Detection of Circulating Crosslinked Fibrin Derivatives by a Heat Extraction–SDS Gradient Gel Electrophoretic Technique

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A technique has been developed to identify and quantitate unique plasmic degradation products of crosslinked fibrin in plasma. In this method, fibrin derivatives are extracted by heat precipitation and dissolved with disulfide bond reduction, after which the crosslinked γ-γ chain remnants are identified by SDS-polyacrylamide gradient gel electrophoresis and quantitated by densitometric analysis. A heterogeneous group of γ-γ chains with molecular weights between 100,000 and 76,000 daltons was identified in lysates of crosslinked fibrin during plasmic degradation in vitro. Three stages of crosslinked fibrin degradation have been arbitrarily defined based primarily on the extent of degradation of these γ-γ polypeptide chains. As little as 20 μg of crosslinked fibrin digest added to 1 ml of normal plasma could be detected by the heat-extraction–gel-electrophoresis technique, identifying the γ-γ derivatives with molecular weights of 98,000, 86,000, 82,000, and 76,000 daltons. Plasmic derivatives of γ-γ chains were not found in normal plasma, but they were identified in the plasma of patients with disseminated intravascular coagulation and deep-vein thrombosis, both before and in increased quantity during successful thrombolytic therapy.

A technique to identify and quantitate in plasma specific degradation products of crosslinked fibrin in distinction to those of fibrinogen would be of value in studying thrombotic disorders and distinguishing between disseminated intravascular coagulation (DIC) and primary fibrinogenolysis. However, because of structural and immunologic similarities between fibrin and fibrinogen, the development of such techniques has been slow. One approach has been to utilize the subtle immunologic differences between plasmic derivatives of fibrinogen and fibrin12 to develop a radioimmunoassay for these products, but none are as yet applicable for clinical use. Using a different approach that combines immunoprecipitation of fibrinogen-related antigen from plasma and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of the precipitate, Gaffney3 was able to demonstrate the fibrin-specific fragment DD in the plasma of a patient with amniotic fluid embolism. Lane and colleagues4 have modified this technique by using solid-phase immunoadsorption of serum followed by SDS-polyacrylamide gel electrophoresis of eluted fibrinogen antigen and identified fragment DD in the serum of patients with DIC. Using the same methods after precipitation of large molecular weight derivatives of fibrin and fibrinogen from plasma with β-alanine,
Graeff and colleagues also demonstrated fragment DD in plasma from a patient with amniotic fluid embolism.

While these studies have clearly demonstrated that fragment DD circulates in patients with DIC, certain technical limitations are evident. First, an immunoprecipitation method depends on careful adjustment of the ratio of antigen to antibody to achieve maximal precipitation of various fibrinogen antigens, some of which may precipitate less completely than others. The use of serum as a test material precludes the identification of fibrin fragments or complexes that are incorporated into the clot. Both of these techniques are, therefore, limited in their ability to quantitate the amount of fibrin derivatives present in patient samples.

We have developed a technique to quantitate plasmic degradation products of crosslinked fibrin in plasma using a simple heat-precipitation method to extract the plasmic derivatives, a sensitive SDS-polyacrylamide gradient gel electrophoretic system to separate the reduced polypeptide chains in the heat precipitate, and densitometric analysis for quantitation of crosslinked γ chain derivatives in the stained gel. This technique has been tested in normal individuals, in two patients during thrombolytic therapy for venous thrombosis, and in two patients with DIC.

**MATERIALS AND METHODS**

Human fibrinogen (grade L) was purchased from AB KABI (Stockholm). Human thrombin (U.S. standard thrombin, Lot. H1) and plasmin were kindly supplied by Dr. David Aronson (Bureau of Biologies Standards, Bethesda, Md). Bovine thrombin (Thrombin, Topical) was obtained through Parke, Davis & Co. (Detroit, Mich.) and aprotinin (Trasylol®) was purchased from Mobay Chemical Corp. (New York, N.Y.). Acrylamide was obtained from Eastman Kodak Co. (Rochester, N.Y.) and recrystallized from a saturated solution of chloroform. Sodium dodecyl sulfate was manufactured by Sigma Chemical Co. (St. Louis, Mo.) and recrystallized for use from a saturated ethanol solution. Phosphorylase A, soybean trypsin inhibitor, carboxypeptidase A, myoglobin, and bovine serum albumin were obtained from Sigma Chemical Co. '251-labeled fibrinogen [Radionuclide-labeled (U25)] Fibrinogen (Human) Sensor®] and urokinase (Abbokinase®) were supplied by Abbott Laboratories (No. Chicago, Ill.). Normal pooled human plasma was obtained from blood collected from 10 normal volunteers and anticoagulated with disodium ethylenediaminetetraacetate (NaEDTA; 0.5% final concentration) in the presence of aminocaproic acid (0.02M final concentration).

**Preparation of Plasmic Degradation Products of Crosslinked Fibrin**

A 50-ml volume of fibrinogen, dissolved in water to a concentration of 20 mg/ml, was added to 350 ml of 0.15 M sodium chloride, 0.05 M tris-hydrochloric acid buffer, pH 7.4, containing 2.5 ml of human plasma fraction 3 (factor XIII activity 900 U/ml) and 6.5 ml of 1.0 M calcium chloride. Clotting was initiated by adding bovine or human thrombin (1.25 NIH U/ml final concentration) and the clot incubated at 25°C for 18 hr. The liquor was then removed by pressing the clot between nylon mesh, after which the clot was washed extensively with 0.15 M sodium chloride, lyophilized, and ground with a glass rod. A 50-mg portion of ground fibrin was suspended in 2.0 ml of buffer to which plasmin was added to a final concentration of 0.15 CTA U/ml, and incubation begun at 37°C with magnetic stirring. Digestion was terminated at desired times by the addition of soybean trypsin inhibitor (final concentration 0.2 mg/ml). For digestions longer than 6 hr, an additional 0.3 U of plasmin were added after the first 6 hr of incubation. After digestion was terminated, the mixture was centrifuged at 2300 g for 15 min to remove...
particulate material. Protein concentration in the supernatant fluid was determined by measuring the optical density at 280 nm with a Beckman Model 24 spectrophotometer, using an extinction coefficient of 15.0.\textsuperscript{10}

**Preparation of Dansyl-Cadaverine-Labeled Plasmic Degradation Products of Fibrin**

This was modified from the technique of Lorand and colleagues.\textsuperscript{11} Fibrin was prepared as above except that the clotting mixture was made in 2.45 mM monodansylcadaverine.\textsuperscript{12} At varying intervals, a 20-μl aliquot of the fluid phase was removed and added to a denaturing solution of 3.75% SDS, 0.38 M sucrose, and 0.025% NaEDTA in 0.1 M boric-acid-tris buffer, pH 8.6, to terminate digestion and prepare the sample for electrophoresis.

**125I-Labeled Plasmic Degradation Products**

\textsuperscript{125}I-labeled plasmic degradation products of crosslinked fibrin were prepared as above except that 25 μCi of \textsuperscript{125}I-labeled fibrinogen (0.4 mg) was clotted with 30.0 mg of cold fibrinogen, then digested with plasmin as noted above.

**Thrombin-Clottable Protein**

Thrombin-clottable protein was determined by the technique of Ratnoff and Menzie.\textsuperscript{13}

**Heat Extraction of Plasmic Derivatives of Fibrin and Fibrinogen From Plasma**

Plasma samples (0.5 ml) were placed in 10 × 75 mm glass tubes and heated at 60°C in a water bath for 5 min. The resultant precipitate was separated by centrifugation at 4100 g for 20 min at 4°C and then washed twice at 27°C with 0.1 M sodium chloride, 0.05 M tris-hydrochloric acid buffer containing 0.1 M NaEDTA, 0.02 M epsilon-aminocaproic acid (EACA), and aprotinin (10 kallikrein inhibitor units/ml) at pH 7.0 with centrifugation at 4100 g for 10 min after each wash. Disulfide bonds were reduced by suspending the final washed precipitate in 0.3 ml of 3.75% SDS, 0.38 M sucrose, 0.025% NaEDTA, 5% β-mercaptoethanol in 0.1 M boric acid–tris buffer at pH 8.6 and heating at 60°C until dissolved, usually within 3 hr.

**Other Extraction Techniques**

Precipitation with protamine sulfate was performed by adding 0.5 ml of protamine sulfate (2.0 mg/ml) to 0.5 ml of plasma at 4°C. After incubation on ice for 20 min, the precipitate was sedimented by centrifugation at 4100 g for 20 min and washed twice at 4°C with buffer. Precipitates were also prepared by adding 0.5 ml of 5.0 M β-alanine to 0.5 ml of plasma at 4°C and incubating on ice for 10 min; centrifugation and washing were performed as for protamine sulfate precipitates. Cryoprecipitates were made by freezing plasma at −40°C, thawing on ice, and then separating and washing the precipitate as above.

**Electrophoresis**\textsuperscript{14,15}

Polyacrylamide slabs (160 × 140 × 1.2 mm) with a linear concentration gradient of polyacrylamide from 5% to 14% were cast at 4°C between glass plates using a gradient former (Model 570, Instrumentation Specialities Co., Lincoln, Nebr.). The composition of the gels was 1 M urea, 0.13 M tris, 0.048 M hydrochloric acid, 0.10% SDS, 0.002% NaEDTA, 0.05% N,N,N',N'-tetramethylene-diamine (TEMED), and 0.05% ammonium persulfate with a sucrose gradient from 0.05 M to 0.25 M and a ratio of acrylamide to bisacrylamide of 29:1. After polymerization at room temperature a stacking gel was applied with a 13-slot sample well former. The composition of the stacking gel was 5% acrylamide, 0.14% bisacrylamide, 1 M urea, 0.03 M tris, 0.05 M sulfuric acid, 0.1% SDS, 0.002% NaEDTA, 0.075% TEMED, and 0.1% ammonium persulfate with the pH adjusted to 6.1–6.2. Samples for electrophoresis were dissolved in 3.75% SDS, 0.38 M sucrose, 0.025% NaEDTA in 0.1 M boric acid–tris buffer at pH 8.6. Disulfide bond reduction was achieved by adding β-mercaptoethanol (5% final concentration) and heating at 60°C for 4 hr. Electrophoresis was performed toward the anode in tris-borate buffer at pH 8.6, containing 0.10% SDS and 0.00035% mercaptoacetic acid. Electrophoresis of two slabs simultaneously was performed in a Model 400 electrophoresis apparatus (Aquebogue...
Machine and Repair Shop, Aquebogue, N.Y.) at 40 mamp constant current until the voltage reached 200 V, then at 200 V constant voltage until \( \frac{1}{2} \) hr after the tracking dye (bromophenol blue) exited. Gels were stained and destained according to the method of Fairbanks and colleagues.\(^{16}\) Molecular weights of reduced polypeptide chains were determined by comparison with a standard curve of migration versus molecular weight, using bovine serum albumin (68,000), phosphorylase A (94,000), ovalbumin (43,000), carboxypeptidase A (34,600), myoglobin (17,000), and the Aa (68,000), Bβ (58,000), and γ (48,000) chains of fibrinogen as standards.

**Quantitative Analysis of γ-γ Chain Remnants**

After electrophoresis of aliquots of dissolved, reduced heat precipitates or of plasmic digests of fibrin, the polyacrylamide slab gel was stained, destained, and cut into strips containing individual samples for densitometric analysis. The portion of the gel strips containing polypeptide chains in the molecular weight range of γ-γ moieties was scanned at 575 nm in a Beckman Model 24 spectrophotometer equipped with a linear scanning device and recorder. From scans of electrophoretic strips of known amounts of purified crosslinked fibrin digests, a standard curve was constructed for each γ-γ band, relating micrograms of digest electrophoresed to maximum optical density of the band. The quantity of crosslinked fibrin digest present in a plasma sample was determined by comparing the optical density of individual γ-γ bands with the appropriate standard curve.

**Autoradiography**

After electrophoresis of samples containing \(^{125}\)I-labeled plasmic digests, the stained and destained gel was placed in a drying solution of 50% methanol, 10% acetic acid, and 20% glycerol for 1 hr, after which it was dried by suction evaporation. The dried gel was apposed to a sheet of x-ray film (Kodak "No-Screen," Eastman Kodak Co., Rochester, N.Y.) for 10 days and the photographic film developed.

![Fig. 1. Protein-stained SDS polyacrylamide gradient (5%-14%) gels of fibrinogen, crosslinked fibrin, and plasmic digests of crosslinked fibrin after reduction of disulfide bonds. The arrow indicates the demarcation between the upper stacking gel and the gradient gel. The inset shows a gel segment containing the bands corresponding in molecular weight from 96,000 to 76,000 in a plasmic digest of dansylated crosslinked fibrin that has been photographed for fluorescence and stained for protein.](image)
RESULTS

Figure 1 shows the SDS polyacrylamide gradient gel pattern of the polypeptide chains of soluble derivatives released during plasmic digestion of crosslinked fibrin, in comparison with the Aα, Bβ, and γ chains of fibrinogen and the γ-γ, β, and α polymer chains of crosslinked fibrin. The 5%–14% linear polyacrylamide gradient spreads the region containing the bands of 60,000–100,000 dalton polypeptide chains, allowing for distinct separation of the heterogeneous group of γ-γ derivatives. In stage 1 crosslinked fibrin digests, the predominant γ-γ chains were those of molecular weight 100,000 and 96,000. The γ-γ derivatives of lower molecular weight were present in smaller amounts, and variable amounts of intact β chain (58,000) were present in addition to a derivative of 38,000 daltons. The γ-γ chain derivatives in stage 2 digests of crosslinked fibrin demonstrated more extensive degradation, with a heterogeneous distribution corresponding in molecular weight from 100,000 to 76,000; only a small amount of intact β chain was present. Stage 3 crosslinked fibrin digests had γ-γ chains of only 76,000 and 82,000 and no intact β chains. Fibrinogen clotted in the presence of the fluorescent lysine analogue dansyl-cadaverine shows fluorescence at the glutamine crosslink acceptor sites on α and γ chains and their remnants. The inset in Fig. 1 shows fluorescence of the bands between 96,000 and 76,000 in a reduced stage 2 digest of dansylated fibrin, indicating that these bands represent chains that contain the crosslink site. The molecular weights and presence of crosslink sites confirmed the identity of these electrophoretic bands as the remnants of γ chain dimers.

Plasmic digests of crosslinked fibrin were added to normal plasma, the solution heated, and the precipitate dissolved with disulfide bond reduction, after which the polypeptide chains were separated and analyzed by SDS polyacrylamide gradient gel electrophoresis (Fig. 2). Bands that corresponded in mobility to γ-γ polypeptide chains of 82,000 and 86,000 were visible in the sample of normal plasma containing either stage 1 or stage 2 crosslinked fibrin digests; only a hazy background staining was present in this area in the control plasma extract. In Fig. 2, the other γ-γ bands overlay partially or completely with bands present in the normal plasma extract and

![Fig. 2. Segments of a protein-stained SDS polyacrylamide gradient (5%–14%) slab gel. The γ-γ bands in stage 1 and stage 2 plasmic digests of crosslinked fibrin are compared with the corresponding regions in dissolved, reduced heat extracts of pooled normal plasma, with and without digest added prior to heat precipitation. The amount of crosslinked fibrin digest added to plasma (0.5 ml) was 600 μg of stage 1 and 1000 μg of stage 2. Molecular weights corresponding to the γ-γ bands are noted, and the lines indicate the location of those bands in the electrophoresed plasma extract.](image-url)
cannot be identified clearly. In other electrophoretic runs, however, sufficient separation was achieved to allow identification of the γ-γ bands of molecular weights 96,000 and 76,000.

Two techniques were employed to verify that the additional bands seen in gel patterns of extracts of normal plasma containing fibrin digests (Fig. 2) were γ-γ remnants. First, 125I-labeled plasmic degradation products of crosslinked fibrin were added to normal plasma, heat extracted, and electrophoresed, after which the positions of protein-stained bands were compared to those demonstrated by autoradiography (Fig. 3A). Confirming the results shown in Fig. 2, the γ-γ derivatives of 82,000 and 86,000 molecular weight were clearly separated on the protein-stained gel and coincided with the position of bands in the autoradiograph. The location of the other γ-γ bands shown by the autoradiograph completely or partially overlapped with bands in the control plasma extract. In the second approach, dansyl-cadaverine-labeled stage 2 fibrin digests were added to normal plasma, heat extracted, and electrophoresed (Fig. 3B). The γ-γ derivatives with molecular weights of 76,000–92,000 in this digest were visualized with ultraviolet illumination, and holes were cut in the fluorescent bands to mark their positions. Bands corresponding to γ-γ derivatives greater than 92,000 molecular weight were present in low concentrations and therefore could not be clearly seen, but the correspondence of perforated, fluorescent bands from 76,000 to 92,000 daltons with protein-stained bands confirmed their identification as γ-γ polypeptide chain remnants.

The recovery of fibrin digests in precipitates was determined by adding 125I-labeled fibrin digest to plasma, preparing the precipitates, and comparing radioac-

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Fig. 3. (A) Segments of a dried SDS polyacrylamide gradient (5%-14%) slab gel showing the γ-γ bands of a 125I-labeled stage 2 plasmic digest of crosslinked fibrin and the corresponding electrophoretic region of a dissolved, reduced heat extract of 0.5 ml of pooled normal plasma containing 400 μg of the same digest. Molecular weights of individual γ-γ bands are shown to the left. (B) Segments of an SDS polyacrylamide gradient (5%-14%) slab gel showing the γ-γ bands in a stage 2 digest of dansylated crosslinked fibrin and the corresponding region in a heat extract of 0.5 ml of pooled normal plasma containing 600 μg of the digest. Unstained gels were viewed with ultraviolet light and holes cut in fluorescent bands to mark their position, after which the gel was stained for protein.
tivity in the precipitates and supernatant fluids (Table 1). The heat extraction method was compared with other extraction techniques to precipitate crosslinked fibrin derivatives from plasma, namely protamine sulfate or β-alanine precipitation, and freezing followed by thawing at 4°C. The addition of 10% trichloroacetic acid (TCA) resulted in nearly complete precipitation of fibrin digests or of fibrinogen. The other techniques were less efficient and extracted fibrinogen more completely than crosslinked fibrin digests, and stage 1 digests more completely than stage 2 or stage 3 digests. Except for 10% TCA, heating at 60°C for 5 minutes.

Table 1. Percent Recovery by Different Extraction Techniques of Radiolabeled Fibrinogen and Plasmic Digests of Crosslinked Fibrin Added to Normal Plasma

<table>
<thead>
<tr>
<th>Extraction Technique</th>
<th>Percent Radiolabeled Protein Recovered*</th>
<th>Crosslinked Fibrin Digest</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fibrinogen</td>
<td>Stage 1</td>
</tr>
<tr>
<td>Heat</td>
<td>82</td>
<td>35</td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>74</td>
<td>33</td>
</tr>
<tr>
<td>β-alanine</td>
<td>86</td>
<td>30</td>
</tr>
<tr>
<td>Freeze-thaw</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
<td>Trichloroacetic acid</td>
<td>95</td>
<td>95</td>
</tr>
</tbody>
</table>

*Values for heat extraction are the average of 5 experiments; for protamine sulfate, β-alanine, and freeze-thaw, 3 experiments each; and trichloroacetic acid, 1 experiment.

![Graph](https://via.placeholder.com/150)

**Fig. 4.** Recovery of crosslinked fibrin digests at varying fibrinogen concentrations. Fibrinogen was added to pooled normal serum containing 100 U/ml of heparin, after which 125I-labeled crosslinked fibrin digest (80 μg/ml final concentration) was added. Extraction was performed by heat precipitation at 60°C for 30 min (see Materials and Methods). Percent recovery was determined by measurement of radioactivity in the precipitates and supernatant fluids. Each point represents the average of two experiments.
## Table 2. Recovery of Radiolabeled Crosslinked Fibrin Digests After Addition to Urokinase-Treated Normal Plasma

<table>
<thead>
<tr>
<th>Plasma Urokinase (IU/ml)</th>
<th>Clottable Protein</th>
<th>Recovery*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/dl</td>
<td>Stage 1</td>
</tr>
<tr>
<td></td>
<td>Percent Control</td>
<td>Percent</td>
</tr>
<tr>
<td>0 (control)</td>
<td>39.6</td>
<td>35</td>
</tr>
<tr>
<td>100</td>
<td>44.1</td>
<td>100</td>
</tr>
<tr>
<td>250</td>
<td>68.1</td>
<td>20</td>
</tr>
<tr>
<td>500</td>
<td>68.1</td>
<td>16</td>
</tr>
<tr>
<td>750</td>
<td>8.2</td>
<td>15</td>
</tr>
<tr>
<td>1000</td>
<td>20.5</td>
<td>13</td>
</tr>
</tbody>
</table>

*Normal pooled citrated plasma was incubated for 1 hr at 37°C with urokinase at various concentrations, after which 13H-labeled crosslinked fibrin digest (40 ìg in 0.01 ml) was added to 0.5 ml of the treated plasma. Heat precipitates were made and percent recovery of digest in precipitate was determined by measurements of radioactivity in the supernatant fluids and precipitates. Thrombin-clottable protein was determined by the technique of Ratnoff and Menzie.13

![Graph](image)

**Fig. 5.** The log-log relationship between maximum optical density of a Coomassie Blue-stained band and the quantity of stage 2 crosslinked fibrin digest electrophoresed in the gel. The inset shows the protein-stained gel segments that were densitometrically scanned to construct the graphs. Three individual γ-γ remnants are quantitated and represented by circles (mol wt 86,000), triangles (mol wt 82,000) and squares (mol wt 76,000); the correlation coefficients for the individual lines are 1.00, 0.92, and 0.93, respectively.
gave the greatest recovery of fibrin digests. More prolonged heating at 60°C did not improve recovery, and heating at higher temperatures resulted in gelation rather than precipitation of the sample.

The recovery of crosslinked fibrin digests in heat precipitates was affected by the plasma fibrinogen concentration (Fig. 4). Recovery was less than 3% at a fibrinogen concentration of 50 mg/dl, but was nearly maximal at concentrations of 200 mg/dl or more, with stage 1 digests recovered more efficiently than stage 2 or stage 3 digests. Table 2 shows the effect of urokinase treatment of plasma on the recovery of radiolabeled fibrin digests by heat precipitation. At a final plasma urokinase concentration of up to 250 U/ml, the plasma clottable protein concentration decreased to 11%–17% of control, and at higher concentrations of urokinase, the clottable protein concentration was 5% or less of control. Recovery of fibrin digests from plasma containing 100 U of urokinase/ml was impaired, yielding about 60% of expected for stage 1 or 2 digests and 89% for stage 3 digests. Even with urokinase levels of 1000 U/ml, the yield was still about 40% of control values, although only 5% of initial clottable protein remained. This relatively small effect of fibrinogenolysis on digest recovery contrasts with the effect of a low absolute fibrinogen concentration (Fig. 4), in which case a plasma-clottable protein of 50 mg/dl allowed for only a 10% of expected yield of digest.

Quantitation of the amount of crosslinked fibrin digest present in an extract was based on the observation that the staining intensity of individual γ-γ bands was directly proportional to the amount of digest that had been applied to the gel and electrophoresed (Fig. 5). This same relationship between optical density and concentration was obtained for γ-γ derivatives when they were electrophoresed after recovery from plasma samples by heat extraction (Fig. 6). At present, the minimum amount of fibrin digest that could be added to normal plasma, extracted, and subsequently identified by protein stain after electrophoresis was 20 μg/ml of plasma.

Figure 7 illustrates the results on plasma samples from patients with DIC or venous thrombosis and during thrombolytic therapy. The extract of pooled normal plasma showed no band corresponding in molecular weight to the 96,000, 86,000, or 82,000 γ-γ chains and only a faint band corresponding in position to the 76,000

![Figure 6. Segments of SDS polyacrylamide gradient (5%-14%) gels showing the protein stained γ-γ bands in a stage 2 crosslinked fibrin digest and in heat extracts of normal plasma and normal plasma to which the digest had been added. The arrows point to the bands representing γ-γ derivatives of molecular weight 82,000 and 86,000.](Image)
FIBRIN DERIVATIVE IN PLASMA

Fig. 7. (A) Segments of protein-stained SDS polyacrylamide gradient gels showing the γ-γ bands in a stage 1 crosslinked fibrin digest and the corresponding areas in gels of heat extracts of pooled normal plasma and of plasma from two patients with DIC. The arrows (top to bottom) point to bands corresponding to molecular weights of 96,000, 86,000, 82,000, and 76,000. (B) Segments of protein-stained SDS polyacrylamide gradient gels of heat extracts of normal plasma, normal plasma incubated with urokinase in vitro, and plasma from two patients before and during thrombolytic therapy. The urokinase incubation (750 CTA U/ml for 1 hr at 37°C) was performed 3 times and bands of 82,000 and 86,000 molecular weight were not observed on any of these occasions. The arrows indicate bands as shown in (A).

remnant. The same normal pattern was seen in 3 other pools of 10 normals each and in 10 individual normal donors. Patients 1 and 2 suffered DIC in the course of pneumococcal or meningococcal septicemia. In the sample from patient 1, bands corresponding to molecular weights of 86,000 and 82,000 were clearly seen, and a heavy band of molecular weight 96,000 was present. The plasma extract of patient 2 clearly showed the 96,000 and 86,000 bands; that of 82,000 molecular weight was
very faint, but a heavy band corresponding to molecular weight 76,000 was present.

Incubation of normal plasma in vitro with urokinase resulted in decreased staining of fibrinogen Aα chains, greater clarity of two bands corresponding in position with the 76,000 γ-γ remnant, and the appearance of a faint band corresponding to that of the 96,000 γ-γ chain (Fig. 7B). No bands appeared in the 86,000 or 82,000 regions. Patient 3 had deep-vein thrombosis treated with a thrombolytic agent and showed marked improvement in the follow-up venographic study. In the plasma extract prior to treatment, heavily stained bands corresponding to molecular weights of 96,000 and 76,000 and lighter bands at 86,000 and 82,000 were seen. In the sample during therapy, the 86,000 band became more prominent, while little change occurred in the 96,000, 86,000, or 76,000 bands. Patient 4 showed similar electrophoretic patterns before and during thrombolytic therapy. Prior to treatment, bands of molecular weight 96,000 and 76,000 were clearly distinguished and that of 86,000 was demonstrable; during therapy, the 86,000 and 76,000 molecular weight bands became more prominent, that of 82,000 became visible, and the 96,000 band remained unchanged.

DISCUSSION

We have sought to directly identify in plasma all fibrin derivatives containing a crosslinked γ-γ chain, whether derived from circulating crosslinked fibrin polymers, from fragment DD,17-19 or from larger fragments released during fibrin lysis.20 In the early phase of plasmic action on crosslinked fibrin, both intact and degraded γ dimers and β chain were found in the soluble lysate (Fig. 1). The presence of intact γ-γ and β chains implies that fragments are released from the digesting fibrin that have not yet been cleaved between D and E domains10 and are, therefore, larger than fragment DD. With more prolonged plasmin action, degradation of the γ-γ chains results in intermediate remnants of molecular size between that of the intact chain and that of the 76,000 dalton derivative.17-19,21 Heretofore, studies of patient samples have been concerned with the detection of only fragment DD,1-3 but in vitro studies (Fig. 1)20 suggest that larger fragments could be liberated from crosslinked fibrin thrombi in vivo. The identification of individual γ-γ derivatives in patient samples would be useful in understanding basic mechanisms of thrombolysis in vivo, and requires not only a discriminating assay system but also an appropriate nomenclature of the relevant crosslinked fibrin digests. Therefore, we have divided the crosslinked fibrin degradation sequence into three stages, patterned after that used for fibrinogen degradation10 and based on the electrophoretic demonstration of progressive γ-γ and β chain degradation (Fig. 1).

The heat-extraction method is much simpler and reproducible than the immuno-precipitation1 or immunoadsorption3 techniques for precipitating fibrin degradation products from plasma, and more efficient than other simple nonimmunologic extraction procedures (Table 1). Although heating to 60°C precipitates proteins other than fibrinogen and fibrin derivatives, the technique of SDS polyacrylamide gradient gel electrophoresis achieves the necessary resolution to separate the γ-γ derivatives from other polypeptide chains in the dissolved reduced plasma extract, allowing their identification and quantitation (Fig. 2). Clear-cut evidence has been presented with dansyl-cadaverine-labeled derivatives to demonstrate that the bands
between 76,000 and 100,000 daltons in crosslinked fibrin digests are of \( \gamma-\gamma \) chain origin (Fig. 1, inset). Furthermore, both dansylated and radiolabeled digests show that the heat-extracted remnants with these same molecular weights are derived from \( \gamma-\gamma \) chains (Fig. 3). Although bands present in extracts of normal plasma may overlap with some \( \gamma-\gamma \) derivatives, the \( \gamma-\gamma \) chains of 96,000, 86,000 and 82,000 daltons can be clearly identified in the gels because only a faint background staining is present in normal plasma samples (Fig. 2). A 76,000 band can often be distinguished, but the presence of a faint band in normal plasma extracts makes quantitation of this \( \gamma-\gamma \) remnant more difficult. The 92,000 \( \gamma-\gamma \) band cannot be demonstrated in plasma extracts by this technique.

Densitometric analysis of the polyacrylamide gels and comparison with a standard curve allows for quantitation of the amount of fibrin digest added to normal plasma (Fig. 6). Calculations of the concentration of fibrin digest in test samples of plasma must include corrections for (1) the percent recovery of digests by heating plasma (Table 1), (2) the types of \( \gamma-\gamma \) remnants that are present (Figs. 1 and 4, Table 1), and (3) the variation in yield relative to the plasma fibrinogen or clottable protein concentration (Fig. 4, Table 2).

The correction for incomplete heat precipitation of digest considers a 20% yield of stage 2 or 3 digests and a 35% yield of stage 1 digests from plasma (Table 1). Standard curves would be based on the optical density of specific \( \gamma-\gamma \) remnant bands. The correlation of band intensity with concentration of digest will differ according to the stage of degradation because of the variation in relative proportions of \( \gamma-\gamma \) remnants present (Figs. 2, 5, and 6). In the fourth patients tested here (Fig. 7), the 96,000 band was more prominent than that of 86,000, and the 82,000 band was only faintly visible. This pattern most resembled a stage 1 digest, indicating that quantitation of these samples would most reasonably use a standard curve and percent recovery calculations relevant for a stage 1 crosslinked fibrin digest. However, the issue is not simply resolved when a heavy 76,000 band is also present (Fig. 7), since this pattern indicates that some stage 3 digest circulates; a separate standard curve may be necessary for quantitation of this \( \gamma-\gamma \) remnant. Since extracts of normal plasma may have a faint band in the region of the 76,000 \( \gamma-\gamma \) remnant, these calculations would also require subtraction of a baseline absorbancy prior to reading from the stage 3 digest standard curve.

A low fibrinogen concentration appears to markedly reduce the yield of fibrin digest (Fig. 4). However, this is not likely to seriously hinder the assay in clinical samples, since most patients with acquired hypofibrinogenemia will have significant levels of fibrinogen or fibrinogen degradation products, which tend to increase the yield of heated plasma extract (Table 2). Thus, patients with DIC or spontaneous or iatrogenic pathologic fibrinogenolysis would be expected to have at least 40% of expected yield of fibrin digest even when the clottable protein is reduced to less than 5% of control values. In such cases, calculations can either be adjusted to variable recovery according to the concentrations of plasma fibrinogen and serum degradation products, or preferably, an additional measurement can be performed after normalizing the fibrinogen concentration by the addition of purified fibrinogen prior to extraction.

In heat extracts of plasma from 10 normal individuals and from 4 pools of 10 normal plasmas, no bands were seen corresponding in mobility to the 96,000,
86,000, and 82,000 dalton γ-γ remnants, indicating a concentration of stage 1 or stage-2 plasma fibrin digest below 20 μg/ml. The faint band at 76,000 may represent a small amount (approximately 20 μg/ml) of stage 3 fibrin digest present in normal individuals, but confirmation is required. Significantly stronger bands of γ-γ remnants were seen in plasma extracts of the 4 patients who were tested, indicating that clinically relevant concentrations of fibrin digests can be detected and that clear-cut distinctions between individual γ-γ remnants can be accomplished. A comparison of the samples from the two DIC patients (Fig. 7, top) demonstrates a preponderance of intact γ-γ chains in both, compatible with the circulation of either crosslinked fibrin/fibrinogen polymers or of large molecular weight complexes of lysed crosslinked fibrin. Variations in DIC states are indicated by the identification in only one of the samples of a heavy band of 76,000, possibly representing an independent pathologic process of fibrin lysis down to the smallest fragment DD derivative. Other studies have shown significant quantities of D dimer in the plasma of patients with DIC, but the patterns shown in Fig. 7 allow for quantitation as well as for more subtle distinctions between patients.

Figure 7B shows γ-γ remnants circulating in patients with deep vein thrombosis, with a prominence of both the largest (96,000) and smallest (76,000) γ-γ derivatives. Horizontal studies are needed to elucidate the reasons for their simultaneous presence that could represent both stage 1 and stage 3 digests or simultaneous circulation of prethrombotic fibrin polymers and fully degraded macromolecular remnants of fibrin, respectively. The assay can also be applied as a tool for assessing the response to thrombolytic treatment inasmuch as increases in the γ-γ remnants of 86,000 (patient 3) and of 86,000 and 76,000 (patient 4) were demonstrated in the plasma during treatment that was known to have effectively dissolved venous thrombi. Prospective studies assessing this technique in patients treated with such agents are feasible, given the respectable yield of digests from plasma treated with high concentrations of urokinase (Table 2) and the minimal changes in electrophoretic pattern produced by urokinase on plasma proteins (Fig. 7B). The slight change noted in the 76,000 band after in vitro urokinase treatment of plasma may be secondary to background changes coincident upon lysis of the Aα chain of monomeric fibrinogen, which migrates in proximity to the smallest γ-γ remnant. The capacity of this procedure to distinguish different γ-γ remnants is well suited for studies of basic pathologic processes of thrombotic disease, especially in conjunction with other relevant assays.

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