ENHANCEMENT OF BLOOD COAGULATION BY SOLUBLE FIBRIN COMPLEXES*

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In recent years increasing attention has focused on soluble fibrin complexes as molecular markers for intravascular fibrin formation. These high molecular weight soluble fibrin intermediates have been produced under a variety of experimental conditions in vitro and in vivo (1–8).

The demonstration of soluble fibrin complexes in the circulation of patients through assay procedures, such as ethanol and protamine sulfate gelation, (9, 10) assays for cryofibrinogen (11), and more recently through agarose gel chromatography, (12, 13) has been claimed to be of prime importance in the diagnosis of localized intravascular thrombosis, as well as disseminated intravascular coagulation. Indeed, the technique of agarose gel chromatography for the detection of soluble fibrin complexes has been claimed by Fletcher and co-workers (12) to afford a highly specific and sensitive means of detection of subclinical leg vein thrombosis, a claim substantiated by the high degree of correlation between abnormal agarose gel chromatograms and localized leg vein isotope accumulation utilizing the 125I-labeled fibrinogen scanning technique. In spite of these preliminary observations which delineate the diagnostic significance of soluble fibrin complexes, information regarding the biological properties and the possible pathophysiological role of these macromolecules is lacking.

In this communication we wish to report on a species of highly aggregated, high molecular weight, soluble fibrin complexes which differ in regard to biophysical characteristics from those originally described by Fletcher et al. (12) and which possess significant biological properties. These complexes were first detected in model systems in vitro; they were later produced in animal experiments; and they were finally detected in a limited number of patients with a variety of thrombotic disorders.

Materials and Methods

Reagents

Normal Plasma. Normal plasma was withdrawn from healthy male subjects (9 vol blood into 1 vol 3.8% trisodium citrate). The blood was immediately centrifuged at 7,000 rpm for 10 min;

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supernatant plasma was used immediately or was immediately frozen and stored at -21°C. All plasmas utilized in these studies were analyzed by agarose gel chromatography (see below). A normal chromatographic pattern, as defined in the following, was demonstrated for all plasmas, whether they were used immediately or utilized after storage in the frozen state for different time periods.

**Purified Human Fibrinogen.** Purified human fibrinogen was prepared according to the methods of Kazal et al. (14) and Mosesson and Sherry (15). The resultant preparations, 96-100% clottable, were homogeneous by polyacrylamide gel electrophoresis and agarose gel chromatography.

**¹³¹I-Labeled Fibrinogen.** ¹³¹I-labeled fibrinogen was prepared by the iodine monochloride technique according to Izzo et al. (16) incorporating less than 0.5 atoms of iodine/molecule of protein. The labeled fibrinogen preparation utilized in this series of experiments contained 97% clottable radiiodine.

**Antihuman Fibrinogen Antisera.** Antihuman fibrinogen antisera were prepared in our laboratory in goats and made monospecific through adsorption with normal human serum.

**Thrombin.** Thrombin was Parke-Davis topical thrombin (Parke, Davis & Co., Detroit, Mich.) used without further purification.

**Hirudin.** Hirudin with a sp act of 3,800 U/ml protein was kindly supplied by Dr. Z. S. Latallo, Department of Radiobiology, Institute of Nuclear Research, Warsaw, Poland.

**Heparin.** Heparin was sodium heparin (Eli Lilly & Co., Indianapolis, Ind.) containing 1,000 U.S.P. U/ml.

**Techniques**

**Agarose Gel Chromatography.** Chromatographic studies were performed using either 0.9 x 30-cm or 1.5 x 90-cm siliconized glass columns packed with Bio-Gel® A-5m (Bio-Rad Laboratories, Richmond, Calif.), an agarose medium with an exclusion limit of 5,000,000 mol wt. In most experiments the elution buffer was Tris, 0.06 M in 0.3 M NaCl, pH 7.6. In experiments focusing on heparin resistance (see below) the buffer used for calibration and elution was barbital, 0.0165 M in 0.3 M NaCl, pH 7.35. The flow rate varied between 8 and 9 cm/h in small columns (0.9 x 30 cm) and varied between 14 and 15 cm/h in large columns (1.5 x 90 cm). In all instances flow rates were adjusted through gravity feeding to rates sufficiently low to avoid compression of the agarose gel medium. The volume of effluent delivered into individual fractions was determined by weighing all test tubes before and after collection of the effluent. Void volumes of individual columns were estimated with the use of platelet-rich plasma. Before determination of the void volume, columns were re-equilibrated with a modified Tyrode's buffer containing no calcium chloride but trisodium citrate to a final concentration of 0.38%. The effluent volume of the first aliquot containing platelets, as estimated by automated counting (Coulter Electronics Inc., Hialeah, Fla.), was taken as the void volume. Effluent fractions collected in experiments involving small columns (0.9 x 30 cm) measured 0.85 ± 0.5 ml; effluent fractions collected in experiments involving large columns (1.5 x 90 cm) measured 2.75 ± 0.15 ml.

**Four Methods of Determining the Content of Fibrinogen and Fibrinogen-Related Molecules in Effluent Fractions.** (a) Radial immunodiffusion modified from Mancini et al. (17). The diffusion medium was 1% agarose containing 1% specific antifibrinogen antiserum and urea to 2 M. The addition of urea to 2 M to the agarose facilitated diffusion of high molecular weight soluble fibrin complexes and produced significantly greater zones of precipitation for these entities than were observed in agarose without urea added. The presence of urea in the agarose medium did not appreciably reduce the formation of precipitable antigen-antibody complexes. After 24 h incubation, the plates were fixed with 1% tannic acid before measurements of the precipitin zones. (b) An automated nephelometric immunoprecipitin assay (AIP).¹ The automated immunoprecipitation assay was identical to the quantitative protein immunoassays performed by the Technicon Immunoprecipitator (18) but utilized a manifold assembled in our laboratory from Technicon spare parts and an Aminco nephelofluorimeter (American Instrument Co., Inc., Silver Springs, Md.) equipped with a flow cell with a 3 mm light path. (c) A thrombin-clotting time technique modified from Clauss (19). 0.2-ml aliquots from column fractions were admixed with 0.1 ml thrombin (200

¹Abbreviations used in this paper: AIP, automated nephelometric immunoprecipitin assay; FDP, fibrinogen degradation products; IRF, immunoreactive fibrinogen; RID, radial immunodiffusion; SFC, soluble fibrin complex(s); TCT, thrombin-clotting time(s).
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U/ml in saline) and the clotting time recorded with the help of a Fibrometer (Baltimore Biological Laboratories, Cockeysville, Md.). In the presence of these high quantities of thrombin, the clotting times depend largely on substrate concentration and are relatively insensitive to the presence of fibrinogen-fibrin degradation products. (d) A clottable protein assay, according to Ratnoff and Menzie (20), was utilized in some experiments; to provide sufficient fibrinogen, 2.0-ml aliquots of effluent fractions were clotted.

Preparation of High Molecular Weight Soluble Fibrin Complexes In Vitro (Thrombin-Treated Plasma). Platelet-poor plasma was incubated with 0.04 U of thrombin/ml at room temperature for 5 min (method I). Residual thrombin activity was blocked with hirudin, added to a final concentration of 0.4 U/ml plasma. The ethanol gelation test (9), a rapid test to demonstrate soluble fibrin complexes, was strongly positive for all plasmas prepared in this fashion.

To provide sufficient material to allow for clottable protein analysis of soluble fibrin complexes, platelet-poor plasma was treated more extensively with thrombin (method II). Platelet-poor plasma was incubated with 0.08 U of thrombin/ml at room temperature for 15 min. Residual thrombin activity was blocked with hirudin added to a final concentration of 1 U/ml of plasma. The small clots, which occasionally formed during incubation of these mixtures, were removed before chromatography.

In experiments involving the use of purified fibrinogen as the starting material for soluble fibrin complexes, fibrinogen (5 mg/ml) in 0.06 M Tris, 0.3 M saline, pH 7.6, was incubated with thrombin (0.02 U/ml) at room temperature for 5 min. Residual thrombin activity was again blocked with hirudin added to a final concentration of 0.2 U/ml incubation mixture. 1 ml of these incubation mixtures was separated by Bio-Gel chromatography on small columns (0.9 x 30 cm); 5 ml of these incubation mixtures were utilized in experiments using large columns (1.5 x 90 cm).

Thrombin Sensitivity. Appropriate dilutions were prepared from selected effluent fractions using 0.06 M Tris, 0.3 M saline, pH 7.6, as a diluent. Aliquots (0.2 ml) of undiluted and diluted effluent fractions were admixed with 0.1 ml of thrombin (200 U/ml in saline) and the clotting times of the mixtures recorded.

In additional experiments, 0.2-ml aliquots of undiluted effluent fractions were admixed with 0.1 ml of thrombin in saline containing 0.1, 0.2, 0.3, 0.4, and 0.5 U. All clotting times were recorded with the aid of the Fibrometer.

The influence of effluent fractions on the thrombin-clotting time of normal plasma was assayed in the following manner. Normal plasma, 0.1 ml, was admixed with 0.2 ml of appropriate column fractions. The mixture was clotted with 0.1 ml thrombin (20 U/ml). Clotting times were recorded in the Fibrometer. The control consisted of 0.1 ml normal plasma and 0.2 ml column buffer (Tris, 0.06 M, 0.3 M NaCl, pH 7.6) clotted with 0.1 ml thrombin (20 U/ml).

Heparin-sensitized Thrombin-Clotting Time Assay. Assay conditions are specified in Fig. 9.

In Vivo Experiments. Male or female albino rabbits (1.5-2 kg) were utilized. All rabbits received 50 U of thrombin dissolved in 10 ml of normal saline through slow intravenous infusion in the ear vein over a period of exactly 5 min. At 1, 2, 4, 6, 8, 10, 20, and 30 min after the termination of thrombin administration, blood samples were withdrawn by heart puncture for analysis for soluble fibrin complex content by agarose gel chromatography.

Patient Studies. Patients included in these studies were admitted to Indiana University School of Medicine hospitals. Two groups of patients were evaluated.

The first group included 12 patients, all admitted with a clear-cut diagnosis of major thromboembolic disease. Nine of these patients presented with unequivocal clinical evidence of a recent onset of extensive deep-vein thrombosis. In five of these nine patients there was also strong evidence of pulmonary embolism by clinical criteria, blood gases, and lung scans. Three additional patients in this group presented with a picture of major completed thrombotic cerebrovascular accidents. Blood samples with withdrawn from all patients within 24 h after their admission. In no instance were the patients on anticoagulant drugs at the time of blood sampling.

The second group encompassed 20 patients undergoing major surgery. 13 patients underwent major abdominal surgery; 2 patients had radical mastectomies; and 5 patients underwent thoracic surgery (lobectomies or pneumonectomies). 13 of these 20 patients underwent surgery for malignant disease. Blood samples were obtained between 12 and 48 h after surgery in all instances. Platelet-poor plasma was prepared, as outlined above. Plasmas were either analyzed immediately by fibrinogen gel
chromatography or were immediately frozen, stored at -21°C, and analyzed later. Agarose gel chromatography was performed on small columns for all patients' samples.

Results

The chromatographic behavior of normal plasma applied to a large column of Bio-Gel A-5m is demonstrated in Fig. 1. 5 cm$^3$ of normal plasma was applied to this column calibrated at a void volume of 47 cm$^3$, and fibrinogen can be seen to elute in a sharply defined symmetrical peak around an effluent volume of 72 cm$^3$.

![Diagram](image)

**Fig. 1.** Bio-Gel® A-5m chromatography of normal plasma (large column). Effluent fractions assayed for fibrinogen by RID, AIP, and TCT.

Fig. 1 illustrates the comparison of three assays for quantifying fibrinogen in the effluent: radial immunodiffusion (RID), the AIP assay, and the thrombin-clotting time (TCT) assay. It can be seen that the two immunoassays show excellent agreement and also that the thrombin-clotting times performed on individual fractions closely parallel the fibrinogen values obtained by the two immunoassays.

In the experiment depicted in Fig. 2, 5 ml of thrombin-treated plasma, prepared according to method I, was subjected to Bio-Gel chromatography. The tracing designated IR$\phi$ (immunoreactive fibrinogen) in this and the following figures represents the average values for the two immunoassays. The tracing designated TCT represents the thrombin-clotting times. It can be seen that the
TCT pattern shows a distinct two-peak pattern with an early peak appearing close to the void volume of this column in an area of effluent were only trace quantities of fibrinogen are detectable by immunoassays. Based on the chromatographic behavior of fibrinogen and a variety of fibrinogen derivatives, previously published from our laboratory (21), we estimated the mol wt of this early-peak material to be in excess of 1 million.

Similar differences could be demonstrated by agarose gel chromatography on smaller columns calibrated at a void volume of 6.4 ml, the type of column originally suggested by Fletcher and co-workers for diagnostic purposes (12, 13). As seen in the upper graph depicted in Fig. 3, elution patterns for IRφ and TCT were in close agreement for normal plasma applied to this column in a volume of 1 cm³; whereas, the TCT for thrombin-treated plasma (method I) applied to the column in a 1-cm³ vol, as displayed in the lower graph, showed a distinct two-peak pattern with the early peak eluting close to the void volume where only traces of IRφ were demonstrable.

Gel chromatography of 25 mg of thrombin-treated highly purified fibrinogen applied to a large column at a 5-cm³ vol again produced fibrin complexes in the effluent region close to the void volume demonstrable by TCT assays (Fig. 4). Under these circumstances, one could also detect an early peak of IRφ exhibiting an elution peak a few milliliters later than the TCT peak.
To exclude methodological artifacts as the reason for low values of fibrinogen obtained in effluent regions in which a shortening of the TCT was noted, we conducted additional experiments. Depicted in Fig. 5 is an experiment in which $^{125}$I-labeled fibrinogen was added to normal plasma before treatment with thrombin, according to method I.

As can be seen, the material contained in the early effluent peak in this experiment incorporated only 6% of the total radioactivity present in the thrombin-treated plasma subjected to Bio-Gel chromatography. This confirms the impression that the highly aggregated soluble fibrin complexes (SFC) demonstrable by the TCT technique contain only small quantities of fibrinogen.

In three experiments (Table I) the fibrinogen content from fractions exhibiting the shortest TCT in the SFC and native fibrinogen effluent regions was quantified as clottable protein, as well as by immunoassays. The plasmas utilized in these experiments were treated more extensively with thrombin according to method II providing sufficient material to allow for accurate clottable protein esti-
mates in SFC fractions. There is a good agreement between fibrinogen values by clottable protein assays and by immunoassays in both the SFC and native fibrinogen peak fractions. Although SFC peak fractions by either assay contain considerably less protein than native fibrinogen peak fractions, TCT of soluble complex fractions are always shorter than in native fibrinogen fractions.

To account for these results, one could speculate that SFC are more sensitive to thrombin action than regular fibrinogen. A comparison of TCT of several dilutions of fractions containing SFC and dilutions of native fibrinogen supported this concept (Fig. 6). Appropriate dilutions of purified fibrinogen and SFC, separated from thrombin-treated fibrinogen by agarose gel chromatography, were clotted with thrombin.

A linear relationship between fibrinogen concentration and TCT was obtained for native fibrinogen between 2,880 and 720 μg/ml. Below 720 μg/ml the relationship was no longer linear, and at concentrations of 280 μg/ml or less, TCT were infinitely long. In contrast, 280 μg/ml of a SFC fraction exhibited a TCT of 24 s. There was a linear relationship between protein concentration and TCT for SFC fractions between 280 and 95 μg/ml; at the latter protein concentration, a TCT of 62.5 s was recorded. At protein concentrations of 70 μg/ml and below, TCT for SFC fractions went to infinity.

Further support for the hypothesis of enhanced thrombin sensitivity of SFC was obtained in experiments in which the substrate concentrations were kept
Fig. 5. Bio-Gel® A-5m chromatography of thrombin-treated normal plasma. $^{131}$I-labeled human fibrinogen was added before thrombin treatment. Effluent fractions assayed for radioactivity and TCT (large column).

**Table I**

_Fibrinogen Determined by a Clottable Protein Assay, Immunoassays, and TCT for SFC Fractions and Main-peak Fibrinogen Fractions_

| SFC fractions | Clottable protein | IRφ | TCT | Main-peak fibrinogen fractions |
|---------------|------------------|-----|-----|--------------------------------|
|               | Clottable protein | IRφ | TCT | Clottable protein | IRφ | TCT |
| Exp. 1        | 114 μg            | 125 μg | 35.5 | 376 μg            | 420 μg | 51.7 |
| Exp. 2        | 163 μg            | 160 μg | 30.2 | 297 μg            | 310 μg | 140.0 |
| Exp. 3        | 310 μg            | 285 μg | 25.0 | 490 μg            | 470 μg | 78.0 |

constant but enzyme concentrations were varied (Fig. 7). Clotting times at different thrombin concentrations for main-peak fibrinogen and the SFC fraction were compared. The starting material in this experiment was 5 ml of plasma treated with thrombin, according to method I, and subjected to Bio-Gel chromatography.

As can be seen from Fig. 7, the early-peak material clotted significantly more
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Fig. 6. TCT as a function of protein concentration in native fibrinogen solutions and SFC fractions. Aliquots (0.2 ml) of fibrinogen or SFC fractions contained in 0.06 M Tris, 0.3 M saline, pH 7.6, were diluted with the same buffer and admixed with 0.1 ml of thrombin (200 U/ml in saline). Clotting times at 37°C were recorded on the fibrometer.

rapidly at this range of thrombin concentrations (0.1-0.5 U/ml) than did the main-peak fibrinogen, although the concentration of IRδ of the SFC utilized in this experiment was only one-fourth of the concentration of IRδ from the main-peak material.

Similar results to those depicted in Fig. 7 were obtained in three additional experiments using thrombin-treated plasma as a starting material to produce the soluble complexes and in an additional three experiments in which purified fibrinogen treated with thrombin was separated by agarose gel chromatography to prepare soluble complexes.

SFC fractions usually produce visible gels upon lowering the temperature or upon lowering the ionic strength through dialysis against distilled water. For this reason we examined the possibility that the enhanced rate of coagulation observed for SFC fractions reflects discreet changes in the ionic milieu of the sample, rather than the addition of thrombin. Discreet, but significant, lowering of the ionic strength of the sample is introduced in our standard thrombin-clotting assay (0.2 ml of sample fraction in 0.06 M Tris, 0.3 M NaCl, pH 7.6 is “diluted” with 0.1 ml of thrombin in 0.15 M NaCl). This possibility was excluded, since we observed that the dilution of two volumes of soluble fibrin complex fractions with one vol of 0.15 M saline did not produce spontaneous gelation of the sample over a 24 h period of observation at 37°C. Furthermore, the clotting times obtained for SFC fractions were identical, irrespective of whether the thrombin used in the assay was dissolved in 0.15 M saline or whether it was dissolved in 0.06 M Tris, 0.3 M saline, pH 7.6. The pH optimum for the reaction lied between 7.6 and 8.0; a change of the pH to 8.5 or to 6.0 produced considerably longer clotting times than was observed at pH 7.6. It would, therefore, appear that the “abnormally short” TCT observed for SFC fractions are unlikely to be caused by nonspecific ionic strength and pH effects.

Fig. 8 depicts the results of eight experiments aimed at comparing the effects of
soluble fibrin complexes and fibrinogen from the main peak on the TCT of normal plasma. In four experiments soluble complexes and fibrinogen were harvested from agarose gel chromatography of thrombin-treated plasma (method I), and in four experiments thrombin-treated purified fibrinogen was used as the starting material. Under the assay conditions employed, it can be seen that main-peak fibrinogen produced little or no shortening of the TCT; whereas, SFC from the early peak, even at very low concentrations, produced a substantial shortening of the TCT of normal plasma, as illustrated by the lowest bar in Fig. 7.

The addition of highly aggregated soluble complexes to normal plasma also produced a decrease in heparin sensitivity, as illustrated by the heparin-sensitized thrombin-clotting assay (Fig. 9). The data presented here again includes eight experiments, four utilizing thrombin-treated plasma and four utilizing thrombin-treated purified fibrinogen for the preparation of SFC. The top bar in Fig. 9 represents the mean normal plasma TCT without heparin in the system. It can be seen from the lowest bar in the diagram that the normal plasma TCT in the presence of SFC is prolonged only insignificantly by the addition of a
standard amount of heparin in comparison to the substantial prolongation in the controls in which either main-peak fibrinogen or buffer was admixed with normal plasma before the addition of an identical amount of heparin (the two middle bars).

To assess the pathological implications of these findings, it seemed critical to demonstrate the presence of biologically active SFC in vivo. In the experiment shown in Fig. 10, a plasma sample was obtained from a rabbit 1 min after the administration of 50 U of thrombin, intravenously, and subjected to Bio-Gel chromatography. As can be seen from Fig. 10, the TCT of effluent fractions showed a broad distribution representing a wide range of different molecular weight soluble complexes with significant amounts of material eluting close to the void volume in an effluent zone where once again only trace quantities of IRφ were demonstrable.

In additional studies in rabbits we obtained data to indicate that this early-peak material is short-lived. In timed studies we were able to demonstrate thrombin-sensitive SFC with a broad molecular weight distribution in decreasing quantities at 1, 2, 4, 6, 8, and 10 min after the infusion of 50 U of thrombin. At 20 and 30 min after the thrombin infusion the fibrinogen gel chromatograms had resumed their normal symmetrical shape.

Fig. 11 illustrates an agarose gel elution pattern for plasma fibrinogen in a patient who presented at Marion County General Hospital with the unquestionable clinical diagnosis of multiple pulmonary emboli. A characteristic two-peak pattern according to the TCT assay emerged with a large early peak of SFC not demonstrable by immunoassays. Our clinical experience so far encompasses 12 patients with well-documented thromboembolic disease; and this thrombin-sensitive highly aggregated polymeric material was demonstrated in 3 patients in this group. In addition, we examined 20 patients within 48 h after major
abdominal or thoracic surgery but could demonstrate thrombin-sensitive polymeric material in only two of these patients, an incidence substantially lower than the incidence of gel chromatographic abnormalities, previously noted by Fletcher et al. (12), in patients undergoing surgery (25 abnormal chromatograms among 91 assays on 55 patients).

Discussion

Our studies demonstrated an unexpected property of SFC, an ability to enhance the rate of normal fibrinogen-to-fibrin conversion. This curious characteristic was first suspected in experiments in which significant discrepancies arose between fibrinogen estimates by immunochemical techniques and fibrinogen estimates by a TCT technique performed in the presence of excess thrombin. We observed that polymeric fractions of high molecular weight exhibited short TCT, although the estimated content of fibrinogen in these fractions was low by immunochemical assays.

Several possibilities were considered to explain these discrepancies. The immunological techniques utilized in these studies for the quantification of...
Fig. 10. Bio-Gel® A-5m chromatography of rabbit plasma withdrawn 1 min after the completion of infusion of 50 U of thrombin, intravenously (large column).

Fibrinogen could conceivably underestimate the true fibrinogen content in polymeric fractions because of the limited capability of these macromolecules for diffusion in gel media. However, we considered this unlikely; since the results of the immunodiffusion assays for fibrinogen incorporating urea to 2 M in the agarose medium closely agreed with the results obtained through a nephelometric immunoprecipitation assay, the latter assay being independent of the diffusion constant of the antigenic material.

IRΦ could be underestimated if antigenic sites of the fibrinogen molecule were obscured through conformational changes arising during intermediate polymer formation. We considered this possibility unlikely in view of our experiments in which the distribution of radiolabeled fibrinogen between higher and lower molecular weight fractions paralleled the distribution of fibrinogen and SFC estimated by the two types of immunochemical assays.

Moreover, in additional experiments we observed that the values for fibrinogen estimated by a clottable protein assay, closely paralleled the lower values observed by immunochemical assays in SFC fractions, as well as the higher values obtained through immunoassays on "main-peak" fibrinogen fractions.

We next considered the possibility that the discrepancies between levels of immunoassayable fibrinogen and TCT estimates of fibrinogen in soluble polymer fractions could reflect enhanced thrombin sensitivity of higher molecular weight
SFC. We confirmed this hypothesis in experiments in which substrate concentrations were varied at constant enzyme concentrations and experiments in which enzyme concentrations were varied in the face of constant substrate concentrations. In both instances, clotting times utilized over the range of substrate and enzyme concentrations were significantly shorter for SFC than for native fibrinogen. Nonspecific effects arising from a change in the ionic environment of the SFC, as a consequence of the test procedure, were excluded; since it was established that TCT were identical, irrespective of whether the thrombin added was dissolved in 0.15 M saline (the standard test procedure) or was dissolved in the standard column buffer (0.06 M Tris, 0.3 M saline, pH 7.6).

We also observed that the soluble complex fractions in small quantities appreciably enhanced the gelation rate of normal fibrinogen, as evidenced by a shortening of the TCT or a heparin-sensitized TCT of normal plasma. The biochemical basis for these findings is obscure. In experiments involving plasma treated with thrombin, fractions containing SFC also contained significant quantities of antihemophilic globulin and alpha, macroglobulin, in addition to varying amounts of low-density beta lipoproteins. The possibility that these proteins in some way accelerate the thrombin-catalyzed fibrinogen-to-fibrin conversion can largely be excluded, since experiments utilized highly purified fibrinogen containing no detectable beta lipoproteins and only trace quantities of antihemophilic globulin.
gave essentially the same results, as did experiments in which normal plasma was used as a starting material to produce soluble complexes. Nevertheless, one cannot exclude the possibility of participation of nonfibrinogen proteins in soluble complex formation. Indeed, we have recently obtained preliminary evidence suggesting that cold insoluble globulin incorporates to a significant degree into SFC in thrombin-treated plasma (22). However, the functional significance of this observation remains obscure.

Soluble fibrin macromolecules react with thrombin, implying that these polymers retain some thrombin-sensitive sites, i.e. soluble polymers must contain monomeric units still possessing A or B peptides; but it remains obscure why such soluble aggregates even at very low concentrations are capable of an enhanced rate of gelation upon incubation with thrombin. Similarly, the ability of soluble complex fractions to shorten the TCT of normal plasma or fibrinogen and to render normal plasma heparin resistant cannot be explained at the present time on the basis of enzyme kinetics. Soluble fibrin complexes could conceivably enhance normal fibrin gelation, as they may constitute a nidus around which enhanced polymerization could occur.

Our demonstration of highly aggregated SFC in the plasma of rabbits after thrombin infusions confirmed earlier observations reported by Lee (23) and Lee and McCluskey (24). These authors first pointed out that intravenous injections of endotoxin or the slow intravenous infusion of thrombin does not result in massive disposition of fibrin in tissues but rather converts circulating fibrinogen into soluble fibrin aggregates ("heparin-precipitable fibrinogen"), which are subsequently rapidly removed by the reticuloendothelial system in the liver and in the spleen. The demonstration of similar, highly aggregated, thrombin-sensitive SFC in occasional patients suggests a pathophysiological role for these aggregates in thrombotic states in man. In a subsequent clinical series to be reported elsewhere and which focuses on postoperative patients with phlebographically documented deep-vein thrombosis, we have not again encountered these highly aggregated, thrombin-sensitive complexes in one single instance. The chromatographic abnormality encountered in these patients with considerable frequency is an early shoulder on the fibrinogen peak in an estimated mol wt range of 500,000-700,000 consistent with a smaller aggregate, presumably a dimer.

Recently, Alkjaersig et al. (25) applying computer analysis to fibrinogen gel chromatograms, obtained from patients with thrombotic states, have postulated the existence of two species of fibrinogen-fibrin complexes possessing minimum mol wt of 430,000 and 550,000 but apparently have not detected the highly aggregated complexes of higher molecular weight described in this communication. In extensive in vitro studies in our laboratory, we have been unable to produce aggregates in the dimeric size range through the addition of thrombin to plasma or fibrinogen using a wide range of concentrations of enzyme and substrate. Similarly, we have been unable to produce such aggregates by incubating mixtures of fibrinogen and a variety of plasmic fibrinogen degrada-

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^Hansen, M. S., T. Kim, E. T. Yin, N. U. Bang, and J. Glover. Comparison of different in vitro and in vivo approaches toward detection of subclinical thrombosis. Manuscript in preparation.
tion products (FDP) with thrombin (21, 26). Under these circumstances, the aggregates which formed are of the same type as the aggregates described in this publication possessing apparent mol wt somewhere in excess of 1 million. Recent work in our laboratory (27, 28), as well as a preliminary report by Alkjaersig and Fletcher (29), suggests that the complexes in the dimeric size range can occur as a consequence of aggregation between higher molecular weight degradation products of noncross-linked fibrin and unaltered fibrinogen. This reaction may occur in the absence of thrombin. Thus, the fibrinogen gel chromatographic abnormalities, which have been frequently observed in clinical situations by Fletcher et al. (12, 13) and by ourselves, could be the direct consequence of fibrinolysis rather than ongoing generation and fibrin deposition. However, this hypothesis assumes that part of the fibrin in intravascular thrombotic deposits remains noncross-linked, an assumption which awaits experimental verification. The highly aggregated, thrombin-sensitive polymers which we have observed in only a few patients may reflect ongoing thrombin generation and fibrin deposition.

Our rabbit experiments showing that such aggregates are cleared rapidly and effectively from the circulation, presumably by the reticuloendothelial system, provide a reasonable explanation why this type of polymer is only infrequently observed in clinical thrombotic states. Thus, this particular type of thrombin-sensitive fibrin complexes is probably too short-lived in the circulation to be of practical diagnostic significance, in contrast to smaller aggregates in the dimeric size range. It is conceivable, however, that these particular species of high molecular weight thrombin-sensitive SFC may have a significant pathophysiological role in the short time span during which they circulate. Since these macromolecules are capable of enhancing the rate of blood coagulation, they may contribute to a temporary “hypercoagulable state” and may accelerate the buildup and extension of thrombotic deposits.

Summary
We have detected a species of soluble fibrin complexes with significant biological properties. Agarose gel chromatography of normal plasma or purified fibrinogen previously incubated with small amounts of thrombin revealed the presence of a species of high molecular weight soluble fibrin complexes, which contained only small quantities of fibrinogen by immunological assays but which exhibited enhanced sensitivity to thrombin. In addition, these complexes substantially shortened the thrombin-clotting time of normal plasma and enhanced the resistance of normal plasma to heparin action. Similar thrombin-sensitive soluble fibrin complexes were demonstrated in vivo in rabbits for up to 10 min after the infusion of 50 U of thrombin. Thrombin-sensitive soluble fibrin complexes were also demonstrated in 3 of 12 patients with documented thromboembolic disease and in 2 of 20 patients after major surgery. High molecular weight soluble fibrin complexes, which exhibit enhanced thrombin sensitivity and which are capable of increasing the rate of normal fibrinogen-to-fibrin conversion by thrombin, may appear consequent to clinical thrombosis and situations involving trauma (e.g., major surgery). Such soluble complexes, although they have no proven role in the primary pathogenesis of intravascular
thrombosis, may contribute to a temporary "hypercoagulable state" and may accelerate the build-up and extension of already existing thrombotic deposits.

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References

1. Shainoff, J. R., and I. H. Page. 1960. Cofibrins and fibrin intermediates as indicators of thrombin activity in vivo. Circ. Res. 8:1013.

2. Shainoff, J. R., and I. H. Page. 1962. Significance of cryoprofibrin in fibrinogen-fibrin conversion. J. Exp. Med. 116:687.

3. Sasaki, T., I. H. Page, and J. R. Shainoff. 1966. Stable complex of fibrinogen and fibrin. Science (Wash. D. C.). 152:1069.

4. Lipinski, B., Z. Wegrzynowicz, A. Budzynski, M. Kopec, Z. S. Latallo, and E. Kowalski. 1967. Soluble unclottable complexes formed in the presence of fibrinogen degradation products (FDP) during the fibrinogen-fibrin conversion and their potential significance in pathology. Thromb. Diath. Haemorrh. 17:65.

5. Marder, V. J., and N. R. Shulman. 1969. High molecular weight derivatives of human fibrinogen produced by plasmin. II. Mechanism of their anticoagulant activity. J. Biol. Chem. 244:2120.

6. Kierulf, P., and U. Abildgaard. 1971. Studies on soluble fibrin in plasma. Scand. J. Clin. Lab. Invest. 28:231.

7. Bachman, F., and O. Pichairut. 1971. Hypercoagulability syndrome in patients with renal disease. Fed. Proc. 30:424.

8. Donati, M. B., J. Vermylen, R. Verhaeghe, and D. Culasso. 1972. Agarose-gel filtration for the separation and identification of fibrinogen derivatives. Transactions of the Third Congress of the International Society on Thrombosis and Haemostasis. Seeman Printery Inc., Durham, N. C. 84.

9. Godal, H. C., and U. Abildgaard. 1966. Gelation of soluble fibrin in plasma by ethanol. Scand. J. Haematol. 3:342.

10. Niewiarowski, S., and V. Gurewich. 1971. Laboratory identification of intravascular coagulation. The serial dilution protamine sulfate test for the detection of fibrin monomer and fibrin degradation products. J. Lab. Clin. Med. 77:676.

11. Pindyck, J., H. Lichtman, and S. Kohl. 1970. Cryofibrinogenemia in women using oral contraceptives. Lancet. 1:51.

12. Fletcher, A., N. Alkjaersig, J. O'Brien, and V. G. Tulevski. 1970. Blood hypercoagulability and thrombosis. Trans. Assoc. Am. Physicians Phila. 83:159.

13. Fletcher, A., and N. Alkjaersig. 1972. Blood hypercoagulability, intravascular coagulation and thrombosis. New diagnostic concepts. Thromb. Diath. Haemorrh. 45:389.

14. Kazal, L. A., S. Amsel, O. P. Miller, and L. M. Tocantins. 1963. The preparation and some properties of fibrinogen precipitated from human plasma by glycine. Proc. Soc. Exp. Biol. Med. 113:989.

15. Mosesson, M. W., and S. Sherry. 1966. The preparation and properties of human fibrinogen of relatively high solubility. Biochemistry. 5:2829.

16. Izzo, J. L., W. F. Bale, M. J. Izzo, and A. Roncone. 1964. High specific activity labelling of insulin with 111I. J. Biol. Chem. 239:3743.

17. Mancini, G., A. O. Carbonara, and J. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry. 2:235.

18. Operation manual for the Technicon® automated immunoassay-precipitin system. 1971. Technicon® Technical Publication No. TA1-02363-00.
19. Clauss, A. 1957. Gerinnungsphysiologische schnellmethode zur bestimmung des fibrinogens. Acta Haematol. (Basel). 17:237.
20. Ratnoff, O. D., and C. Menzie. 1951. A new method for the determination of fibrinogen in small samples of plasma. J. Lab. Clin. Med. 37:316.
21. Smith, G. F., and N. U. Bang. 1972. Formation of soluble fibrin polymers: fibrinogen degradation fragments D and E fail to form soluble complexes with fibrin monomer. Biochemistry. 11:2958.
22. Bang, N. U., M. W. Mosesson, M. S. Hansen, R. Patterson, and L. E. Mattler. 1972. Participation of cold insoluble globulin in the formation of soluble fibrin polymers in plasma. Transactions of the Third Congress of the International Society on Thrombosis and Haemostasis. Seeman Printery Inc., Durham, N. C. 64.
23. Lee, L. 1962. Reticuloendothelial clearance of circulating fibrin in the pathogenesis of the generalized Schwartzman reaction. J. Exp. Med. 115:1065.
24. Lee, L., and R. T. McCluskey. 1962. Immunohistochemical demonstration of the R. E. clearance of circulating fibrin aggregates. J. Exp. Med. 116:611.
25. Alkjaersig, N., L. Roy, and A. P. Fletcher. 1973. Analysis of gel exclusion chromatographic data by chromatographic plate theory analysis: Application to plasma fibrinogen chromatography. Thromb. Res. 3:525.
26. Mattler, L. E., Z. S. Latallo, M. L. Chang, M. S. Hansen, and N. U. Bang. 1973. Analysis of soluble fibrin monomer complexes by agarose gel chromatography and protamine sulfate gelation. Fed. Proc. 32:260.
27. Chang, M. L., Z. S. Latallo, L. E. Mattler, M. S. Hansen, and N. U. Bang. 1973. Copolymerization of fibrin fragments D and E with fibrin monomer in soluble fibrin monomer complex formation. Fed. Proc. 32:314.
28. Bang, N. U., M. S. Hansen, G. F. Smith, Z. S. Latallo, M. L. Chang, and L. E. Mattler. 1973. Molecular composition and biological properties of soluble fibrin polymers encountered in thrombotic states. Ser. Haematol. 6:494.
29. Alkjaersig, N., and A. P. Fletcher. 1973. Biophysical properties of plasma "fibrinogen" during thrombolysis and limited activation of coagulation system. Clin. Res. 21:870.