Quantitative Estimation of Split Products of Fibrinogen in Human Serum, Relation to Diagnosis and Treatment

By CLARENCE MERSKEY, GEORGE J. KLEINER AND ALAN J. JOHNSON

PRIMARY SPONTANEOUS FIBRINOLYSIS or fibrinolysis secondary to systemic intravascular coagulation (dethrombosis) may produce large quantities of circulating split products. Similarly, fibrinogen split products in plasma or serum provide incontrovertible evidence for both primary and secondary fibrinolysis. These split products may result from the in vivo or in vitro digestion of fibrinogen or fibrin by plasmin,1,2 by trypsin and other proteolytic enzymes3 or even perhaps by hydrolytic enzymes (cathepsins) derived from neutrophilic leukocytes.4 In this report these split products are considered to be partially digested fibrinogen or fibrin fragments which react with antifibrinogen (or antifibrin) sera and are incoagulable with thrombin. They inhibit platelet aggregation and the thrombin-fibrinogen reaction,5 thromboplastin formation,2,6 and fibrin polymerization.7 The effects on fibrin clot formation are clinically significant since they prolong the thrombin clotting time. Salmon8 and Nussenzweig and Seligmann9 using both immunodiffusion and immunoelectrophoresis in agar gel, showed that split products occur with fibrinogen digestion in vitro. Ferreira and co-workers10,11 first reported finding these split products in the sera of a large number of patients with different diseases. Using a “fibrin degradation flocculation test” as well as immunoelectrophoresis,
they observed an abnormal precipitin reaction in most but not all of the sera of patients with such diverse conditions as abruptio placentae, uterine rupture, dead fetus syndrome, myocardial infarction, disseminated cancer, snake bite, and during extracorporeal circulation. No abnormalities were found in the sera of normal subjects.

In a study employing comparative electrophoresis in agar gel, Nilehn and Nilsson electrophoresed fibrinogen split products and subsequently diffused split product antiserum and the test sample on opposite sides of the center well. They detected fibrinogen split products in the sera of patients with myocardial infarction and pulmonary edema, thrombosis, leukemia, liver disease, obstructive jaundice, mental disease, and during normal labor, as well as in 5 of 28 normal subjects. Split products could readily be induced in the sera of healthy and diseased individuals by injecting nicotinic acid or pyrogens, by electroshock, or by prolonged venous occlusion.

Split products were detected by immunoelectrophoresis in serum from one patient in our series with disseminated cancer and multiple thrombi, but not in all others, even though readily detected by the precipitin and latex agglutination tests as well as the tanned red-cell hemagglutination inhibition immunoassay (TRCHII). The TRCHII was found to be the most sensitive and specific method and yielded the best quantitative results. In this type of assay, first described by Boyden, various proteins are conjugated with tannic acid-treated erythrocytes and the red cells are agglutinated by the corresponding antisera. Hemagglutination is inhibited if the antisera are incubated with and neutralized by the homologous antigen before the protein-conjugated cells are added. Chorionic gonadotrophin, growth hormone, fibrinogen and other antigens have been quantitated by this technic. Large amounts of split products in the blood of patients with defibrination syndrome and/or fibrinolysis could be detected by any of the various immunologic methods described above and could be quantitated by the TRCHII. When small amounts were present, they could not be detected as precisely by methods other than the TRCHII.

The present report is concerned primarily with the test findings on serum samples from 97 patients in whom primary or secondary fibrinolytic disease might be suspected and 33 normal controls. Secondary fibrinolysis was of special interest since it was found in patients with defibrination syndrome, pulmonary embolism and during and immediately following the delivery of normal and dead fetus. The finding of split products in the sera of many of these patients confirmed the qualitative results of Ferreira and co-workers and of Nilehn and Nilsson. Up to 768 μg./ml. was found in sera from patients with defibrination syndrome and up to 576 μg./ml. in primary fibrinolysis. Lesser amounts, up to 96 μg./ml., occurred in other disease states and approximately 2.0-5.0 μg./ml. split products was found in 10 per cent of normal sera. Ferreira found no split products in normal sera whereas Nilehn and Nilsson found 18 per cent of sera from normal subjects containing split products. The sensitivity of their technic, however, was probably 100 μg./ml., making it difficult to correlate their findings with the trace amounts found in our series.
SPLIT PRODUCTS OF FIBRINOGEN

MATERIALS

1. Latex particles sensitized with antifibrinogen antibody (Fi test).*  
2. Microcapillary tubes (1.4-1.6 mm. O.D.).†  
3. Sheep red blood cells (SRBC) 1-14 days old in Alsever’s solution.‡  
4. Formalin (3 per cent) in normal saline (0.85 per cent w/v); pH adjusted to 7.0-7.5 with 0.1 N NaOH.  
5. Acid buffer: 0.15 M sodium phosphate dibasic—0.35 volume; 0.15 M potassium phosphate monobasic—0.65 volume; 0.15 M sodium chloride—1.0 volume; pH adjusted to 6.4.  
6. Citrate buffer; same concentrations as acid buffer except that 0.1 M sodium citrate is substituted for sodium chloride; pH adjusted to 6.4.  
7. Sodium azide stock solution 100 mg./ml. in water.  
8. Tannic acid, 10 mg./ml. in water, is diluted 1:400 in acid buffer.  
9. Bovine thrombin.†  
10. Glycerol-activated human plasmin, 165 units/ml.§  
11. Kunitz pancreatic trypsin inhibitor, 1.0 mg./ml. (each mg. is equivalent to 3100 Christensen units of plasma).‖  
12. Normal horse serum adsorbed with SRBC. Five parts serum to one part washed SRBC is incubated 15 minutes at 37 C. and centrifuged at 1000 g for 10 minutes after which the cells are discarded. The adsorbed serum is diluted 1:250 in citrate buffer.  
13. Normal human plasma: Blood is collected from healthy donors and 1/10 volume of 3.8 per cent sodium citrate and Trasylol (25 Christensen units of aniplasmin/ml.) is added immediately. Plasma is obtained by centrifuging the mixture at 12,000 g for 15 minutes at 4 C.  
14. Normal human serum: Blood from healthy donors is mixed with Trasylol (100 units/ml.). allowed to clot for eight hours at 37 C., and the serum separated. Other normal serum samples were aged for 24 hours at room temperature, lyophilized and used for antisera adsorption.  
15. Antifibrinogen serum: Rabbits were immunized with highly purified, plasminogen-poor fibrinogen.‡‡ Such fibrinogen preparations usually showed one component in the ultracentrifuge, on immunoelectrophoresis and in starch gel electrophoresis. Clottable protein, 250 μg., in Freund’s complete adjuvant was injected into the foot pads and neck muscles. Two weeks later, 100 μg. of clottable protein in 0.25 ml. of aluminum hydroxide** was injected intravenously. Intramuscular injections were repeated at 10-14 day intervals until high-titer antiserum was obtained. The antiserum was adsorbed three to six times with lyophilized, aged human serum (1.0-2.0 mg./ml.) at 37 C. for 4 hours, then at 4 C. for 12-18 hours. The stock antiserum, with 1.0 mg./ml. sodium azide added, was stored at -60 C., and the working antiserum for daily use was stored at 4 C. When tested against normal human plasma or purified fibrinogen, only one precipitin band could be demonstrated by either immunodiffusion or immunoelectrophoresis. When the antiserum was mixed with an equal volume of plasma in a microcapillary tube, a heavy flocculation was observed in 3 minutes, but the precipitate was negligible in 18 hours when a similar mixture was made with normal human serum. Commercial antisera†† are suitable for TRCHII and im-

*Hyland Laboratories, Los Angeles, Calif.  
†Yankee microhematocrit tubes #A 2931, Clay-Adams, Inc. N. Y.  
‡Thrombin Topical, Parke Davis & Co., Detroit, Mich.  
‖Worthington Biochemical Co., Freehold, N. J.  
§§Preparation A 128 (protease inactivator, Farbenfabriken Bayer A. G., Leverkusen, Germany.  
**Aluminum hydroxide suspension, Cutter Laboratories, Berkeley, Calif.  
††Hyland Laboratories, Los Angeles, Calif.; Behringwerke, A. G., Marburg-Lahn, Germany.
munodiffusion in agar gel but may produce insufficient precipitate for use in the microcapillary precipitin test. Results in the TRCHII were similar whether antisera against fibrin or human fibrinogen or the split products resulting from the in vitro digestion of this fibrinogen by plasmin, were used. While we have not fully evaluated all these antisera in the TRCHII against pathologic plasma or serum, no striking differences are apparent to date.

METHODS

A. Precipitin Test: A microcapillary tube was filled with fibrinogen antiserum to a depth of 2.0 cm., an equal volume of test material added, the tube sealed with heat, and the mixture incubated for 18 hours at 37 C. The amount of precipitate was observed periodically, and after 18 hours measured in mm. The capillary tubes were then centrifuged at 11,000 r.p.m. for 5 minutes and the deposit measured with 40X magnification.

B. Fi Test: The sample was diluted in 0.1 M glycine buffer containing 1.0 per cent NaCl, pH 8.2.24 One drop of diluted sample was mixed with two drops of Fi test reagent on a glass slide, and the mixture was observed for agglutination. Macroscopic or microscopic evidence of agglutination within 120 seconds was considered a positive reaction.

C. Immunodiffusion and Immunoelectrophoresis: Immunodiffusion was performed in agar gel on microslides by the method of Ouchterlony and immunoelectrophoresis by the method of Scheidegger.

D. Tanned Red Cell Hemagglutination Inhibition Immunoassay (TRCHII): (1) Sheep red blood cells (SRBC) were washed four times in 50 volumes of saline. (2) One volume of 8 per cent SRBC in saline was mixed with one volume of 3 per cent formalin in saline (pH 7.0-7.5). (3) Cells were agitated gently for 18-24 hours at 37 C. with a magnetic stirrer. (4) Formalin SRBC were washed four times with 50 volumes of distilled water, and a 10 per cent suspension was made in distilled water containing 1.0 mg./ml. of sodium azide. Cells could then be stored at 4 C. for months.

Sensitization Procedure: (1) One volume of red cells was centrifuged (1000 g for 5 minutes), washed three times with 50 volumes of acid buffer, and a 2 per cent suspension was made in acid buffer. (2) One volume of these cells was mixed with one volume of freshly prepared tannic acid (1:40,000) and incubated at 56. C. for 60 minutes with occasional stirring. (3) The mixture was centrifuged (1000 g for 5 minutes); the cells were washed three times in 50 volumes of acid buffer and a 4 per cent suspension was made in citrate buffer. (4) The red cell suspension was then divided. Four-fifths volumes was sensitized by adding an equal volume of 1:250 diluted normal plasma or, alternatively, purified fibrinogen, 1.0 µg./ml. of clottable protein. A similar proportion of aged normal serum was added to the remaining one-fifth volume as a control. The cell suspensions were incubated at 37 C. for one hour, and the tubes were inverted every 15 minutes. The cells were sensitized at 37 C. rather than 56 C.17,20 since fibrinogen denatures at the higher temperature. As indicated above, the cells were coated with either purified fibrinogen or plasma; serum is used as a control since it does not sensitize the cells. The amount of antigen used for cell suspension is not critical, for we found no significant differences with plasma dilutions ranging from 1:100 to 1:5000, or approximately 25 µg. to 0.5 µg. of fibrinogen/ml. (5) The cells were centrifuged (1000 g for 5 minutes), washed three times in 50 volumes of citrate buffer, and a 2.5 per cent suspension was made in citrate buffer with 1:250 adsorbed horse serum and 1.0 mg./ml. sodium azide. The final concentration of cells was checked by microhematocrit.

Dilution Technic: Citrate buffer containing 0.4 per cent of adsorbed horse serum was used as a diluent throughout the test. Reagents mixed in round-bottomed test tubes provide readable settling patterns, but a microtitration kit is much more convenient, yielding accurate results more quickly and requiring very small quantities of reagents.

*Hyland Laboratories, Los Angeles, Calif.
Antiserum Titration: Antiserum was diluted serially in the buffer (doubling dilutions). One volume of sensitized (or control) cells and one volume of buffer were added to one volume of diluted antiserum. Results were read after storage at 4 C. for 12-18 hours. At present the antiserum can be diluted to approximately 1:200,000 before the first negative tube appears. A dilution of 1:2000 is currently used in our laboratory for the inhibition test. A positive reaction is shown by the formation of a coarse agglutinate or loosely stippled mat at the bottom of the tube, and a negative reaction by a button- or doughnut-shaped ring at the bottom of the tube.17

Adsorption of Serum or Other Test Material: Occasionally, samples of serum or thrombin-treated plasma showed a nonspecific agglutination up to a titer of 1:8, which could be usually reduced to 0 or 1.0 by prior adsorption of the sample for 2 hours at 4 C. with 1/5 volume (or more) of fresh or formalized washed packed SRBC.

Inhibition Assay: Doubling dilutions of adsorbed normal plasma or other test material were made in citrate buffer, and an equal volume of diluted antiserum was added to each tube. After they had stood at 4 C. for 10 minutes, one volume of cold (4 C.) cells was added. The tubes were shaken, left at 4 C. for 12-18 hours, then read. The last negative tube was regarded as the end point. Suitable controls with each assay included dilutions of normal plasma, serum, or other test material with sensitized and control cells but no antibody, as well as control cells with antibody.

Cells: SRBC maintained their reactivity for at least 21 days after sensitization. If not used within 2 hours of washing, they should be rewashed 3 times in fresh buffer.

Buffers: Citrate buffer was used exclusively to prevent clot formation, a possibility when acid buffer17 is used with plasma or fibrinogen.

Heparin and Thrombin: Heparin (± 20 units/ml.) or thrombin (100 NIH units/ml.) did not inhibit specific hemagglutination of sensitized cells.

Sensitivity and Precision: Dilute antiserum improved the sensitivity of the assay but too high a dilution made the end point difficult to define. The assay’s precision was increased by using additional intermediate dilutions. The concentration of red blood cells in the final mixtures must be similar in all tubes.

Reproducibility: The inhibition assay yielded more sensitive and consistent results when performed at 4 C. Table 1 shows variations produced with a standard normal plasma tested 3-5 times a week over a period of 2 months with a constant dilution (1:5000) of antiserum. The use of standard control plasmas permitted control of the daily test results.

RESULTS

Normal Plasma: The TRCIII was used to measure the fibrinogen concentration of the plasma (Table 2). Three mg./ml. of plasma fibrinogen commonly produced a titer of approximately 800; with higher fibrinogen levels the titer was 1600. It was concluded from these data that approximately 3 µg./ml. of fibrinogen (range: 2-5 µg.) neutralized the antiserum used in each assay tube and inhibited red cell agglutination. Thus the sensitivity of the assay was about 3 µg. fibrinogen/ml. of sample, while the inherent error in quantitation primarily due to difficulty in reading the end point was approximately ±2 times this figure.

Normal Serum: Serum was collected from 33 normal subjects and 1/10 volume of thrombin (1000 units/ml.) containing 0.1 mg./ml. of Kunitz inhibitor was added. After incubation for one hour at 37 C., the sera were centrifuged and tested by immunoassay. The titer of 27 was zero and of 3 was 1.0. Results in the other 3 were inconclusive; despite repeated adsorption, nonspecific agglutination in the control tubes prevented readings below 1.0, 1.0 and 4.0, respectively. A titer of 1.0 represents 2.0-5.0 µg./ml. of fibrinogen
Table 1.—Reproducibility of Hemagglutination Inhibition Titer with Same Antiserum Concentration and Standard Plasma Used Throughout

<table>
<thead>
<tr>
<th>Reciprocal of Hemagglutin. Inhib. Titer</th>
<th>Observations (%)</th>
</tr>
</thead>
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<tr>
<td>400</td>
<td>13</td>
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<tr>
<td>800</td>
<td>50</td>
</tr>
<tr>
<td>1600</td>
<td>30</td>
</tr>
<tr>
<td>3200</td>
<td>7</td>
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Table 2.—Hemagglutination Inhibition Titer Relative to Plasma Fibrinogen Concentration

<table>
<thead>
<tr>
<th>Plasma Sample No.</th>
<th>Clottable Protein (mg./ml.)</th>
<th>Reciprocal Hemagglutin. Inhib. Titer</th>
<th>Sensitivity of Assay (µg./ml.) Fibrinogen</th>
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<tr>
<td>1</td>
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<td>800</td>
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<td>2</td>
<td>4.41</td>
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<td>2.8</td>
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<tr>
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<tr>
<td>9</td>
<td>2.90</td>
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<td>10</td>
<td>3.07</td>
<td>1600</td>
<td>1.9</td>
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Sensitivity calculation: clottable protein (mg./ml.) × hemagglutination inhibition titer.

or split products, the maximal amount found in most samples of thrombin-treated normal serum.

**Blood Coagulation:** The TRCHII titer diminishes as blood coagulates and plasma fibrinogen disappears. This was shown by the following experiment: Normal citrate plasma was centrifuged at 14,000 g for 15 minutes; samples were then incubated at 37°C and 1/4 volume of 1/10 M calcium chloride was added. One M sodium citrate (1/8 volume) was added to each plasma sample at successive intervals after the calcium chloride. The tubes were observed for clotting, and the nature of any clots recorded. The clots were removed on wooden applicators one hour after formation. The residual fibrinogen was measured on each specimen by both a standard procedure for fibrinogen and the TRCHII. After the plasma started to clot, the clottable protein decreased; there was a short delay before the TRCHII titer started to fall, then both decreased steadily. Eventually no fibrinogen was detectable by either method.

**Hypocoagulable States:** Residual fibrinogen may be found in shed blood serum of patients with a hypocoagulable state—for example, after heparin or coumarin administration, or with coagulation defects such as hemophilia. The addition of thrombin (100 NIH units/ml in 1/10 volume) removes this type of immunologically reacting material.

**Plasmin-Digested Fibrinogen:** Purified human fibrinogen was digested by plasmin and the reaction monitored by serial thrombin clotting times. The reaction was stopped periodically by adding an excess of Kunitz inhibitor. The TRCHII, the amount of precipitin, and the residual clottable fibrinogen were
SPLIT PRODUCTS OF FIBRINOGEN

Table 3.—Plasmin Digestion of Purified Fibrinogen

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Clottable Protein (mg./ml.)</th>
<th>Thrombin Clotting Time (Sec.)</th>
<th>Reciprocal of Hemagglutination Thrombin</th>
<th>Precipitin (mm.)</th>
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<tr>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>6.8</td>
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<td>5120</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>5.0</td>
<td>16.0</td>
<td>3840</td>
<td>32</td>
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<tr>
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<td>1280</td>
<td>8320</td>
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<td>4096</td>
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<tr>
<td>120</td>
<td>No clot</td>
<td>14.0</td>
<td>640</td>
<td>1536</td>
</tr>
<tr>
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<td>No clot</td>
<td>15.0</td>
<td>960</td>
<td>2176</td>
</tr>
<tr>
<td>315</td>
<td>No clot</td>
<td>13.0</td>
<td>640</td>
<td>512</td>
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<tr>
<td>345</td>
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</tr>
<tr>
<td>545</td>
<td>No clot</td>
<td>10.0</td>
<td>100</td>
<td>96</td>
</tr>
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</table>

Human fibrinogen (83 per cent clottable) was incubated at pH 8.6 with glycerol-activated human plasmin (35 mg. fibrinogen/caseinolytic unit plasmin). Aliquots of digestion mixture were removed at intervals and the reaction stopped with Kunitz inhibitor (1 mg./ml.). The thrombin clotting time was measured at 37 C. using equal volumes of platelet poor normal plasma, a 1/10 dilution of the incubation mixture and thrombin (10 units/ml.). The TRCHII and precipitin tests were performed both before and after the addition of 100 units thrombin/ml. Thrombin-clottable fibrinogen is absent after 18 minutes, but both immunologic assays remain positive for a longer period. The TRCHII (without thrombin) is essentially unchanged for 40 minutes, then the titer falls but remains abnormal; the amount of precipitin diminishes to near normal levels sooner and is normal in the last 3 specimens. Thrombin-treated specimens demonstrate increasing amounts of split products as digestion proceeds, then the amounts diminish. Once again the TRCHII is more sensitive, remaining abnormal even in the 545-minute specimen whereas the precipitin test is normal by 90 minutes.

measured on each sample (Table 3). The addition of thrombin removed undigested fibrinogen. While clottable fibrinogen decreased to zero, both immunologic methods remained positive in high titer; eventually the amount of immunologically reacting material diminished. This experiment demonstrates that both the TRCHII and the precipitin test are sensitive to fibrinolytic split products, the TRCHII being the more sensitive test. The early digestion products with TRCHII seem similar quantitatively to those of undigested fibrinogen and this justified quantitative measurement of these split products in serum as approximately 3.0 μg./ml per dilution of the serum.

Human Defibrination Syndromes and Clinical Fibrinolytic States: The TRCHII was employed to test serum samples from 17 patients with defibrinination associated with malignant disease, abruptio placentae, or acute allergic reaction. All sera had significant quantities of fibrinogen split products up to 768 μg./ml. (Table 4). Three patients with spontaneous primary fibrinolysis had similar amounts of split products in the serum, and up to 96 μg./ml were
found in the sera of patients with various diseases probably associated with secondary fibrinolysis. For example, results were abnormal in 9 of 21 patients with hepatic cirrhosis or necrosis. It is significant that four patients with markedly prolonged thrombin clotting times had no evidence of split products. The test revealed abnormal sera in 1 of 17 patients with acute myocardial infarction; 2 of 12 patients with rheumatoid arthritis; 1 of 7 postoperative patients; 5 of 10 women during, and in the first 2 hours following, a normal delivery; 1 of 2 women with a cesarean section, all 3 women delivering dead fetuses; and 5 patients with pulmonary embolism.

**Fi Test:** When the Fi test was performed on diluted (1:100) normal plasma, agglutination was observed macroscopically within 10 to 20 seconds. No positive reactions occurred within two minutes in any of 20 normal serum samples with added thrombin. Without thrombin, however, only 3 of these sera were negative; 15 were positive at a 1:2 dilution and 2 at a 1:4 dilution. When 36 thrombin-treated sera containing at least 6.0 μg./ml. of split products were evaluated by the Fi test, 13 were negative (6 with 6.0-12 μg./ml. and 7 with 24 μg./ml. of split products); the remainder were abnormal though positive results were obtained by TRCHIII at a greater dilution in most instances (Table 5).

Apparent false-positive reactions were obtained with the Fi test. Anomalous
Table 5.—Comparison of Results with TRCHII, Fi Test, and Precipitin Test on Thrombin-Treated Abnormal Sera

<table>
<thead>
<tr>
<th>TRCHII Split Products (μg./ml., approx.)</th>
<th>Fi Test Serum Dilution</th>
<th>Precipitin (mm.) (Uncentrifuged)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 2 4 8 16</td>
<td>0.5 1.0 1.5 2.0 2.5 3.0 4.0+</td>
</tr>
<tr>
<td>6.0</td>
<td>4 1 1</td>
<td>3 1</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>1 2 1</td>
</tr>
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<td>24</td>
<td>7 3 3 1</td>
<td>2 4 6 2</td>
</tr>
<tr>
<td>48</td>
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<tr>
<td>72</td>
<td>1 1</td>
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</tr>
<tr>
<td>96</td>
<td>1 2</td>
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<td>144</td>
<td>1</td>
<td>1 1</td>
</tr>
<tr>
<td>192</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>288</td>
<td>1 2</td>
<td>1 2</td>
</tr>
<tr>
<td>384</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Zero in Fi test indicates a negative result; other dilutions positive at that dilution. Figures in body of table represent individual serum samples.

Results were encountered in 5 of the 20 patients with hepatic cirrhosis or necrosis and 1 of 4 patients with multiple myeloma. No fibrinogen or split products were demonstrable in the serum of these patients by any other method, yet dilutions of at least 1:16 (and as much as 1:64) were positive with the Fi test. The serum of a seventh patient with only 6.0 μg./ml. of split products shown by TRCHII was diluted 1:10 and still yielded positive results with the Fi test. These false positive reactions are still being investigated.

Precipitin Test (Table 5): Using this in 20 normal serum samples with added thrombin, 16 (measured without centrifugation) were found to have 1.0 mm. of precipitin or less, three had 1.3 mm., and one had 1.5 mm. The 36 serum samples that were abnormal with TRCHII were also evaluated with the precipitin test; normal amount of precipitin were found in 6, with the remainder abnormal. One additional serum sample that was normal with TRCHII was borderline with the precipitin test. In general, there was good correlation between the two tests; the more split products detected by TRCHII, the more precipitin present; borderline values and precise quantitation, however, were more difficult to evaluate with the precipitin test.

Immunodiffusion in Agar (Ouchterlony Plates): With normal plasma, a precipitin line gave a reaction of identity with purified fibrinogen in agar diffusion plates, but no such line was seen with normal serum. Some human serum samples that gave abnormal results with TRCHII had either one or two lines on the agar plates. In some instances, where there were two lines, the inner line (nearer to the antiserum) was similar to the fibrinogen line and had reaction of identity with it. The outer line was generally less intense and situated at a greater distance from the center well (thus nearer to the well originally containing the serum) (Fig. 1). This line usually curved at either end to merge with both the first line and the fibrinogen line. When a single line was seen (usually with lesser amounts of split products), it occurred at a variable distance from the center well, sometimes at a distance similar to
Fig. 1.—Ouchterlony immunodiffusion in agar gel. Center wells contain anti-fibrinogen serum. Wells 2, 4, 6 contain normal plasma. *Left figure:* Well 1 contains normal serum. Well 3 contains serum (without additional thrombin), and well 5 contains the same serum (with additional thrombin) from a patient with defibrination syndrome. Serum in well 3 contains more than 1000 μg. ml. split products; serum in well 5 contains 768 μg. ml. split products. Both double lines merge with the normal plasma (fibrinogen) lines. No reaction is seen with normal serum. *Right figure:* Wells 1, 3, and 5 contain sera from another patient with defibrination syndrome. Sera in wells 1 and 3 (before heparin therapy) contain 144 μg. ml. split products; serum in well 5 (during heparin therapy) contains 36 μg. ml. Double line is seen opposite wells 1 and 3 and a single line opposite well 5. Inner lines all show reaction of identity with normal plasma.

fibrinogen, but not infrequently more peripherally. It too usually merged with the fibrinogen line.

Immunodiffusion proved to be less sensitive than the TRCHII since, while 43 of 46 sera containing more than 12 μg./ml. split products had one or two abnormal lines on agar, only 5 of 32 with 6–12 μg./ml. split products showed an abnormal pattern. On the other hand immunoelectrophoresis was even less sensitive. Sera containing small amount of split products from patients with defibrination syndrome and fibrinolysis might have no band. Sometimes there was a single band which resembled that seen with normal plasma and fibrinogen. If large quantities of split products were present in serum, as occurred in some examples of defibrination syndrome, the band might be split (Fig. 2) and resembled that seen with plasmin-digested fibrinogen or was even identical to it.14

**Discussion**

In these studies, large amounts of fibrinogen or fibrin split products were demonstrated in the blood of patients with defibrination syndrome, especially in cases of abruptio placentae and disseminated cancer with thrombosis. These products were also detected in the sera of pulmonary embolism and, less frequently, in smaller amounts in venous thrombosis or myocardial infarction, rheumatoid arthritis, during and in the first 2 hours following delivery of a normal infant or a dead fetus, or during cesarean section. In most of these conditions characterized by overt thrombosis, the amount of split products
Fig. 2.—Immunoelectrophoresis in agar gel. Both horizontal troughs contain antifibrinogen serum. The center wells contain: Upper panel: normal plasma in both. Lower panel: (above) normal serum; (below) thrombin-treated serum containing 384 μg. ml. split products from patient with defibrination syndrome. Note: Absence of band with normal serum, and abnormal and split band with a large amount of fibrinolytic split products.

found seemed to be correlated with the amount of intravascular fibrin formed. Since there was usually little or no evidence of systemic lysis (as judged by lysis of fibrin plates and euglobulin clots), the split products were attributed to locally activated extracellular

or intracellular enzymes.

The nature of the incoagulable split products is not clear. They may be fragments of fibrinogen and/or fibrin resulting from proteolytic digestion. One of the two major fragments may be present in lesser amounts since it does not diffuse as far in agar gel as does fibrinogen (Fig. 1). On the other hand, this effect might be due to partially polymerized and/or digested fibrin. One or both of these fragments may represent the fraction of incoagulable fibrinogen in plasma reported by Ratnoff, Sharp et al., and Shainoff and Page.

Large amounts of split products were also found in the blood of patients treated with streptokinase or urokinase. Similar or lesser amounts were noted in spontaneous fibrinolysis, either primary (idiopathic) or associated with hepatic cirrhosis or necrosis.
Blood coagulation was defective (incomplete) in some of these clinical states for various reasons: (1) lack of coagulation factors such as platelets, Factors V and VIII, etc., in the defibrination syndrome; (2) the anticoagulant effect of split products in patients with fibrinolysis; and (3) use of anticoagulant drugs in thrombotic disease. In some cases, residual (uncotted) fibrinogen in the “serum” reacted in the immunologic assays and had to be removed before the presence of split products could be ascertained. The prior addition of thrombin effectively countered this variable, enabling us to use plasma as well as serum test samples. (The results with plasma plus thrombin did not differ essentially from those with serum plus thrombin.) Normal serum with added thrombin usually showed no split products. In 3 of 30 such samples a TRCHII titer of 1.0 was found representing 2.0–5.0 µg./ml. of fibrinogen split products and 3 other sera were difficult to evaluate. Ferreira’s group\textsuperscript{10,11} and Allington\textsuperscript{22} found none; Nilehn and Nilsson\textsuperscript{13} reported 100 µg./ml. (?) in 5 of 28 normal serum samples; and Schwick\textsuperscript{33} found about 300 µg./ml. of residual fibrinogen or split products in normal serum. We have no explanation for these widely divergent findings.

Quantitation of split products in disease states varies considerably (Table 4). The largest amounts (up to about 768 µg./ml.) occur with defibrination and secondary fibrinolysis, but in other apparently comparable examples of defibrination only 12 µg./ml. was detected. In idiopathic (primary) fibrinolysis large amounts also occur, but in our experience this is a rare disease. When large amounts of split products occurred, plasma fibrinogen was usually markedly decreased, but low fibrinogen levels were occasionally encountered with only small amounts of split products. Eight of 21 patients with hepatic cirrhosis or necrosis had no detectable split products, while in the 13 other cases that appeared similar the level sometimes reached 24 µg./ml. Variable and generally smaller amounts of split products were also found in other disorders. Thus, all five patients with pulmonary embolism had considerable amounts, as did women during and following normal or abnormal obstetrical delivery. Small amounts were present in a variable proportion of patients with rheumatoid arthritis, myocardial infarction and postoperative states. This list is incomplete since they are likely to be found in other disease states associated with intravascular coagulation.\textsuperscript{34}

It is thus evident from the data that the incidence of primary and secondary fibrinolysis is relatively high in patients with diseases which are frequently encountered and that fibrinolysis is a common disorder. Changes in the patient's clinical status were associated with variable amounts of split products. In a patient with defibrination syndrome who improved clinically with heparin therapy, for example, the split products diminished as the plasma fibrinogen level rose (Fig. 3).

The presence of split products was primarily important to establish the diagnosis of occult fibrinolysis; determination of the actual concentration was mainly useful in assessment of clinical progress.

Our precipitin test differs from that of Ferreira and Murat,\textsuperscript{10} which relies on an immediate precipitin reaction. We observed the reaction over 18 hours and
measured the precipitate before and after centrifugation. When testing normal plasma or thrombin-treated plasma or serum containing considerable amounts of reacting material, flocculation was visible within one or two minutes. Lesser amounts of reacting material might require an hour or two, or even 6–24 hours. Quantitation was not very precise but grossly abnormal sera could be recognized without difficulty; identification of borderline abnormalities was more difficult.

No reaction was observed with normal serum using standard methods of immunodiffusion and immunoelectrophoresis. Normal plasma demonstrated identity with purified fibrinogen, and dilutions up to 1:32 reacted sufficiently to be identified. With large amounts of split products, a double line was noted on the Ouchterlony plates (Fig. 1) and a split, abnormally positioned band was found with immunoelectrophoresis (Fig. 2). A single band or none at all characterized samples containing small amounts of fibrinogen or split products. The two micromethods, while highly specific, also appeared to lack sensitivity.

Both the Fi test and the TRCHII depend upon precipitation of sensitized particles by corresponding antisera. In the Fi test, latex particles coated with the antibody agglutinate directly in the presence of antigen. Conversely, in the TRCHII, fibrinogen is applied to the red cells which are agglutinated by the antiserum unless the antiserum has been neutralized by prior incubation with the antigen. The highest dilution of the antigen which inhibits the reaction
contains the previously established concentration of antigen. The Fi test is rapid and simple, yielding immediate results; the TRCHII, time-consuming but more specific and much more precise, was also reproducible from day to day with ± 1-2 tube dilutions in most instances.

Specificity and sensitivity of the TRCHII for fibrinogen was shown by sensitization of the red cells by only 0.5 μg./ml. of the protein, although this could not be accomplished by dilute serum. The sensitized cells could be agglutinated by a 1:200,000 dilution of antiserum. Their agglutination by even more concentrated antiserum was inhibited by less than 5.0 μg./ml. of fibrinogen but not by whole serum. The specificity of the test was further demonstrated by the loss of reactivity of recalcified plasma as the thrombin-clottable fibrinogen disappeared, while plasmin-digested fibrinogen remained reactive even when no longer clottable by thrombin.

The TRCHII, the most specific and sensitive assay we have used to detect fibrinogen and its split products, often yields positive results where other technics yield negative or equivocal results. The disadvantages are that it is somewhat tedious and requires very careful attention to detail or the results cannot be reproduced. The precipitin test with suitable antiserum is very useful to detect split products but quantitation is not precise. The Fi test shares with the precipitin test the advantages of speed and simplicity and is commercially available; it is also only semiquantitative and may give negative results in the presence of split products or even false positive results. In our experience the gel diffusion methods with or without electrophoresis are specific but time-consuming, lack sensitivity, and are poorly quantitative. The shape and appearance of the bands, however, may provide additional information not otherwise available.

A bedside determination of thrombin-treated blood, plasma, or serum can be obtained within 15 or 20 minutes by the Fi or precipitin test. In the presence of large amounts of split products, a positive result may be obtained immediately by either method. Lesser amounts may be detected immediately by the Fi test or by the precipitin test after several hours' incubation.

**SUMMARY AND CONCLUSIONS**

Split products of fibrinogen and fibrin are found in the sera of patients with defibrination syndrome and/or fibrinolysis. They may result from spontaneous (primary) fibrinolysis or secondary fibrinolysis of intravascular fibrin deposits.

The split products can be detected by several immunologic methods. Both immunodiffusion and immunoelectrophoresis in agar gel show abnormal bands in high-titer pathologic serum samples (usually more than 12 μg./ml.). One of the lines present on immunodiffusion is closer to the point of application than is the other. The position of the closer band might result from the presence of small amounts of fibrinogen-sized molecules or from moderate amounts of partially polymerized or digested fibrin. A precipitin test in a capillary tube offers a simple and sensitive method for demonstrating split products; immediate precipitin occurs with high-titer products but lesser amounts may require
up to 18 hours incubation. The Fi test, agglutination of antibody-coated latex particles, is simple, rapid, moderately sensitive, and commercially available but sometimes yields false-positive results. The precipitin or Fi test on thrombin-treated blood, plasma, or serum may be positive when split products are present in high titer, can be read immediately, and thus provides a rapid bedside test.

Neither the precipitin nor the Fi test is as sensitive as the tanned red-cell hemagglutination inhibition immunoassay (TRCHIII) for the quantitation of fibrinogen and its split products. This test is sensitive to 2.0–5.0 µg./ml. of fibrinogen or split products and much more reliable than the other methods. For example, 13 of 22 samples with up to 24 µg./ml. of split products yielded negative results with the Fi test and positive results with TRCHIII.

Because defective and incomplete coagulation may coexist with fibrinolysis in these clinical syndromes, an excess of thrombin must be added to remove thrombin-clottable fibrinogen and establish the presence of nonclottable split products.

It was necessary to demonstrate split products to diagnose occult fibrinolysis; thrombin-treated normal serum was found to contain up to 2.0–5.0 µg./ml. of split products.

Up to 768 µg./ml. split products were detected in serum from patients with reduced fibrinogen with associated primary fibrinolysis (idiopathic, hepatic disease), induced fibrinolysis (streptokinase, urokinase) or in defibrination syndrome with secondary fibrinolysis (metastatic cancer, abruptio placentae, diffuse allergic vasculitis). In other patients with secondary fibrinolysis, up to 96 µg./ml. were occasionally encountered during and following obstetrical delivery of normal or dead fetus, in pulmonary embolism, myocardial infarction, and rheumatoid arthritis.

The actual quantity of split products was of greatest value in assessing clinical progress. Heparin therapy in patients with defibrination syndrome, for example, was associated with a rise in plasma fibrinogen and a fall in the concentration of split products. The data indicate that trace amounts of fibrinolytic split products may occur in normal serum. Larger amounts are found both in primary and secondary fibrinolysis, which are relatively common disorders.

**Sommario in Interlingua**

Productos fisso de fibrinogeno e de fibrina es incontrate in le seros de patientes con syndrome de disfibrination e/o con fibrinolyse. Ille productos pote resultar ab un spontanee (o primari) fibrinolyse o ab le lyse de depositos intravasular de fibrina.

Le productos fisses pote esser detegite per plure methodos immunologic. Tanto immunodiffusion como etiam immunoelectrophorese in gel a agar monstra bandas anormal in specimen de sero pathologic a alte titro (usualmente plus que 12 µg/ml). Un del lineas revelate per immunodiffusion es plus proxime al punto de application que le alte. Le position del banda
plus proxime resulta forsan ab le presentia de micro quantitates de moleculas del dimension de fibrinogeno o ab moderate quantitates de fibrina partialmente polymerisate o digerite. Un test a precipitina in un tubo capillari representa un simple e sensibile metodo pro le demonstration de productos fisse. Precipitina immediate occurre con productos a alte titro sed minus grande quantitates require usque a 18 horas de incubation. Le test Fi—consistente in le agglutination de partículas de latex revestite de anticorpo—es simple, rapide, moderatemente sensibile, e comercialmente disponibile, sed a vices illo produce resultatos pseudo-positive. Le test a precipitina o Fi in sanguine, plasma, o sero tractate con thrombina pote esser positive quando productos fisse es presente in alte titros; illo pote esser legite immediatemente, e assi illo provide un rapide test effectuabile al latere del lecto del patiente.

Ni le test a precipitina ni le test Fi es tanto sensibile como le immunoessayo a inhibition de hemagglutination con tannate erythrocytos pro le quantification de fibrinogeno e su productos fisse. Iste test es sensibile usque a inter 2,0 e 5,0 μg/ml de fibrinogeno o productos fisse e es plus fidel que le altere methodos. Per exemplo, 13 de 22 specimens con usque a 24 μg/ml de productos fisse rendeva resultatos negative in le test Fi e resultatos positive in le immunoessayo.

Viste que coagulation defective e incomplete pote coexister con fibrinolyse in le mentionate syndromes clinic, un excesso de thrombina debe esser addite pro eliminar productos fisse.

Il esseva necessari demonstrar productos fisse pro diagnosticar fibrinolyse occulte. Sero normal tractate con thrombina revelava un contento de usque a 2,0 a 5,0 μg/ml de productos fisse.

Usque a 768 μg/ml de productos fisse esseva detegite in sero ab patientes con reducet nivellos de fibrinogeno in association con fibrinolyse primari (idiopathic morbo hepatic), con inducete fibrinolyse (streptokinase, urokinase), o in le syndrome de defibrination con fibrinolyse secundari (cancere metastatic, abruption placental, diffuse vasculitis allergie). In altere patientes con fibrinolyse secundari, usque a 96 μg/ml esseva incontrate occasionalmente durante e post le parturition obstetric de fetos normal o morte, in embolismo pulmonar, in infarcimento myocardial, e in arthritis rheumatoide.

Le precise quantitate de productos fisse esseva del plus grande valor in le observation critic del progresso clinic. Per exemplo, heparina in patientes con le syndrome de defibrination esseva associate con un augmento in le fibrinogeno plasmatic e un declino del nivello de productos fisse. Le datos indica que oligoquantitates de fibrinolytic productos fisse pote occurrer in sero normal. Plus grande quantitates es incontrate in casos tanto de primari como etiam de secundari fibrinolyse, le quales es disordines relativamente commun.

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Quantitative Estimation of Split Products of Fibrinogen in Human Serum, Relation to Diagnosis and Treatment

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