Dysproteinemia Induced in Vitro by Plasmin Digestion of Fibrinogen

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During the proteolytic digestion of fibrinogen by plasmin, increasing amounts of trichloracetic acid (TCA)-soluble, tyrosine-containing products are released. It has also been possible to demonstrate large protein fragments among the products of partial proteolysis of fibrinogen. Holmberg suggested, and Seegers demonstrated by electrophoresis, that fibrinogen was split into two large fragments. Sherry and Alkjaersig showed by two dimensional paper chromatography the appearance of four components after 24 hour digestion of fibrinogen. Similarly Shulman showed electrophoretically that fibrin is broken into four or more different components by reaction with plasmin. Niewiarowski and Kowalski were able to isolate a product of partial proteolysis of fibrinogen and to demonstrate its electrophoretic mobility in the range of gamma globulin. This substance possessed antithrombin activity.

In the present study it is shown that proteolysis of fibrinogen, carried out in a medium of plasma, can bring about changes in the electrophoretic pattern of plasma proteins, which resemble the picture seen in dysproteinemia. These findings could be confirmed by plasmin digestion of I\(^{131}\)-labeled fibrinogen.

Material and Methods

Fibrinogen was prepared by the method of Kekwick et al.; 1.2 to 2.5 per cent solutions in oxalate saline (1.8 per cent NaCl, 0.065 per cent sodium oxalate) were used in the experiments.

Plasminogen was prepared by the method of Kline. Plasmin was in the form of spontaneously active Kline preparation. Streptokinase (SK) was purchased from the Warsaw Serum and Vaccine Factory. Thrombin was prepared after separation of prothrombin by the method of Lewis and Ferguson, slightly modified.

Blood was collected by venepuncture from healthy blood donors into 0.1 M ammonium oxalate (1 part of anticoagulant to 9 parts of blood); plasma was obtained by centrifuging for 10 minutes at 1500 rpm.

Fibrinogen-enriched plasma was obtained by addition of fibrinogen solution to oxalated plasma in the proportion of 1 part of fibrinogen solution to 3 parts of plasma.

Digestion of fibrinogen and fibrinogen-enriched plasma was performed on a water-bath at 37 C. in the system composed of 1 ml. of plasma or fibrinogen, 0.1 ml. plasminogen and 0.1 ml.SK. In some of the experiments, the fibrinogen was coagulated with thrombin and the products of fibrinolysis examined. Samples, 0.1 ml. each, were removed from the incubation mixture at various time intervals, cooled in ice and subjected to paper electrophoresis.

Paper electrophoresis was performed in a ridge-pole type apparatus, using Veronal buffer (pH 8.6 µ - 0.05, 15 hours at 0.2 m A/cm per run).

Electrophoretograms were developed on Whatman no. 1 paper strips of 30 mm. width
and 230 mm. length, treated with 1 per cent aqueous solution of sodium citrate as recommended by Hirsch and Cattaneo.3 Paper strips were stained with Bromphenol blue, washed with 4 per cent Na₂CO₃ in methanol and evaluated planimetrically.

Labeling of Fibrinogen and Properties of I¹³¹-Fibrinogen

Fibrinogen was labeled with I¹³¹ iodination by a slight modification of the method of Shulman and Tagnon.12 To 100 μL. Na¹³¹ in a volume of about 50 μL. was added 30 μL. 0.002 M NaI, 30μL. 0.02 M NaNO₂ and 20 μL. 0.5 M HCl. The total volume of the mixture was brought to 750 μL. with 1/15 M phosphate buffer, pH 7.6. To this mixture was added 750 μL. of a 2.1 per cent solution of human fibrinogen. After one hour the mixture was dialyzed against 0.01 M phosphate in physiologic saline, pH 7.6, containing 0.1 per cent ml. sodium citrate. The labeled fibrinogen preparations contained 23.6 per cent of initial radioactivity, and 1 atom I¹³¹ per 35,000 molecules.* The specific activity of the fibrinogen preparations was expressed in terms of μC. per Gm. protein. The labeled fibrinogen solutions were clear, possessed a single radioelectrophoretic peak, and showed the following coagulation and fibrinolysis times, compared with the starting unlabeled fibrinogen.

Coagulation time of unlabeled fibrinogen 30 sec.
Coagulation time of labeled fibrinogen 45 sec.
Lysis time of unlabeled fibrin clots 2.5 min.
Lysis time of labeled fibrin clots 3 min.

Lysis times of fibrin clots were estimated in the following incubation mixture: 100 μL. fibrinogen, 100 μL. plasma; 4 vol. suitably diluted plasmin; and 1 vol. thrombin. Clottable protein in the labeled preparations was not estimated.

Fibrinolysis Assay with Labeled Fibrinogen

For purposes of comparison of the results of various experiments, the preparations employed had to be standardized. A 30 minute lysis time of the formed fibrin clots was arbitrarily chosen for the incubation mixtures to be examined. This was done by adequate dilution of the plasmin preparations.

The incubation mixtures for standardizing lysis time were: (I) 4 vol. labeled fibrinogen; 4 vol. plasma; 4 vol. suitably diluted plasmin; and 1 vol. thrombin; (II) 4 vol. labeled fibrinogen; 0.4 vol. adequate diluted plasmin; and 1 vol. thrombin.

The so-called 30 minute plasmin preparations seemed weak enough to prevent further digestion in the course of electrophoresis.

The mixtures to be tested by electrophoresis were obtained by incubation of fibrinogen with 30 minute plasma preparations at 37 C. for 30 minutes, 1, 2 and 4 hours. After each time interval 20 μL. samples were submitted to paper electrophoresis. Simultaneously samples were precipitated with TCA 7.5 per cent final concentration or sodium tungstate. The activities of the supernatants of these precipitates in 30 μL. volumes were estimated with the same counter geometry as the radioelectrophoretograms.

Radioelectrophoretograms and other radioactive samples were taken with the use of an apparatus described previously1 through a slit of 2 mm. width.

RESULTS

Digestion of fibrinogen.—Figures 1A and 1B illustrate the results obtained with two different fibrinogen preparations, after digestion with SK-activated plasminogen at various times. It can be seen that high molecular weight, split products of different electrophoretic mobilities are obtained. The relative concentrations of the split products depend on the time of incubation and on the strength of the plasminogen preparations.

*And 1 atom of carrier iodine per 3 molecules fibrinogen.
Digestion of fibrinogen-enriched plasma.—For more accurate electrophoretic identification of the split products of fibrinogen or fibrin digestion, the incubation was performed in a plasma medium. The results can be seen in figures 2A and 2B. Accompanying the decrease of the fibrinogen peak there can be observed an increase of the alpha, beta and gamma globulin fractions. An increase of the number of fractions which could be differentiated by electrophoresis was evident in some experiments.

Digestion of labeled fibrinogen.—Figure 3 represents a typical experiment concerning digestion of $^{131}$I-labeled fibrinogen. It can be seen that in the course of the digestion the activity of the starting material, represented by a steeply rising curve with a single high peak, is changed into a broad and low curve with splitting into two or three peaks. (The "stepping" of the curve can be interpreted as due to adsorption of fibrinogen on the filter paper at the starting point.) The newly formed peaks possess electrophoretic
mobilities nearer the gamma and beta globulin range. In this experiment increase of radioactivity in the alpha globulin range can be observed. Some of the activity is dispersed in the region of mobility faster than that of the albumin fraction.
Digestion of labeled fibrinogen by plasmin in plasma medium. Abscissa, distance on electrophoretogram; ordinate, activity in cpm; - - - - - , control; - - - - , ½ hr. incubation time; - - - - - , 1 hr. incubation time; - - - - - , 2 hr. incubation time; - - - - - - - , 3½ hr. incubation time.

Digestion of labeled, fibrinogen-enriched plasma.—For more accurate interpretation of the electrophoretic localization of the newly formed fractions, the digestion was performed in a plasma medium. The plasma medium corresponds more adequately to the biologic conditions in which proteolysis
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Fig. 5.—Increase of TCA-soluble (○) and tungstate-soluble (△) activities during digestion. Abscissa, incubation time (hr.); ordinate, per cent of released activity.

of fibrinogen occurs in the organism. The existence of a potent plasmin inhibitor in serum may influence the dynamics of fibrinogen digestion. Figure 4 represents the distribution of activities of the split products. Fibrinogen is digested, leaving a “core” with the same electrophoresis mobility as the undigested fibrinogen. Radioactivity appears in the range of alpha, beta and gamma globulins. Gamma globulin radioactivity seems to be the highest at the beginning of digestion and decreases in the course of digestion, while the alpha and beta activities increase.

Simultaneously with the above two radioelectrophoretic experiments, TCA- and tungstate-soluble radioactivities were determined. At various intervals TCA and tungstate were added to the reaction mixture, and the activities of the supernatant counted. The results of these determinations are shown in figure 5. It can be seen that from the beginning of the digestion, TCA-soluble-activity is released, whereas the tungstate-soluble activity is negligible.

For comparison of the relative amounts of the various digestion products formed in the course of proteolysis, the radioactive balance (tables 1 and 2) is presented.

The radioactive balance permits the conclusion that about 45 per cent of the initial fibrinogen activity is left as the “core,” about 10 per cent after 1 to 2 hour digestion is changed into “gamma globulin,” from 8.8 to 16.4 per cent is changed into “beta globulin,” relatively small amounts are changed into “alpha globulins” and faster-moving products. The amounts of TCA-soluble substances are relatively high.

For better understanding of the mutual interrelationships of the newly formed fractions figures 6 and 7 are presented. The radioactivities of the areas in figure 3 and 4, respectively are plotted as differences of increases or decreases, as compared with the initial area, representing undigested fibrinogen.
TABLE 1.—Radioactive Balance of Fibrinogen Digestion

<table>
<thead>
<tr>
<th>Split products</th>
<th>Incubation time</th>
<th>Control Fibrinogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>½ h</td>
<td>1 h</td>
</tr>
<tr>
<td>&quot;core&quot;</td>
<td>10678</td>
<td>10939</td>
</tr>
<tr>
<td>gamma</td>
<td>4391</td>
<td>4770</td>
</tr>
<tr>
<td>beta</td>
<td>305</td>
<td>678</td>
</tr>
<tr>
<td>alpha</td>
<td>106</td>
<td>246</td>
</tr>
<tr>
<td>faster products</td>
<td>—</td>
<td>18</td>
</tr>
<tr>
<td>TCA-soluble</td>
<td>2361</td>
<td>2577</td>
</tr>
<tr>
<td>Sum</td>
<td>17841</td>
<td>19228</td>
</tr>
<tr>
<td>% deviation</td>
<td>-11.2</td>
<td>-3.7</td>
</tr>
</tbody>
</table>

19,975 cpm fibrinogen was used for the experiment. During digestion of fibrinogen at various time intervals the activities of split products have been estimated and expressed as cpm.

TABLE 2.—Radioactive Balance of Fibrinogen Digestion in Plasma Medium

<table>
<thead>
<tr>
<th>Split products</th>
<th>Incubation time</th>
<th>Control fibrinogen in plasma without plasmin incubation at 37 °C for 2 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>½ h</td>
<td>1 h</td>
</tr>
<tr>
<td>alpha (core)</td>
<td>2050</td>
<td>1695</td>
</tr>
<tr>
<td>gamma</td>
<td>1678</td>
<td>1488</td>
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<tr>
<td>beta</td>
<td>737</td>
<td>866</td>
</tr>
<tr>
<td>alpha</td>
<td>94</td>
<td>119</td>
</tr>
<tr>
<td>faster products</td>
<td>74</td>
<td>62</td>
</tr>
<tr>
<td>TCA-soluble</td>
<td>806</td>
<td>850</td>
</tr>
<tr>
<td>Sum</td>
<td>5439</td>
<td>5080</td>
</tr>
<tr>
<td>% deviation</td>
<td>-2.8</td>
<td>-9.2</td>
</tr>
</tbody>
</table>

5594 cpm fibrinogen was used for the experiment. During digestion at various time intervals the activities of split products have been estimated and expressed as cpm.

From these figures it can be seen that gamma globulin activity is formed at the beginning of the digestion. In the course of the digestion, gamma globulin activity seems to be found in the beta globulin and TCA-soluble fraction.

In the curve representing digestion of pure fibrinogen, the degradation of fibrinogen occurs suddenly; in the plasma medium this process is slower.

DISCUSSION

From the experiments performed with unlabeled fibrinogen it is possible to conclude that products of fibrinogen digestion by plasmin in vitro possess the electrophoretic mobilities of other plasma proteins.

The results obtained with iodinated fibrinogen are in accordance with the above findings. The procedure is, however, considerably more accurate and convenient.

As a result of digestion of iodinated fibrinogen by plasmin, TCA-soluble, I\(^{131}\)-containing, low molecular products are continually released. At the same time high molecular products with electrophoretic mobilities of alpha, beta and gamma globulins are formed as well as albumin-like and faster-moving substances.

TCA-soluble activity can be released directly from the fibrinogen molecule.
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Fig. 6.—Mutual interrelationships of the newly formed fractions after fibrinogen digestion. The radioactivities of the split products are plotted as differences of increases or decreases compared with the starting fibrinogen. Abscissa, incubation time (hr.); ordinate, activity in cpm.

It is also possible that plasmin digestion of the intermediates can release TCA-soluble radioactivity. This possibility has been proved by Niewiarowski and Kowalski, who showed that the antithrombin derived from fibrinogen, which possesses the electrophoretic mobility of gamma globulin, can be further digested by plasmin.

The results obtained when labeled fibrinogen is digested in the plasma medium, in the presence of alpha, beta and gamma globulins, could of course be equally well explained on the basis of an adsorption of split products on the globulins, which would then act as carriers of the split product activity, conferring on them the observed electrophoretic mobilities. Nevertheless, this explanation seems unlikely, since fragments of similar mobilities are found when digestion is allowed to proceed in the absence of globulins with pure fibrinogen.

The electrophoretic pattern of plasma proteins obtained in our experiments are similar to those seen in patients with dysproteinemia.

On the basis of these findings, it might be suggested that in states of protracted enhanced fibrinolysis with compensatory increased production of fibrinogen, digestion of fibrinogen or fibrin may be the mechanism for the origin and development of dysproteinemia in some pathologic states. If the speed of digestion of intermediary products is slower than the digestion of fibrinogen, these products may accumulate and give the dysproteinemia picture. In many pathologic conditions with dysproteinemia an enhanced fibrinolysis has been described, e.g., in cirrhosis of the liver. It is interesting to note that the increased globulin peak observed in this and other diseases
is "broad," with ill defined margins, in contrast with the globulin peaks in myeloma.

In our experiments we have often observed a quick changing globulin pattern, depending on the speed of fibrinogen digestion. By comparison of successive radioelectrophoretograms it may be concluded that there exists a possibility of mutual interconversion of the high molecular products.

The remaining "core" after digestion of the fibrinogen molecule shows an electrophoretic mobility which is indistinguishable from that of fibrinogen. This occurs at the time when clottable fibrinogen cannot be demonstrated. It may correspond to the so-called "peak," described by electrophoretic investigators.

However, further work, performed in vivo, is needed for a fuller elucidation of these problems.

**Summary**

1. Electrophoretic investigation of split products of plasmin digestion of pure fibrinogen and of fibrinogen in a plasma medium was performed. It was shown that split products possessed electrophoretic mobilities of alpha, beta and gamma globulins.

2. It could be shown that by digestion of 1\textsuperscript{31}I-labeled fibrinogen, radioactivity in the range of alpha, beta and gamma globulins is released.

3. Simultaneously with the formation of high molecular intermediates, low molecular, TCA-soluble substances are released.

4. Radioactivity balance calculation of the relative amounts of the newly formed fractions has been performed.

5. A possible influence of plasmin-induced fibrinogen proteolysis on the electrophoretic pattern of plasma proteins in dysproteinemia has been suggested.
6. The electrophoretic pattern of plasma proteins, obtained in the above experiments is similar to those seen in patients with dysproteinemia.

SUMMARIO IN INTERLINGUA

1. Esseva effectuate investigationes electrophoretic del productos de fission de fibrinogeno pur a fibrinogeno in un medio de plasma, resultante del digestion proteolytic per plasmina. Esseva monstrate que le productos de fission possede mobilitates electrophoretic de globulina alpha, globulina beta, e globulina gamma.

2. Il esseva possibile monstrar que le digestion de fibrinogeno a $^{131}$I resulta in le liberation de radioactivitate in le area de globulina alpha, globulina beta, e globulina gamma.

3. Simultaneemente con le formation de intermediarios a alte peso molecular, substantias a basse peso molecular que es solubile in TCA es etiam liberate.

4. Esseva effectuate calculationes de balancia de radioactivitate pro determinar le quantitates relative del novemente formate fractiones.

5. Es stipulate le possibilitate de un influentia del proteolyse de fibrinogeno que es inducite per plasma super le configuration electrophoretic del proteinas del plasma in dysproteinemia.

6. Le configuration electrophoretic de proteinas de plasma, obtenite in le supra-describebte experimentos, es simile al configurationes correspondente que es vidite in patientes con dysproteinemia.

REFERENCES


Dysproteinemia Induced in Vitro by Plasmin Digestion of Fibrinogen

E. KOWALSKI, A. BUDZYNSKI, M. KOPEC and K. MURAWSKI