. Upper Panel: Agarose gel chromatogram of ^{125}I-labeled early fibrin degradation products. This preparation was obtained from lysis of fibrin by plasmin after 12 minutes incubation at 37°C. Experimental conditions otherwise identical to those described in Figure 8. Hirudin was added to the digest to 100 units/ml and 3 mg/ml digest incubated with 3 mg/ml of ^{131}I-labeled fibrinogen.

Lower Panel: Agarose gel chromatogram of this incubation mixture demonstrating significant peak shifts for both ^{131}I fibrinogen and components of ^{125}I-labeled early fibrin degradation products.

stable (acid-insoluble) dimer in patients with renal allografts or necrotizing vasculitis of varying origin, these authors attempted to simulate formation of such dimers from native fibrinogen in the absence of thrombin. When fibrinogen was incubated with purified, activated FSF under physiologic conditions, one-third of

the fibrinogen became incorporated into 11S acid-stable dimers within 30 minutes. The physical properties and chemical composition of these dimeric macromolecules arising from the direct crosslinking of native fibrinogen were clearly distinguishable from the SFC formed in the course of coagulation by thrombin. The authors suggested that both types of derivatives may occur *in vivo* and that the distribution between the two, i.e., thrombin-derived SFC and SFC derived from direct crosslinking of intact fibrinogen, may prove of value in differentiating between alternate pathways of deposition of fibrinogen in vascular lesions. However, direct proof has not been furnished that such SFC produced by crosslinking of fibrinogen unaltered by thrombin is identical to the acid-stable dimers detected in patients [128].

PHYSIOLOGY AND PATHOPHYSIOLOGY OF SFC

Whereas a number of animal experiments have dealt with the occurrence of SFC in a variety of pathologic conditions, the presence of these macromolecules under normal physiologic conditions has been described convincingly in only one publication. Sherman [130], upon injection of rabbit ^{125}I-labeled fibrinogen of low solubility (Blombäck fraction I_4 [16]) demonstrated multiple pathways of catabolism for this material. At timed intervals plasma samples were withdrawn from these rabbits and separated into a low solubility fibrinogen fraction (I_4) and the high solubility fibrinogen fraction (I_8) of Mosesson [102], presumably arising from limited proteolysis of low solubility fibrinogen [105, 129]. In addition, SFC were isolated through precipitation in the cold at low ionic strength. With time, as the specific activity of low solubility fibrinogen declined, there was a concomitant increase in the specific activity of high solubility fibrinogen and SFC fractions. After 3 days the specific activity of SFC and high solubility fibrinogen slightly exceeded that of low solubility fibrinogen. In separate experiments the T-½ of low solubility fibrinogen was 47 hours; the T-½ of high solubility fibrinogen and SFC was 26.5 and 13 hours, respectively.

Sherman interpreted these results to suggest two normal catabolic pathways for fibrinogen: (1) low-grade plasmin hydrolysis converting low solubility to high solubility fibrinogen and (2) conversion of low solubility fibrinogen to SFC through thrombin action. In this publication Sherman also quotes indirect evidence by other investigators suggesting similar mechanisms for fibrinogen catabolism.

McFarlane [99] noted that fibrinogen of very low solubility prepared by differential ammonium sulfate fractionation was rapidly cleared from the circulation (70% cleared in 6 hours). Using the same technic, Regoeczi [116] has described a two-phase disappearance of low solubility fibrinogen in rabbits. An initial fall-off of over-all radioactivity to 10% at 24 hours was followed by a slower second phase with a T-½ of 47.9 hours. Considering their preparative technic, the suggestions made by McFarlane and Regoeczi that the material contained large quantities of SFC appeared to be correct, although no attempts were made to quantify the amounts of SFC present in the preparations.

The presence of SFC in plasma under pathologic circumstances and the possible role of this material in causing deposits of "fibrinoid" in renal glomerular capillaries and other organs have been studied in animal models using endotoxin or thrombin

infusions. Thomas and Good [55, 141] noticed that the earliest pathology evident in the generalized Shwartzman phenomenon is the appearance of intravascular deposits of homogeneous eosinophilic material with the staining properties of "fibrinoid." They also observed that the deposition of this material in glomerular capillaries was prevented by heparin, thus suggesting that the coagulation mechanism may be involved in the development of the Shwartzman reaction.

In later studies Thomas et al. [141], investigating the possible precursors of fibrinoid, demonstrated a cold-precipitable gelatinous protein fraction in heparinized plasma samples obtained from rabbits between 1 and 6 hours after injection of endotoxin. This heparin cold-precipitable protein was not demonstrable in serum. Its relationship to fibrinogen or SFC was further documented by its electrophoretic behavior, by the fact that it was partially clottable by thrombin and from the observation that this material disappeared from the blood within 10 minutes following the I.V. injection of Liquoid®, an acidic polymer known to be capable of precipitating fibrinogen. Subsequently, the same authors [142] further characterized this cold-precipitable protein and suggested, but did not conclusively prove, that this material was fibrinogen partly polymerized on its way toward conversion into fibrin.

Solid proof that this material was fibrinogen acted upon by thrombin was first obtained by Shainoff and Page [126] with the observation that the cryoprecipitable material contained 30% less fibrinopeptide A than did fibrinogen.

However, at that point the direct association between circulating SFC and vascular "fibrinoid" deposits observed in the generalized Shwartzman reaction had not been presented. To confuse the picture, the I.V. administration of thrombin or thromboplastin [14, 101, 115, 124] failed to produce lesions resembling those of the generalized Shwartzman reaction, although these manipulations produced profound fibrinogenopenia. However, when thrombin was infused directly into the renal artery rather than intravenously, classic renal cortical necrosis resulted, associated with massive fibrin deposition in glomerular capillaries [119]. The important studies by Lee and Lee and McCluskey [88, 89] provided a sound explanation for these discrepancies.

These authors, confirming previous observations, noted that infusions of thrombin or a single dose of endotoxin produced macroscopic and microscopic evidence of fibrinoid deposition in only a small number of rabbits. When thrombin or endotoxin was given in combination with epsilon amino caproic acid, classical microscopic and macroscopic evidence for bilateral renal cortical necrosis was observed in a high percentage of the animals. When rabbits were first infused with thorotrast, a radio-opaque medium known to block the function of the reticuloendothelial system, and subsequently given a single dose of endotoxin, the classic generalized Shwartzman reaction developed in the majority of the animals.

It would appear, therefore, that SFC, which were demonstrated in all animals as "heparin-precipitable fibrinogen," are cleared by the reticuloendothelial system or by the fibrinolytic enzyme system.

Concrete evidence for the important role of the reticuloendothelial system in clearing SFC was provided in experiments in which rabbits were given an intravenous infusion of thrombin or an injection of endotoxin. Immunohistochemical examination of tissues, using fluorescein conjugated antiserum against

rabbit fibrin, showed bright intracytoplasmic staining in many of the phagocytic cells of the liver and spleen. In normal rabbits, as well as in animals injected with large doses of heparin prior to thrombin or endotoxin, no such intracellular staining was observed.

In our laboratory [25] we have recently observed that SFC produced by a different mechanism also can give rise to microcirculatory fibrin deposits. ^{125}I-labeled high molecular weight degradation products of fibrin consisting mainly of Fb X and Y, when injected into rabbits, disappeared from the circulation with a T-½ of 5 hours and rapidly accumulated in the spleen. When rabbits previously injected with ^{125}I fibrinogen were subsequently given "cold" early fibrin degradation products, deposition of radio-labeled fibrin-like material in the lungs was significantly higher than in control animals receiving only ^{125}I-labeled fibrinogen. We postulate that these deposits represent complexes of components of the early fibrin digest with fibrinogen similar to those we had previously demonstrated *in vitro*. Thus, there appears to be a clear association between circulating SFC and microcirculatory thrombosis.

A recent study in dogs [24] aimed at clarifying the relationship between SFC and major venous thrombosis. The experimental thrombosis model of Wessler [145] was utilized and measurements for SFC were performed to determine whether these arise as a consequence of local thrombus formation or are a manifestation of a "systemic hypercoagulable" state, or both.

Three experimental models were studied in groups of dogs. The included pathophysiologic changes were: (1) local thrombus formation by stasis and injection of serum locally into a clamped-off venous segment, (2) the injection of homologous serum as a source of activated clotting factors, and (3) combined homologous serum infusion and local stasis thrombosis. Semiquantitative estimates of SFC were performed by fibrinogen gel chromatography [3] and the serial dilution protamine sulfate test [108]. Serial studies demonstrated that local thrombosis in the absence of circulating activated clotting factors did not generate significant amounts of SFC. The injection of serum in the absence of stasis thrombosis resulted in only transient increases in circulating SFC. The infusion of serum combined with the formation of local stasis thrombi gave rise to persistent and substantial quantities of circulating SFC. Thus, it would appear from these experimental models that SFC are most likely to occur in association with intravascular thrombosis combined with a "hypercoagulable state," defined as the presence of activated procoagulants in the circulation.

In our laboratory we recently addressed ourselves to the question whether SFC possess biologic properties in their own right, in addition to their function as molecular markers for intravascular fibrin deposition [11, 12, 64].

In initial *in vitro* experiments we isolated high molecular weight SFC fractions from thrombin-treated plasma or purified fibrinogen. We noted that purified SFC fractions exhibited a significantly greater sensitivity to thrombin than did fibrinogen; i.e., these macromolecules clotted significantly more rapidly over a wide range of thrombin concentrations than did fibrinogen. We also noted that these high molecular weight SFC substantially shortened the thrombin clotting time of normal plasma and enhanced the resistance of native plasma to heparin action in vitro.

To assess the pathologic implications of these findings we attempted to demonstrate biologically active SFC *in vivo*. In the experiment shown in Figure 10a plasma sample was obtained from a rabbit 1 minute following the administration of 50 units of thrombin, I.V., and subjected to agarose gel chromatography.

Effluent fractions were monitored for fibrinogen by immunochemical assays and by thrombin clotting times in the presence of excess quantities of thrombin [29]. Thrombin-clottable material showed a broad distribution representing fibrinogen and a wide range of different molecular weight SFC. The enhanced thrombin sensitivity of these SFC can be appreciated by comparing the thrombin clotting times and the quantities of immunoreactive fibrinogen in the effluent zone close to the void volume representing high molecular weight SFC. These high molecular weight fractions, which contained only trace quantities of fibrinogen by immunoassays, showed substantially shortened thrombin clotting times.

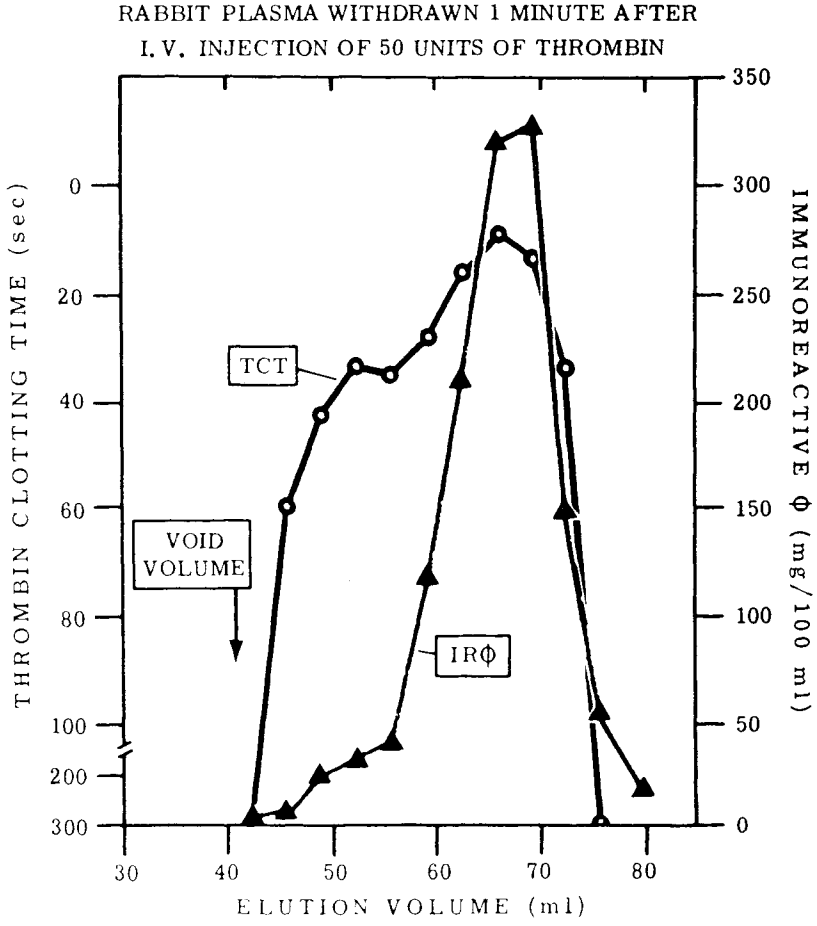

FIG. 10. Agarose gel chromatogram of plasma sample obtained from a rabbit 1 minute following the administration of 50 units of thrombin, I.V. 5 ml of rabbit plasma was applied to the column and effluent fractions monitored for immunoreactive fibrinogen (IRØ) and thrombin clotting times (TCT).

In additional studies in rabbits we obtained data to indicate that this high molecular weight material is short lived. In timed studies we demonstrated thrombin-sensitive SFC by agarose gel chromatography at 1, 2, 4, 6, 8 and 10 minutes following the infusion of 50 units of thrombin but not at 20 and 30 minutes. These observations fully confirm the observations of Lee [88], indicating that SFC are very rapidly cleared by the reticuloendothelial system. We postulate on the basis of these experiments that the SFC which we demonstrated *in vitro* and *in vivo*, since they are capable of enhancing the rate of blood coagulation, may contribute to a temporary "hypercoagulable state" and may accelerate the buildup and extension of thrombotic deposits.

A series of recent preliminary observations [50] point toward additional significant biologic effects of SFC. Measurements of blood viscosity at low shear rates in 150 patients suffering from acute thromboembolic disorders indicated that SFC significantly influenced blood rheology. After correcting viscosity in these blood samples for hematocrit and fibrinogen content, viscosity at low shear stress was shown to be significantly and positively correlated with the content of SFC quantified by agarose gel chromatography. The approximate ratio of rheologic activity in centipoises was calculated as 10 for SFC, 5 for native fibrinogen and 1 for "fibrinogen first derivative," i.e., Fg X, at shear rates of 0.2 cm^{-1}. These findings may have clinical importance, since the presence of SFC may substantially alter microcirculatory blood flow characteristics.

TESTS FOR THE DETECTION OF SFC AND THEIR CLINICAL APPLICATION

The following test systems have been suggested as useful for the detection of SFC in clinical thrombotic states:

(1) gelation tests through the addition of protamine sulfate or ethanol; or chilling the patient's plasma to +4°C;

(2) N-terminal analysis of fibrinogen fractions purified from plasma by a modified Cohn procedure;

(3) incorporation of ^{14}C-labeled glycine-ethyl ester into SFC;

(4) the indirect demonstration of SFC through specific radioimmunoassay for fibrinopeptide A;

(5) agarose gel chromatography.

Gelation Tests

Principle. Certain manipulations, such as the addition of protamine sulfate, ethanol or chilling the patient's plasma to +4°C, may produce visible gels or solid fibrin strands in plasmas containing SFC. The mechanism for this "paracoagulation" phenomenon is not completely clarified. As previously mentioned, several authors have suggested that SFC is produced through the interaction between fibrin monomer, Fg X or Fb X, and nonclottable degradation products of fibrinogen or fibrin [81, 85, 90, 94, 107, 108, 144]. Most of these investigators proposed that

positive gelation tests occur because the addition of substances such as protamine sulfate or ethanol or lowering the temperature of plasma produces the dissociation of this type of hypothetical complexes, liberating fibrin monomer from the complex and allowing it to undergo nonenzymatic polymerization to form a fibrin gel.

Recent experiments in our laboratory [86] cast some doubt on the validity of this theory. Figure 11 demonstrates an experiment in which an early fibrinogen digest was treated with thrombin and the mixture chromatographed on Bio Gel A-5m. The elution profile depicted in the upper diagram of Figure 11 shows a large peak appearing close to the void volume and representing SFC. Aliquots of individual fractions were incubated with protamine sulfate and additional aliquots were incubated with thrombin and protamine sulfate at 37°C and the distribution of radioactivity between precipitate and supernatant estimated. As can be seen in the lower portion of Figure 11, greater than 90% of radioactivity in the fractions containing SFC was precipitable by protamine sulfate with a rapid fall-off of values to less than 10 to 15% in the area of effluent containing unpolymerized degradation products. The amount of radioactivity precipitable with thrombin plus protamine sulfate treatment closely paralleled the amount of radioactivity precipitable by protamine sulfate alone. Specifically, the radioactivity precipitable by thrombin plus protamine sulfate in the area where fibrinogen and clottable fragment X normally elute is negligible, indicating substantial incorporation of these components of the digest into SFC fractions. As previously mentioned, we established in additional experiments [86, 136] that nonclottable Fg Y, D and E under identical experimental circumstances failed to incorporate into SFC; therefore, the SFC fraction depicted in Figure 11 can consist only of FM and/or Fb X. Thus, the widely accepted notion that the "paracoagulation" phenomenon results from the splitting of complexes between fibrin monomer and nonclottable degradation products of fibrinogen or fibrin clearly needs revision.

Clinical Applications. Although there is clearcut evidence from *in vitro* [18, 31, 53, 108, 125, 142] and animal experimental studies [24, 106] that either the ethanol or protamine sulfate tests readily detect SFC in plasma and although these rapid and simple tests have been widely accepted by clinicians as a means of screening patients for diffuse intravascular coagulation, their precise role as useful diagnostic tools in clinical medicine is far from established.

The Ethanol Gelation Test

Evidence has been presented by Kierulf et al. [77] in one major clinical series suggesting that positive ethanol gelation tests may be associated with "hypercoagulable states" and microcirculatory thrombosis. Among 1,948 unselected patients admitted to an internal medicine department, 11.4% demonstrated positive ethanol gelation tests. Among the patients with positive tests there was a statistically significant preponderance of malignant neoplasms, pneumonia and diseases of the blood vessels, including pulmonary embolism, arterial thrombosis and venous thrombosis. This study also included a control population of 275 healthy individuals aged between 18 to 80 years, all showing a negative ethanol gelation test.

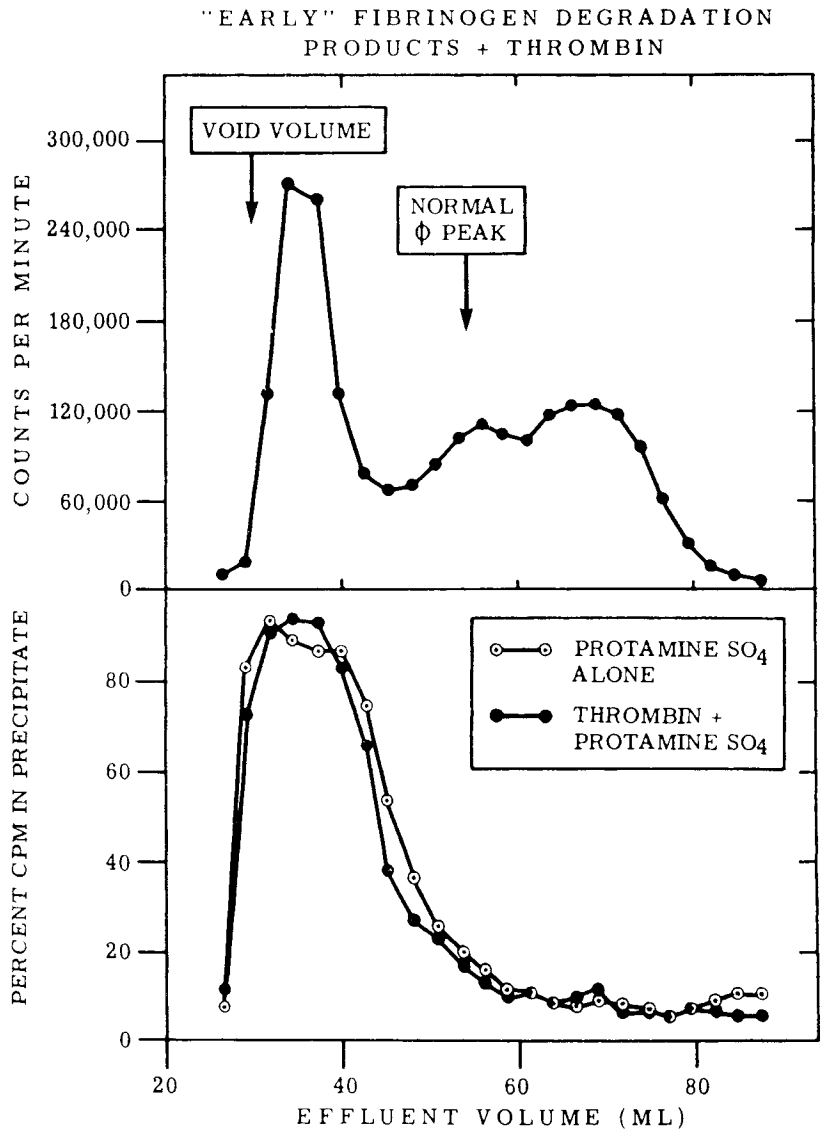

FIG. 11. Agarose gel chromatography of early fibrinogen digest treated with thrombin. 9 mg of digest mixture trace-labeled with ^{125}I was treated with 0.4 units of thrombin for 10 minutes at 37°C followed by 3 units of hirudin.

Upper Panel: Effluent pattern for mixture.

Lower Panel: Per cent protein in individual fractions precipitated by protamine sulfate alone and by thrombin plus protamine sulfate. Protamine sulfate was added to aliquots from each column fraction to 1 mg/ml, the mixture incubated for 60 minutes at 37°C. Samples were centrifuged and the supernatant and precipitate counted separately for ^{125}I. To additional 1 ml aliquots of each fraction were added 10 units of thrombin. After 5 minutes incubation of the samples at 37°C protamine sulfate was added to 1 mg/ml and the mixtures incubated an additional 60 minutes at 37°C. Precipitate and supernatant were separated and counted separately for ^{125}I.

Breen and Tullis [18] reported on a small series of 5 patients with clinically documented diffuse intravascular coagulation and found positive ethanol gelation tests in all 5. For comparison, they studied 36 other patients with "diverse medical and surgical disorders," including 4 with "primary fibrinolysis." In all these control patients the ethanol gelation test was negative.

In a preliminary communication Arneson et al. [5] have found evidence suggesting that the ethanol gelation test may be of some prognostic value in acute myocardial infarction. Plasmas from 80 consecutive patients with acute myocardial infarction were tested, and, among these, 17 showed a positive and 63 a negative ethanol gelation test. The mortality rate among patients with a positive ethanol gelation test was 47%, as opposed to 14% among patients with a negative gelation test.

In contrast, Hedner and Nilsson [67] found the ethanol gelation test far less specific and useful. These authors performed this test in 305 patients with malignant disease, postoperative complications, sepsis, multiple fractures, liver disease, normal pregnancy and postpartum states, renal diseases and different blood disorders. Although the over-all incidence of positive ethanol gelation test was 10%, this study was disappointing in that only 5 of 17 patients exhibiting all the classic clinical and laboratory evidence of diffuse intravascular coagulation exhibited positive ethanol gelation tests.

The Protamine Sulfate Gelation Test

Clinical studies utilizing protamine sulfate, rather than ethanol, to demonstrate SFC in patients' plasma are equally inconclusive and confusing at this point.

Seaman [125] summarized his experience with this test in a series of case reports. During a one year period all citrated blood specimens collected for coagulation studies for a variety of reasons were also examined by the protamine sulfate gelation test. Two hundred positive tests were encountered among a total of 2,921 specimens examined. Eight case reports fulfilling all the criteria for disseminated intravascular coagulation were reported to have positive protamine sulfate gelation tests.

No patients with negative tests and with other clinical and laboratory evidence for disseminated intravascular coagulation were encountered. Two patients with evidence of deep vein thrombosis and pulmonary embolism and one patient with retinal vein thrombosis had positive protamine sulfate gelation tests; however, an additional 14 patients with venous thrombotic disorders had negative tests, including 3 patients with diagnosed pulmonary emboli. In addition, Seaman also noted a very high incidence (53/105) of positive protamine sulfate gelation tests after open-heart surgery, particularly in association with prosthetic valve replacement, suggesting fibrin deposition on the artificial valve as a source of SFC.

Palester-Chlebowczyk et al. [112] conducted protamine sulfate gelation tests on 36 healthy persons and on 392 persons with a variety of diseases. Whereas the incidence of positive tests among healthy persons was 6%, the incidence varied between 6 and 60% in different disease states, the highest incidence being noted among patients with malignancies (50%) and patients with kidney diseases (60%). A low incidence of positive protamine sulfate gelation tests was observed in patients

with congenital heart diseases or acquired valvular heart diseases; however, the incidence rose significantly during and immediately following open heart surgery. It was also noted that the incidence of positive tests was significantly higher among patients who suffered from generalized and local bleeding complications, as opposed to patients who did not bleed; however, the number of patients among the group of bleeding patients showing definitive laboratory signs of disseminated intravascular coagulation was not given.

Niewiarowski and Gurewich [108] found positive protamine sulfate gelation tests in all of 6 patients with findings consistent with disseminated intravascular coagulation on clinical and laboratory grounds. In addition, they examined 8 cirrhotic patients with evidence of "primary fibrinolysis" without clinical evidence of diffuse intravascular coagulation and observed a negative protamine sulfate gelation test in all of these 8 patients. Similarly, normal protamine sulfate gelation titers were observed in 75 normal plasmas.

An additional group of 8 patients undergoing therapeutic thrombolysis with streptokinase was examined. In 7 patients in whom no significant evidence of radiographic thrombolysis occurred, protamine sulfate gelation tests remained negative throughout the treatment. In contrast, in one patient in whom a large thrombus was lysed, an initial negative test became positive during the streptokinase infusion providing *in vivo* confirmation supporting the concept that plasmin degradation of fibrin can produce SFC [27, 33].

Gurewich and Hutchinson [58] reported that 9 of 10 patients with pulmonary embolism had strongly positive protamine sulfate tests. The protamine sulfate test in the hands of these investigators was not affected by heparin and tended to become negative only after 2 to 4 days of heparin therapy. In 21 of 25 patients with deep-vein thrombosis, the test was positive. For comparison, 5 of 50 normal subjects had positive tests; 33 of 75 patients with cirrhosis of the liver, 15 of 42 with metastatic neoplasms, 5 of 17 patients with chronic renal failure, 4 of 4 patients with acute arterial thromboembolism, 5 of 21 patients with chronic obliterating arteriosclerosis. 7 of 15 patients on oral contraceptives, and 10 of 10 patients with documented disseminated intravascular coagulation showed positive tests.

Gurewich et al. [59] later studied a total of 82 patients suspected of having deep-vein thrombosis and/or pulmonary embolism. In 46 of these patients the venogram was positive and in another 36 patients with classic symptoms or signs suggestive of deep-vein thrombosis the venogram was normal. Among patients with positive venograms, 29 were symptomatic and 17 were asymptomatic. An incidence of 86% positive protamine sulfate gelation tests were found in symptomatic patients with positive venograms. Patients with positive venograms who were asymptomatic at the time showed an incidence of 24% positive protamine sulfate gelation tests. In 36 patients in whom the venogram was negative, positive gelation tests were found in 11%. Among 21 patients with documented pulmonary embolism, 16 had positive protamine sulfate tests. In contrast, among 17 patients in whom the diagnosis of pulmonary embolism was excluded by lung scans and the subsequent clinical course, one positive protamine sulfate gelation test was found.

Other investigators have been less enthusiastic about this test. Hedner and Nilsson, in a series referred to previously [67], found a positive protamine sulfate

gelation test in only 1 of 17 patients with clinical and laboratory evidence for diffuse intravascular coagulation. Indeed, in their whole study, including 305 patients, this test was found positive in only this one patient.

In a recent study [65] we ourselves encountered 12 cases of venographically demonstrable thrombi among 38 postoperative patients, but we found only one positive protamine sulfate gelation test in the whole group.

Similar negative conclusions concerning the specificity and sensitivity of the ethanol and protamine sulfate gelation tests have been reached by other investigators [34, 96].

Comparison of Ethanol and Protamine Sulfate Gelation Tests

In the few studies in which protamine sulfate and ethanol gelation tests have been run in parallel in the same plasma [67, 106], a poor correlation between these two tests has been observed. These discrepancies may be explained in part on the basis of *in vitro* observations [60, 80], suggesting that the ethanol gelation test is sensitive mainly to SFC formed from FM; whereas, the protamine sulfate test is sensitive also for the demonstration of SFC arising from lysing fibrin.

Different authors have utilized different assay conditions in performing either the protamine sulfate or the ethanol gelation test. Thus, the ethanol gelation test, according to Breen and Tullis [18], appears to be sensitive to pH showing greater specificity and a lower incidence of false-positive results at more alkaline pH's.

Similarly, it appears that the ethanol gelation test may produce false-positive results at very high fibrinogen levels [54]. The protamine sulfate test is sensitive to the amount of protamine sulfate added, the pH, temperature and fibrinogen concentration, being more specific at higher temperatures (37°C) and at lower concentrations of protamine sulfate. One method described [91], which depended on the reading of the optical density of plasmas to which excess protamine sulfate was added at room temperature, has later been disputed as reflecting fibrinogen levels, rather than the levels of SFC [60, 108].

The high number of false-positive test results and possibly false-negative test results in different studies strongly suggest that optimum conditions for maximum sensitivity and specificity of these two tests have not been established and that further studies are needed before the true value of these simple and rapid assays can be established in the diagnosis of ongoing fibrin deposition.

Cryofibrinogen

The appearance of enhanced levels of cold-precipitable fibrinogen was first reported by Korst and Kratochvil [82] in a patient with migratory thrombophlebitis associated with carcinoma. Additional reports focusing on isolated cases or a small series of patients [20, 52, 62, 68, 72, 74, 103, 117, 118, 120, 138] have since appeared. In most of these cases abnormally high levels of cryofibrinogen were found in patients with carcinoma; diffuse intravascular coagulation; severe, acute and chronic infections; and major vessel thrombo-occlusive disease.

In a major study, McKee et al. [100] detected abnormally high levels of cryofibrinogen in 28 out of 665 patients. This abnormality was usually associated

with cancer, lymphomas, leukemia, myeloma, collagen disease and thromboembolic disease. Among 135 normal controls, none had elevated levels of cryofibrinogen. There was a positive correlation between the levels of cryofibrinogen and thrombosis and bleeding. No correlation was found between the appearance of cryofibrinogen and other cryoglobulins or between cryofibrinogen and plasma fibrinogen levels.

More recently, Pindyck et al. [113] noted a 26% incidence of cryofibrinogenemia in 78 women taking oral contraceptive drugs, a 12% incidence in 60 women using IUD's and an 8% incidence among 66 women using neither. It appears, therefore, that the cryofibrinogen reaction, which historically was the first assay system used in the demonstration of SFC, may show clinical correlation with disseminated intravascular coagulation and thrombotic disorders. However, the exact value of this simple test in screening for diffuse intravascular coagulation or subclinical thrombosis has not as yet been evaluated in large series, nor has the correlation between an abnormal cryofibrinogen reaction and other tests for the detection of SFC been established.

N-Terminal Analyses of Modified Cohn Fraction I from Patients' Plasmas

Principle. When plasma is treated with ethanol in the cold in the presence of TAMe, a fibrinogen fraction greater than 90% clottable can be harvested. In the course of thrombin action on fibrinogen, fibrinopeptides are removed, exposing N-terminal glycine. A comparison of N-terminal glycine of the total precipitated fraction, the clottable proteins and the clot liquor is performed, allowing for quantification of N-terminal glycine in clottable SFC [76].

Clinical Application. This test was used in studying 10 patients with positive ethanol gelation tests and with signs of intravascular coagulation and fibrinolysis [78]. In all 10 patients, N-terminal glycine was demonstrated in large amounts. In addition, some patients showed increased amounts of several other N-terminal groups, suggesting that SFC in these patients arise from the joint action of plasmin or other proteases and thrombin. Although this test system appears to be a promising tool in the detection of SFC, the complex nature of the assay probably precludes its widespread use.

Detection of SFC through Incorporation of ^{14}C-labeled Glycine Ethyl Ester into SFC

Principle. When ^{14}C-labeled glycine ethyl ester is incubated with SFC in the presence of activated fibrin stabilizing factor and calcium, enzymatic incorporation of this amino acid ester occurs. In contrast, the mean incorporation of ^{14}C-labeled glycine ethyl ester into fibrinogen or plasmin split products of fibrinogen is negligible [79, 92].

Clinical Application. The test has been reported to produce abnormally high results in 7 patients: 2 with meningococcal septicemia with clinical and laboratory evidence suggesting disseminated intravascular coagulation, 2 patients with Rocky Mountain spotted fever associated with disseminated intravascular coagulation, and

3 patients in whom therapeutic abortions induced by the intrauterine injection of hypertonic saline produced changes in clotting factors consistent with disseminated intravascular coagulation.

This test system, although somewhat cumbersome, does represent a logical approach to the detection of SFC; however, its definitive diagnostic role remains to be established in a large series.

The Indirect Demonstration of SFC through Specific Radioimmunoassay for Fibrinopeptide A

The feasibility of quantifying circulating fibrinopeptide A through specific radioimmunoassays has been convincingly demonstrated [109]. In theory, such tests should correlate well with ongoing intravascular fibrin formation and the appearance of SFC in the circulation.

In preliminary studies, Nossel et al. [110] employed such assays in clinical situations. The A peptide cleaved by thrombin and the amino terminal fragments of the alpha chain cleaved by plasmin could be distinguished immunochemically. Plasma samples from 20 healthy men showed total immunoreactivity equivalent to 0.1 to 0.8 ng/ml, part of which was thought to represent the plasmin-cleaved amino terminal portion of the alpha chain. Plasma from patients with intravascular coagulation contained peptide immunoreactivity equivalent to 4 to 80 ng/ml, almost all of which was thought to be A peptide. Based on evidence that the A peptide plasma T-½ is 3 minutes, it was estimated that thrombin degrades fibrinogen at the rate of 0.5 to 10 g/24 hrs in patients with gross intravascular coagulation and at the rate of less than 0.05 g/24 hrs in normals. Heparin infusion was associated with a sharp decrease in A peptide levels, suggesting that this assay may provide an index of therapeutic efficacy of anticoagulant therapy.

Demonstration of SFC through Gel Exclusion Chromatography

Principle. Plasma is separated on large-pore agarose gels, and effluent fractions are assayed for fibrinogen by immunochemical assays or the equally sensitive staphylococcal clumping test [66, 87]. These agarose columns separate fibrinogen, SFC and fibrinogen-fibrin degradation products on the basis of molecular size and, particularly, individual molecular Stokes radius.

When carefully calibrated columns packed with agarose gels are used, one can obtain a symmetrical peak for plasma fibrinogen at an effluent volume which does not vary from assay to assay.

In pathologic plasmas, SFC would appear in an effluent position earlier than the fibrinogen peak, and fibrinogen-fibrin degradation products of molecular weights lower than fibrinogen would appear in effluent fractions subsequent to the fibrinogen peak.

A computer program recently described by Alkjaersig et al. [3], using chromatographic plate theory analysis, makes it possible to arrive at a semi-quantitative estimate of the contents of SFC, fibrinogen and fibrinogen-fibrin split products from poorly resolved chromatographic patterns.

Clinical Applications. Utilizing the technic of agarose gel chromatography, Fletcher, Alkjaersig and co-workers, in a series of preliminary communications, have made some important claims for the sensitivity and specificity of this technic in identifying SFC in patients.

In an initial collaborative investigation, Fletcher et al. [41, 43] studied the plasmas from patients participating in a trial conducted by the British Medical Research Council and focusing on the use of ^{125}I-labeled fibrinogen scanning technic for diagnosing subclinical deep-vein thrombosis in postoperative patients. Subclinical thrombophlebitis by this technic is diagnosed if isotope accumulation is detected in accessible leg veins in high-risk patients previously given ^{125}I fibrinogen.

The *in vivo* ^{125}I fibrinogen scans and the *in vitro* fibrinogen gel chromatograms correlated well. A total of 91 assays was performed on 55 patients. Complete agreement of both positive and negative findings for the two methods was found in 43 patients, both assays being normal in 27 and both assays being abnormal in 16. In 10 patients, abnormal gel chromatograms were detected in the face of normal ^{125}I fibrinogen scans, and 2 patients exhibited a normal gel chromatogram and abnormal ^{125}I fibrinogen scans. The significant numbers of abnormal gel chromatograms in the face of normal ^{125}I scans were interpreted by Fletcher et al. to suggest that agarose gel chromatography is capable of detecting fibrin deposits not demonstrable by ^{125}I scanning of leg veins.

Alkjaersig et al. [1, 46] studied the incidence of abnormal gel chromatograms in random sample groups of 770 women of whom 575 had been receiving oral contraceptive therapy for varying periods. The studies concluded that shortly after oral contraceptive therapy was begun, the incidence of abnormal plasma fibrinogen gel chromatograms rose from 3.7% in the untreated women to 16.2% in those receiving medication. After 3 months, 26.5% abnormal gel chromatograms were detected and the incidence then stabilized at approximately 20% for the remainder of the year. Data for long-term continuous oral contraceptive therapy indicated that the chromatographic abnormality varied in different groups between 20 and 29%. Although increased duration of treatment (1 to 8 years) apparently was associated with an increased incidence of chromatographic abnormalities, these results were not statistically significant. In several patients, sequential studies were performed which demonstrated that the gel chromatograph abnormality in most instances was transient in nature. Only 10% of the patients with abnormal chromatographic findings were clinically symptomatic; however, association of symptomatology with abnormal gel chromatograms was highly significant on statistical grounds.

The data quoted have all been derived from an uncontrolled trial and await confirmation in a rigidly controlled, double-blind prospective study currently underway [49].

The same group also provided preliminary evidence on the usefulness of fibrinogen gel chromatography in the study of acute cerebrovascular syndromes [2, 19, 45].

An initial study [19] focused on 43 patients with acute cerebrovascular accidents of thrombotic origin, 13 of whom were treated with urokinase. Initially abnormal gel chromatograms revealing circulating SFC were demonstrated in 31 patients. Seven patients had normal findings, and 5 patients revealed a chromato-

graphic pattern characteristic of fibrinolysis. These latter patients with partial hemiplegia made excellent recoveries.

The 13 urokinase-treated patients–5 with cerebral venous sinus thrombosis, 3 with partial hemiplegia and 5 with completed hemiplegia–were treated with sufficient urokinase to convert circulating SFC into lower molecular weight breakdown products, as evidenced by sequential agarose gel chromatograms. Three patients with partial hemiplegia did excellently, and an impressive response was seen in 3 patients with cerebral venous sinus thrombosis, all of whom remained free of residual neurological sequelae, even though in 4 repeated angiography failed to show improvement.

It was implied from these studies that agarose gel chromatography may be of significant value in assessing the prognosis in thrombotic strokes and in assessing efficacy of therapeutic regimens, such as fibrinolytic enzyme treatment.

Subsequently, serial studies on 20 patients with an acute cerebral thrombosis were reported [2]. In addition, serial agarose gel chromatography, serial assays for plasminogen, fibrinogen, alpha-1 antitrypsin, alpha-2 macroglobulin and antithrombin III were also obtained on all samples. Forty-nine per cent of the total plasma samples examined showed the presence of circulating SFC. The samples showing abnormal fibrinogen gel chromatograms also showed significantly depressed levels of antithrombin III, plasminogen, alpha-1 antitrypsin and alpha-2 macroglobulin, indicative of activation of blood coagulation, as well as the fibrinolytic enzyme system. These observations suggested that a blood hypercoagulable state follows the acute thrombotic stroke in a high proportion of patients.

Patients suffering from acute myocardial infarction were also studied [44, 47]. Ninety-six patients suffering from unequivocal acute myocardial infarction and 19 patients who were monitored for, but did not develop, myocardial infarction were studied. Sixty-one per cent of samples from patients with proved myocardial infarction examined on days 1 through 12 showed abnormal gel chromatograms, compared to a 12% incidence in the controls. Four patients who developed acute myocardial infarction while under observation showed the immediate appearance of abnormal chromatograms indicative of circulating SFC. Patients who improved clinically showed a less frequent appearance of chromatographic abnormalities than those who remained unchanged or deteriorated (48% vs. 68% ($p < 0.01$)). Patients who remained unchanged or deteriorated also demonstrated a significantly lower fibrinolytic response, as evidenced by agarose gel chromatographic effluent patterns. These results suggested to the authors that patients with myocardial infarction frequently develop intravascular coagulation and blood hypercoagulability and that persistence of this complication is clinically deleterious. The development of plasma fibrinogen chromatographic abnormalities over the first 12 days in patients with acute myocardial infarction was uninfluenced by anticoagulant therapy.

In a preliminary report [28], agarose gel chromatography was applied in a prospective study of 3 patients with recurrent, painful sickle cell crises. During the course of these observations, 4 major, 2 moderate and 8 mild crises occurred. A highly characteristic pattern of coagulation changes was observed, consisting of an early elevation of SFC and a concomitant decrease in fibrinogen-fibrin breakdown

products, followed by striking elevations of total fibrinogen and plasminogen and moderate elevations of fibrinogen-fibrin degradation products. The earliest changes preceded the onset of pain on at least two occasions, and the entire sequence of changes required 4 to 6 weeks to evolve. The magnitude of the changes appeared directly correlated to the severity of sickle cell crises. These findings provided impressive suggestive evidence for a role of activation of blood coagulation in sickle cell crises.

The validity of the technic of agarose gel chromatography in the study of thrombotic disorders has been confirmed in other laboratories.

Graeff et al. and von Hugo and Graeff [56, 57, 143], in view of the high incidence of intravascular fibrin deposits in stillbirth and newborns dying in the perinatal period, examined cord vein plasma samples from 14 newborns with positive ethanol gelation tests. Using two technics, agarose gel chromatography and polyacrylamide gel electrophoresis, and intragel immunoprecipitation, they were able to demonstrate SFC with a higher molecular weight than fibrinogen in the cord bloods from these infants. SFC were not observed in cord vein plasma samples of 14 newborns with a negative ethanol gelation test. Two types of SFC were identified, one of lower molecular weight dissociable in 5 M urea presumed to be formed from noncovalently linked components and one of higher molecular weight consisting of predominantly covalently linked components.

Carvalho et al. [21] studied a group of 17 patients with Type II hyperlipoproteinemia, noting the high incidence of thrombotic complications in this type of patient. In comparison to 26 normal subjects, high molecular weight SFC detected by agarose gel chromatography was increased 10-fold in hyperbetalipoproteinemic patients.

In these patients, plasma prekallikrein, kallikrein inhibitor and Factor XII were significantly lower than normal, suggesting that activation of the intrinsic pathway may be the mechanism responsible for intravascular coagulation. Clofibrate abolished the activation of the intrinsic pathway and decreased intravascular coagulation in Type II patients, despite the lack of significant plasma lipid response to this therapy.

Recent data from our own laboratory [65] have confirmed that the detection of SFC through agarose gel chromatography represents a sensitive means of detecting subclinical thrombosis in postoperative thrombosis.

In our studies, 12 of 38 patients undergoing major surgery had clear-cut venographic evidence of leg vein thrombosis. Only one of these patients developed clinical signs of deep vein thrombosis and pulmonary embolism. All 12 patients showing phlebographic evidence of leg vein thrombosis had abnormal chromatograms postoperatively. An additional 8 patients with abnormal chromatograms had normal venograms. Eighteen patients had normal venograms, as well as normal chromatograms. Among the 20 patients with abnormal gel chromatograms postoperatively, 7 patients exhibited abnormal chromatograms preoperatively, as well. In no instance did we encounter an abnormal gel chromatogram before surgery returning to normal in the postoperative sample. Thus, in our series we have confirmed the observations by Fletcher et al. [41] indicating that agarose gel chromatography accurately detects subclinical deep vein thrombosis in leg veins, and we have also confirmed that abnormal test results can be demonstrated in a

high number of instances in which evidence for lower extremity deep vein thrombosis was lacking. Results similar to those reported by Fletcher et al. and by our laboratory have recently been obtained in one additional study [69].

A preliminary report suggesting that abnormal fibrinogen gel chromatographic patterns frequently occur in acute or chronic renal glomerular disorders [71] has been confirmed by ourselves [13] and by Bachman and Pichairut [6].

SUMMARY AND CONCLUSIONS

We have reviewed the evidence indicating that SFC, which were first discovered and studied under highly artificial experimental conditions, can exist *in vitro* under physiologic or near physiologic conditions.

Considerable controversy remains concerning the molecular mechanisms involved in SFC formation, and much work remains to be done in identifying the exact biochemical events which favor SFC formation *in vivo*.

Next, we have presented available animal experimental evidence which unequivocally associates circulating SFC with microcirculatory thrombosis, as well as major thrombo-occlusive vascular disease.

Finally, we have attempted to review the rapidly growing clinical literature suggesting the substantial diagnostic potential of various technics for SFC detection.

The specificity and sensitivity of the different assay procedures are subjects of considerable controversy at this point; nevertheless, a review of existing literature allows for certain tentative conclusions.

The simpler rapid screening tests for SFC, such as the ethanol and protamine sulfate gelation tests, may be helpful in detecting intravascular fibrin deposition when used in conjunction with other assay procedures; however, the number of false-positive and false-negative results plaguing most clinical series would in our judgment make it inadvisable to rely solely on these tests for the diagnosis of intravascular coagulation.

Using available evidence and our own clinical experience, we concur that the more sophisticated methods for the identification of SFC, such as agarose gel chromatography and radioimmunoassays for fibrinopeptide A, have provided us with new tools which have distinct advantages over existing methods for thrombosis detection but also some disadvantages.

We conclude that the presence of SFC in plasma reflects either ongoing intravascular fibrin deposition or the lysis of pre-existing fibrinous deposits. Agarose gel chromatography, the method with which we are most familiar, seems to be sensitive enough to detect small thrombi not apparent by clinical criteria, and it thus may be helpful in identifying high-risk patients.

However, most available clinical evidence accumulated so far indicates that this method may be too sensitive to have major diagnostic importance in individual patients; i.e., it may frequently reflect fibrin deposits which are too small to be clinically significant.

The method from all existing evidence has no predictive value; it indicates merely that fibrin is being formed, not that fibrin deposition will occur at some future date. In contrast to sensitive radiographic and radioisotope technics (e.g., the ^{125}I fibrinogen scanning method), this method does not localize thrombi. It merely

indicates that fibrin deposition is presently occurring somewhere in the circulation.

It appears that the major potential of agarose gel chromatography and other equally sensitive assays (e.g., radioimmunoassay for fibrinopeptide A) lies in the area of large-scale studies focusing on epidemiologic factors, risk factors and prognosis in thrombotic disorders. In addition, these assays should be well suited to assess the efficacy of established, as well as newer, forms of therapy in thromboembolic disease in man.

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