Fibrinogen Bethesda: a Congenital Dysfibrinogenemia with Delayed Fibrinopeptide Release

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ABSTRACT A dysfibrinogenemia (fibrinogen Bethesda) was detected in a 9 yr old male of Mexican-English extraction who had a lifelong history of mild bleeding diathesis. The prothrombin and partial thromboplastin times were moderately prolonged; the thrombin and Reptilase times were markedly prolonged. The plasma fibrinogen level was normal by conventional methods but was markedly reduced by the Clauss method. Results of all other tests for clotting factors, fibrinolysis, antithrombin levels, clot stabilization, and fibrin(ogen) degradation products were normal. The patient's plasma and fibrinogen inhibited the clotting of normal plasma or fibrinogen by thrombin. Family studies revealed that the propositus' mother and two siblings exhibited these abnormalities to a lesser degree and indicated an autosomal dominant inheritance. Fibrinogen Bethesda was similar to normal fibrinogen in the following respects: metabolic turnover time (measured in the propositus' mother); immunodiffusion, ultracentrifugal, electrophoretic (on cellulose acetate or polyacrylamide gel), and chromatographic (on DEAE-cellulose) characteristics; sialic acid content; and aggregation of fibrin monomers. By contrast, fibrinogen Bethesda gave an abnormal immunoelectrophoretic pattern especially when whole plasma (as opposed to purified fibrinogen) was examined, and it showed a pronounced decrease in the rate of fibrinopeptide release by thrombin. This decrease, which was shown to involve both fibrinopeptides A and B, distinguishes fibrinogen Bethesda from previously reported dysfibrinogenemias.

INTRODUCTION

Congenital abnormalities related to the function of fibrinogen have been reported in detail in eight families (1–9). These abnormal fibrinogens have usually been detected by the finding(s) of a prolonged prothrombin time and/or thrombin time although the concentration of fibrinogen and other coagulation factors have been normal. The inheritance of these defects is consistent with an autosomal dominant pattern.

The functional defect in three fibrinogens has been related to abnormal aggregation of fibrin monomers. Immunoelectrophoresis of these three fibrinogens seems to establish them as three separate entities although the actual site of the structural defect has been reported in only one.

The present report describes a family of Mexican-English descent with a congenital qualitative defect of fibrinogen (10). The fibrinogen abnormality was first noticed in a 9 yr old male who had a prolonged prothrombin time. Family studies revealed an autosomal dominant pattern of inheritance. The functional defect was localized to impaired fibrinogen-thrombin interaction with a delayed release of fibrinopeptides. This type of functional abnormality distinguishes this dysfibrinogenemia, fibrinogen Bethesda, from other reported dysfibrinogenemias.

METHODS

Unfractionated plasma. Blood from family members (family H) and normal individuals was collected in 1/100 volume of 40% sodium citrate or 1/50 volume of 20% potassium oxalate. Plasma was separated after centrifugation at 4000 rpm (3000 *g* at tip) at 4°C for 15 min. All coagulation studies were performed immediately or the sample was frozen at −30°C and tested within 24 hr.

Preparation of fibrinogen. Blood from normal individuals and members of family H was collected in acid citrate dextrose solution or 4% sodium citrate. Plasma was separated by centrifuging twice at 3000 rpm for 15 min, and then recentrifuged after dilution with an equal volume of 0.09 M sodium citrate. Protein was precipitated by adding saturated $(NH_4)_2SO_4$ (pH 5.5) to 25% saturation at room temperature. After 70 min the precipitate was harvested by centrifugation at 4°C. It was washed three times with 1 M

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$(NH_4)_2SO_4$, each time in a volume approximately equal to twice the original plasma volume. After the third wash the precipitate was dissolved in 0.005 M sodium citrate and reprecipitated with $(NH_4)_2SO_4$ (25% saturation). This precipitate was washed once in 1 M $(NH_4)_2SO_4$ and redissolved in 0.005 M sodium citrate. A portion was tested for clottable protein, and the rest was frozen at −60°C until used. The clottability varied between 90 and 96%.

Another sample of normal fibrinogen, prepared from a large donor pool, was used after it had been shown to possess the same characteristics as normal fibrinogen obtained by $(NH_4)_2SO_4$ fractionation. This was Blombäck fraction I-2 (11) which had been reprecipitated three times in the presence of epsilon aminocaproic acid to remove plasminogen (12); it was at least 92% clottable.

Thrombin. The human thrombin was prepared by Dr. D. L. Aronson, Division of Biologics Standards. It was kept frozen at a concentration of 40 U.S. units/ml in Tris chloride (0.02 mole/liter)–NaCl (0.15 mole/liter) buffer, pH 7.4. Bovine thrombin[1] was dissolved in 50% (v/v) glycerol in normal saline. Dilutions of thrombin were made with normal saline.

Coagulation studies. Coagulation and fibrinolytic studies of the propositus and his family were performed by the following techniques: Ivy, Nelson, and Bucher method (13) for bleeding time, tourniquet test, Lee-White clotting time (14), platelet count by the method of Bull, Schneiderman, and Brecher (15), the prothrombin time by the method of Quick (16) utilizing rabbit brain thromboplastin,[2] and the partial thromboplastin time by the method of Langdell, Wagner, and Brinkhous (17) using Celite[3] as the activator. The recalcification time was performed at 37°C on one part citrated plasma to which one part 0.85% NaCl was added, followed by one part 0.025 M $CaCl_2$. The thrombin time was performed by the method of Peden and McFarland (18). In some experiments 0.025 M $CaCl_2$ or 0.1% toluidine blue was substituted for the distilled water. The thrombin time was measured with concentrations of bovine thrombin from 1 U/ml to 100 U/ml with and without 0.025 M $CaCl_2$. Reptilase, venom of *Bothrops atrox,* was obtained in powder form as a gift from Pentapharm Ltd., Basel, Switzerland. It was dissolved in distilled water and used at a concentration of 20 μg of venom/ml. Factor V was assayed by the method of Stefanini (19), Factors VII–X by the method of Owren and Aas (20), and Factors VIII, IX, and XI by a modification of the method of Simone, Vanderheiden, and Abildgaard (21). Factor XIII was measured by the method of Losowsky, Hall, and Goldie (22). Fibrinogen was determined by the following four methods: (*a*) gravimetric, (*b*) tyrosine determination on the fibrin clot (23), (*c*) immunodiffusion by the method of Feinberg (24), and (*d*) by the method of Clauss (25).

The euglobulin clot lysis time was performed by the Milstone method (26). The plasminogen assay was modified from the method of Alkjaersig, Fletcher, and Sherry (27); alpha casein[4] was used as the substrate. Fibrin(ogen) degradation products were measured by tanned sheep red cell hemagglutination inhibition (28) and by immunodiffusion (29). Thrombin generation tests were performed using 0.6 ml of oxalate plasma diluted 1:20 in Michaelis' buffer, pH 7.40, added to rabbit brain extract and 0.025 M $CaCl_2$. At intervals portions were removed and added to normal human fibrinogen, and the clotting times were observed. Antithrombin activity was measured using intact plasma (30).

Unless otherwise stated, clottability of purified fibrinogen preparations was determined by the following procedure. Fibrinogen was diluted in 0.005 M sodium citrate, and the absorbance at 280 nm was measured. It was clotted with bovine thrombin (final concentration, 2.5 U/ml) for 2 hr at room temperature whereupon the clot was removed, and the A_{280} of the supernate was measured as was the A_{280} of an appropriate thrombin blank. Clottability was expressed as per cent of total protein.

Fibrin monomer aggregation was studied with four different techniques. One was a modification of the Ferry-Morrison technique (31). For this, whole blood was collected in EDTA. The separated plasma was diluted 1:5 in 0.85% NaCl containing Trasylol[5] (250 U/ml of plasma) and clotted at 37°C for 30 min with bovine thrombin (10 U/ml of diluted plasma). The mixture was then incubated at 4°C overnight. The clot was washed three times at room temperature with 0.85% NaCl, dissolved in 5 M urea, and then dialyzed at 4°C for 72 hr against Michaelis' buffer (pH 4.6, ionic strength 0.1). After dialysis the fibrin monomers were diluted to a concentration of 0.5 mg/ml. To the diluted monomer solution was added 0.7 ml of H_2O and enough alkaline Michaelis' buffer (pH 9.6) to bring the final pH to 6.4. This procedure was performed at final ionic strengths of 0.05, 0.1, 0.2, and 0.3. Fibrin monomers prepared from purified fibrinogen were also tested in this system.

The second method used was based on the technique of Belitser, Varetskaja, and Malneva (32, 33). Fibrinogen in 0.3 M NaCl was diluted with 0.06 M potassium phosphate buffer, pH 6.8, to give a final concentration of 1 mg/ml and was treated with human thrombin. The clot was wound on a glass rod and dissolved in 0.02 M acetic acid. Reaggregation was initiated by diluting a portion of this solution with a large (11- to 31-fold) excess of 0.06 M potassium phosphate buffer, final pH 6.7.

In the third method, the fibrinogen in 0.3 M NaCl was diluted with 0.3 M Tris chloride buffer, pH 7.4, before clotting, and the clot was dissolved in 0.3 M sodium acetate buffer, pH 4.2. Reaggregation was brought about by diluting with 0.06 M potassium phosphate buffer (pH 6.8), final pH 6.2 (with a 16-fold excess) or 6.5 (with a 31-fold excess).

The concentration of fibrin monomer was determined before reaggregation by measuring the A_{280} of a diluted portion. Based on this measurement, the concentration of monomer was adjusted by dilution with the appropriate solvent (e.g. 0.02 M acetic acid) so that aggregation rates of fibrins from different sources could be compared at the same concentration. The progress of reaggregation at 25°C was followed by measuring the A_{350} in rectangular cuvettes of 1 cm light path.

The fourth method of measuring fibrin monomer aggregation was similar to that employed in studying fibrinogen Cleveland (8).

Fibrinopeptide release was investigated by a method patterned after the original procedure of Lorand (34). Fibrinogen solutions were dialyzed against 0.3 M NaCl, and the concentrations were determined by differential refractometry. After dilution to the desired concentration (usually 5 or 10 mg/ml) with 0.3 M NaCl, the pH was adjusted by adding 1/50 volume of 1 M potassium phosphate buffer, pH 6.8. Portions of the resulting solution (0.5 ml each) were placed in 12 × 75 mm test tubes and treated with 0.05 ml of a solution of human thrombin. At timed intervals, 0.5 ml of

[1] Parke, Davis & Co., Detroit, Mich.
[2] Ortho Pharmaceutical Corp., Raritan, N. J.
[3] Warner-Chilcott Laboratories, Morris Plains, N. J.
[4] Worthington Biochemical Corp., Freehold, N. J.
[5] Bayer, A. G., Leverkusen, Germany.

4% (v/v) acetic acid was added to the contents of a tube. As soon as the clot, if any, had dissolved, the mixture was treated with 0.05 ml of 100% (w/v) trichloroacetic acid (TCA). After standing for at least 15 min, the samples were centrifuged, and the clear supernates were taken for analysis. In the case of the zero-time sample, the acetic acid was added before the thrombin solution.

Portions (usually 0.4 ml each) of the TCA supernates were analyzed by the Folin method developed by Lowry, Rosebrough, Farr, and Randall (35) with the following modification. To compensate for the acidity of the samples, the concentration of NaOH in reagent A was doubled. This approach was as effective as prior neutralization, it was more convenient, and it brought about no additional dilution of the samples.

Fibrinopeptides were also determined by the method of Shainoff and Page (36). Two sets of measurements were obtained, one giving the total TCA-soluble arginine in the solutions, and another giving the amounts of each of the fibrinopeptides after electrophoretic separation. For the former, 0.05 ml of the TCA supernate was mixed with 0.4 ml of water, 0.05 ml of 10% KOH, and 0.1 ml of 0.1% 8-hydroxyquinoline (ethanolic) in an ice bath. The mixture was then treated by rapid addition of 0.1 ml of a chilled, freshly prepared solution (0.5%) of *N*-bromosuccinimide (recrystallized from warm acetone). After a carefully timed interval of 15 sec, 0.05 ml of 40% urea was admixed, and the solution was immediately transferred to a microcuvette (1 cm path) for measurement of the absorbance at 520 nm. Appropriate reagent blanks were treated in the same manner. The color generated by the fibrinopeptides was a direct function of their arginine content; the molar absorptivity was 1.89×10^4, the same as free arginine.

Processing of samples (0.2 ml) for electrophoretic separation of the fibrinopeptides involved removal of TCA and salt as described before (36). Each solution was then deposited by drying at a position 5 cm from the cathodal edge of a 2043A mgl filter paper strip. Electrophoresis was performed in a Beckman model R cell with 0.045 M potassium orthoborate at pH 8.6 by applying 350 volts for 5 hr. Strips were dried at 105°C and treated with reagents for Sakaguchi's reaction by the method specified previously (36) for blood and urine. The strips were then scanned with a Joyce, Loebl and Co., Ltd. (Gateshead-on-Tyne, England) Rotoscan equipped with a 500 mm filter. From numerous previous measurements areas under the peaks were known to be proportional to their arginine content. Identification of peptides A, AY, AP, and B were based on known mobilities. A fifth peptide that was consistently found as a minor component (37) proved on the basis of a negative ninhydrin reaction to be a derivative of fibrinopeptide B. Its electrophoretic mobility suggested that it is fibrinopeptide B which is lacking one amide nitrogen. We have therefore designated it peptide BX.

Chemical and physical studies. Sialic acid was measured by the thiobarbituric acid method of Warren (38).

Gradient elution chromatography was carried out at 5°C on a column (2.2 × 33 cm) of DEAE-cellulose prepared by the method of Peterson and Sober (39). The procedure and the concave gradient were those described previously (40).

All spectrophotometric measurements were made in Beckman spectrophotometers. When absorbance at 280 nm was used to compute fibrinogen concentration, the $A^{1\%}_{1cm}$ was taken as 15 (41). When precise protein concentrations were required, differential refractometry was performed on dialyzed samples with a Brice-Phoenix instrument. The final dialysate served as the blank. A specific refractive increment value of 0.188 ml/g was employed (42).

Ultracentrifugal analyses were done at 20°C in a Spinco model E ultracentrifuge. The buffer used was 0.05 mole/liter in Tris, 0.03 mole/liter in acetic acid, and 0.275 mole/liter in NaCl (pH 8.0, ionic strength 0.3).

Plasma and fibrinogen of the members of family H were subjected to immunoelectrophoresis in Ionagar[6] (1.5%) and agarose[7] (1%) at pH 8.2 and at pH 8.6 with 0.05 M barbital buffer by the procedure of Grabar and Williams (43). Double immunodiffusion in agar was performed in both Ionagar and agarose. The antisera used in these experiments were prepared in rabbit and goat.[8]

Intact plasma and purified fibrinogen from family H were subjected to electrophoresis on cellulose acetate[9] with barbital buffer (pH 8.6, ionic strength 0.075) at 250 volts for 1 hr. For comparison normal plasma and fibrinogen were electrophoresed simultaneously with the samples from family H.

Purified fibrinogen from normals and members of family H were electrophoresed on 5% polyacrylamide gels, 8 M urea–5% acrylamide gels, and on 0.1% sodium dodecyl sulfate (SDS)–5% acrylamide gels. The fibrinogen in standard 5% gels and in urea gels was electrophoresed in Tris-glycine buffer at pH 8.4. Samples for electrophoresis in SDS were preincubated in 1% SDS–0.1 M sodium phosphate buffer at pH 7.1 for 3 hr at 37°C and then dialyzed into 0.1% SDS–0.01 M sodium phosphate buffer, pH 7.1, and electrophoresed in the same buffer. All gels were fixed in 20% sulfosalicylic acid and stained with Coomassie blue.

Fibrinogen survival. On separate occasions autologous and homologous fibrinogen were iodinated with ^{125}I by the method of MacFarlane (44) to a ratio of 0.5 mole iodine per mole of fibrinogen. After iodination, excess iodine was removed by precipitation of the fibrinogen with saturated $(NH_4)_2SO_4$ (final concentration, 25% saturated) and repeated washing of the precipitate with 1 M $(NH_4)_2SO_4$ in 0.005 M sodium citrate until the activity of the last wash was less than 0.5% of the activity of the first supernate. The fibrinogen-^{125}I was redissolved in 0.005 M sodium citrate. The final activity was approximately 7 μCi/mg fibrinogen. The iodinated fibrinogen was filtered through Millipore filters[10] (pore size 0.45 μm) before use.

The subject was placed on saturated solution potassium iodide (SSKI) 2 days before and maintained on SSKI throughout the study. A dose of 30 μCi of fibrinogen-^{125}I in 4 or 5 ml of 0.005 M sodium citrate with 1% Normal Serum Albumin (Human) as stabilizing protein was given intravenously. Samples of blood were collected 15 min, 1 hr, 2 hr, 4 hr, 8 hr, and daily thereafter. 24-hr urine collections were made over the duration of the experiment. On each sample the plasma radioactivity, TCA precipitability of the radioactivity, 2- and 24-hr clottabilities, and fibrinogen concentrations were determined. Identical studies were performed on 14 normal individuals for control values. The data were analyzed by a least squares program on a Control Data 3200 computer (45).

Other methods and procedures used are described in the text.

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[6] Colab Labs, Inc., Glenwood, Ill.

[7] Fisher Scientific Co., Pittsburgh, Pa.

[8] Certified Blood Donor Service, Woodbury, N. Y.

[9] Beckman Microzone; Beckman Instruments, Inc., Palo Alto, Calif.

[10] Millipore Corp., Bedford, Mass.

TABLE I

Coagulation Studies in the Propositus with Fibrinogen Bethesda

	Patient	Control
Bleeding time, *min*	4	2–5
Clotting time (glass), *min*	8	6–14
Prothrombin time, *sec*	16.2	11–14
Partial thromboplastin time, *sec*	45.4	30–35
Thrombin time, *sec*	148	<35
Reptilase time, *sec*	106	24
Euglobulin clot lysis, *min*	>180	>180
Plasminogen, *casein units/ml*	2.7	2–4
Platelet count, *platelets/mm³*	355 × 10³	200–400 × 10³
Factors V, VII, VIII, IX, XI	Normal	
Factor XIII (qualitative)	Insoluble clot	Insoluble clot
Fibrinogen, *mg/100 ml*		
Gravimetric	360	200–450
Fibrin tyrosine content	330	200–400
Immunodiffusion	300	250
Thrombin clotting time (Clauss)	36	186–380

Case histories. K. H., a 9 year old, has been in good health since birth except for a lifelong history of bleeding and easy bruisability. Excessive bleeding was noted after circumcision and after minor cuts or abrasions. During early childhood a cardiac murmur was noted, and a diagnosis of coarctation of the aorta was established. The child and his parents have noticed spontaneous ecchymoses over all parts of his body and rare hemarthrosis. In June 1968, cardiac catherization revealed a ventricular septal defect in addition to the coarctation of the aorta. In August 1969, the coarctation of the aorta was surgically corrected. The patient received 2 g of fibrinogen[11] the day before surgery and 4 g during surgery. Blood loss was minimal during the operative and postoperative periods, and the surgical wound healed without any complications.

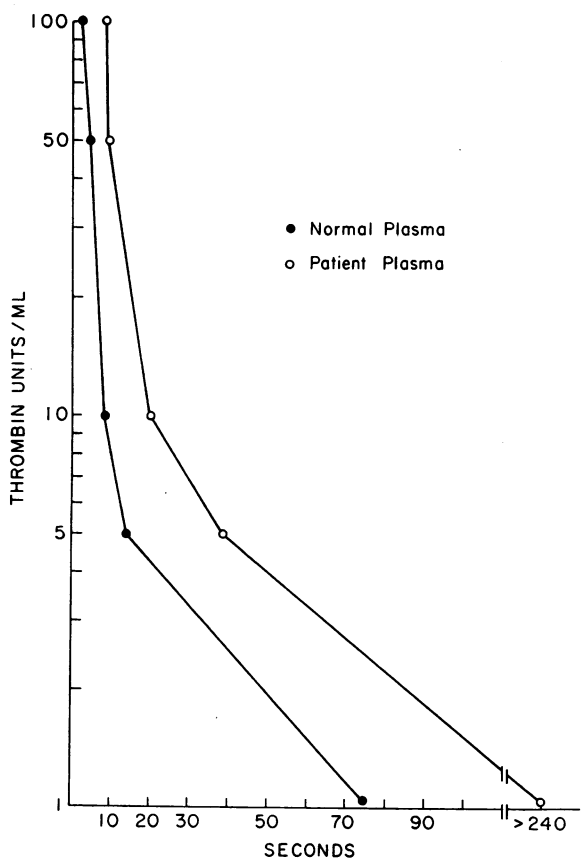

FIGURE 1 Effect of varying thrombin concentration on the clotting time of normal plasma and fibrinogen Bethesda plasma. Bovine thrombin was used at 37°C.

TABLE II

*Thrombin Time of the Propositus' Plasma under Various Conditions**

Addition to plasma	Clotting time	
	Patient	Normal
	sec	
Bovine thrombin	147.5	30.5
Bovine thrombin + $CaCl_2$	70.0	22.5
Bovine thrombin + toluidine blue	76.0	27.0
Human thrombin	14	6

Substances used: 0.2 ml plasma; 0.1 ml distilled water; 0.1 ml thrombin; 0.025 M $CaCl_2$; 0.1% toluidine blue.

* Where $CaCl_2$ or toluidine blue was used, it replaced the distilled water. Final concentrations: bovine thrombin, 0.6 U/ml; human thrombin, 5 U/ml. Temperature: 37°C.

D. H., the 45 yr old mother of the proband, has been in good health with no history of excessive bleeding. She stated that she has bruised easily all of her life. At age 40 she underwent an abdominal hysterectomy without bleeding or transfusion.

R. H., the 19 yr old brother of the proband, has no significant medical history, including an uncomplicated circumcision and tonsillectomy.

F. H., the 21 yr old sister of the proband, is in excellent physical health and has not experienced any unexplained bleeding although she also complained of unexplained ecchymoses.

RESULTS

Only the coagulation tests involving the conversion of fibrinogen to fibrin by thrombin or Reptilase (Table I) yielded abnormal results, namely: marked prolongation of the thrombin time and the Reptilase time, moderate prolongation of the prothrombin time and partial thromboplastin time, and reduced concentration of fibrinogen as measured by the method of Clauss. Addition of toluidine blue to the proband's plasma did not correct the thrombin time, which was prolonged when either bovine or human thrombin was used (Table II). Thrombin and Reptilase times were shortened somewhat by $CaCl_2$, but were still markedly abnormal. Increasing the concentrations of thrombin resulted in a shortening of the proband's thrombin time, but the times given by the ab-

[11] The Cutter Laboratories, Berkeley, Calif.

TABLE III

Family Coagulation Studies: Fibrinogen Bethesda

	Propositus	Mother	Brother	Sister	Father	Normal
Prothrombin time, *sec*	16.2	12.4	13.9	12.6	10.9	11–14
Partial thromboplastin time, *sec*	45.4	30.2	32.4	30.0	34.8	30–35
Thrombin time, *sec*	148	86	90	77	32	<35
Fibrinogen, *mg/100 ml*						
a. Fibrin tyrosine content	330	405	413	418	376	200–400
b. Thrombin clotting time (Clauss)	36	94	95	110	245	186–380
Ratio of b/a, %	11	23	23	26	65	65–120

normal plasma were still prolonged compared with normal plasma (Fig. 1).

The proband's mother, sister, and brother had prolonged thrombin and Reptilase times and low levels of fibrinogen as measured by the Clauss procedure. All other coagulation tests were normal (Table III). The proband's father had normal coagulation values.

Evidence of other coagulation factor deficiencies, platelet abnormalities, or vascular abnormalities was not found. The antithrombin activities and the thrombin generation of the proband's and his mother's plasma were normal. The normal plasminogen level, normal euglobulin clot lysis, and absence of fibrin(ogen) degradation products in the serum excluded a hyperfibrinolytic state.

Immunodiffusion of plasma and fibrinogen from normals and members of family H against anti-human fibrinogen revealed complete lines of identity. Furthermore, immunodiffusion gave no evidence of fibrinogen or fibrinogen related material in serum prepared by allowing blood to clot in glass for 24 hr at 37°C. However, in contrast with serum samples prepared from normal blood, those taken from the propositus' clotted blood after 1, 2, and 4 hr all contained material which was immunologically identical with fibrinogen.

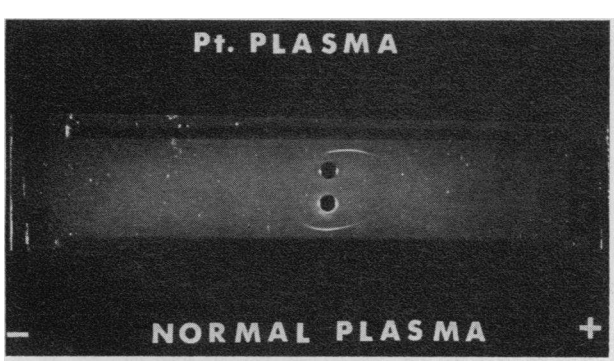

FIGURE 2 Immunoelectrophoretic comparison of normal fibrinogen and fibrinogen Bethesda. Conditions: Ionagar, 1.5%; pH 8.6; antiserum, goat anti-human fibrinogen.

Immunoelectrophoresis of plasma from the propositus and his mother against anti-human fibrinogen resulted in an arc with increased anodal migration compared with that of the arc produced by fibrinogen in normal plasma (Fig. 2). When purified fibrinogen was tested, this abnormality was not as noticeable.

Fibrinogen from family H migrated at the same rate as normal fibrinogen during electrophoresis on cellulose acetate. This was true of the purified fibrinogen or unfractionated plasma. Polyacrylamide gel electrophoresis also revealed no difference in the migration rates of the purified fibrinogens.

The disappearance of homologous and autologous fibrinogen-^{125}I in the proband's mother was normal (Fig. 3). The $t_{\frac{1}{2}}$ of homologous fibrinogen was 3.9 days, and the $t_{\frac{1}{2}}$ for autologous fibrinogen was 3.6 days (the normal value averaged[12] 4.0 ±0.6 days). The fractional catabolic rates with autologous and homologous fibrinogen were normal.

Samples from both the propositus and his mother were tested for their ability to inhibit the clotting of

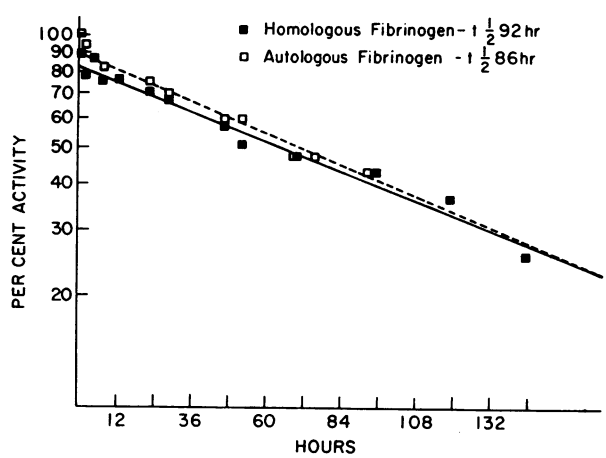

FIGURE 3 Survival of homologous and autologous fibrinogen-^{125}I in the propositus' mother.

[12] All estimates of variance reported are standard deviations $[\Sigma d^2/(N-1)]$.

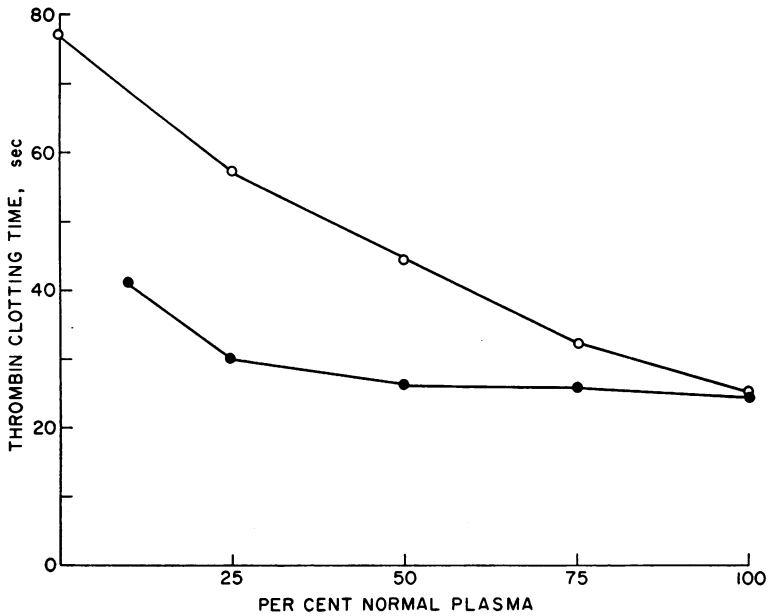

FIGURE 4 Effect of fibrinogen Bethesda on thrombin clotting time of normal fibrinogen. Open symbols represent normal plasma diluted with plasma from the propositus' mother; closed circles, normal plasma diluted with normal saline. The normal plasma used had the same fibrinogen level (fibrin tyrosine content method) as that of the propositus' mother. Bovine thrombin, 2.5 U/ml; temperature, 37°C.

normal fibrinogen. Mixing plasma of members of family H with normal plasma in various ratios resulted in prolongation of the thrombin clotting time in comparison with the clotting times obtained with normal plasma diluted with saline or barbital buffer (Fig. 4). The inhibitory effect was similar when purified fibrinogen was tested. Defibrinated plasma was prepared from members of family H and normals by heating at 56°C for 30 min. The defibrinated plasmas were compared for their ability to prolong the thrombin clotting time of normal plasma. The clotting times were similar at all dilutions.

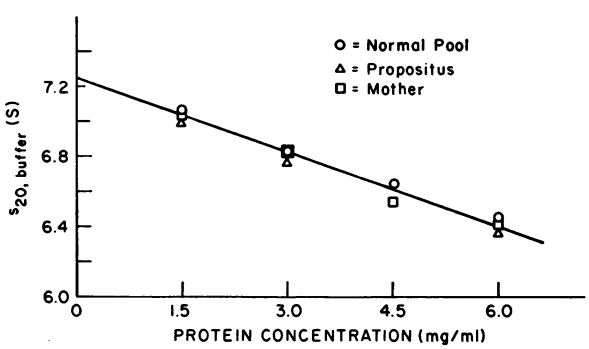

FIGURE 5 Sedimentation coefficient of normal and abnormal fibrinogen as a function of protein concentration. All fibrinogens were prepared by $(NH_4)_2SO_4$ fractionation, dialyzed against Tris acetate–NaCl buffer, analyzed by differential refractometry, and centrifuged at 59,780 rpm.

Analyses for total sialic acid demonstrated that the fibrinogens of the propositus and his mother did not differ from each other or from normal fibrinogen (either pooled or single donor) prepared in the same manner; the values averaged 0.70 ±0.05%. Chromatographically purified normal fibrinogen analyzed simultaneously had a sialic acid content of 0.63 ±0.03%, in good agreement with the figures reported for highly purified human fibrinogen (46, 47), and chromatographically purified fibrinogen from the propositus' mother (see below) had a value of 0.67%.

Ultracentrifugal analyses revealed no differences between the fibrinogens of the propositus, his mother, and normal donors, either with respect to the sedimentation coefficient or its concentration dependence (Fig. 5). The extrapolated sedimentation coefficient ($s^\circ_{20,buffer}$), 7.25S, could be used to compute an $s^\circ_{20,w}$ value of 7.8S. In addition, the apparent diffusion coefficients computed from the schlieren pattern showed no significant differences. In these analyses the relative area under the major schlieren boundary, 94 ±2%, agreed well with the clottability of the samples examined.

The rate of fibrin monomer aggregation of fibrinogen Bethesda was normal. This was true in the case of monomer prepared from either purified fibrinogen or

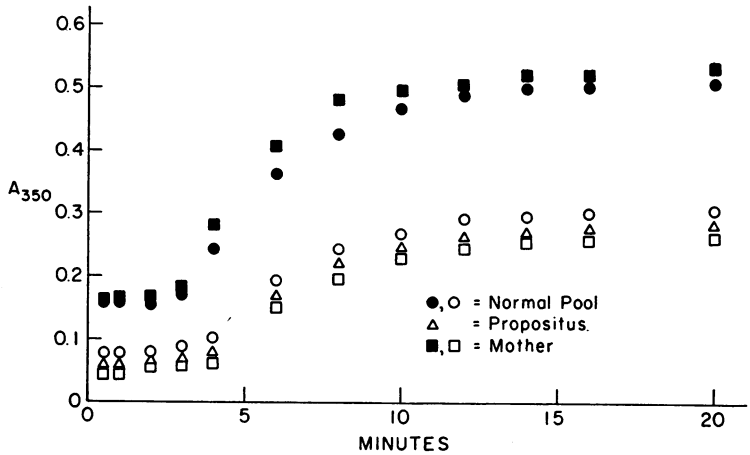

FIGURE 6 Aggregation of fibrin monomers from normal and abnormal fibrinogen. At time zero a solution of fibrin monomer in 0.02 M acetic acid was diluted with a large volume of 0.06 M potassium phosphate buffer. Final pH, 6.7; final ionic strength, 0.12. Final protein concentration: filled symbols, 0.20–0.21 mg/ml; open symbols, 0.13–0.14 mg/ml.

whole plasma, and was demonstrated by each of the methods employed. Typical aggregation patterns are shown in Fig. 6. The duration of the lag period varied with the method, and the steepness of the curve depended on the initial monomer concentration and the final pH; however, the general pattern depicted here was observed under all conditions studied.

Chromatography of fibrinogen from the propositus' mother revealed the major peaks characteristic of normal fibrinogen (48). Both the relative amounts of these chromatographic components and their elution positions agreed closely with those observed for normal fibrinogen (Fig. 7). Two tubes of eluate near the apex of peak 1 were pooled, concentrated by ultrafiltration with a Diaflo[18] membrane, dialyzed, and ultracentrifuged. The single, symmetrical boundary had a sedimentation coefficient consistent with those shown in Fig. 5.

Portions of the chromatographic fractions of fibrinogen Bethesda throughout the elution pattern were dialyzed against 0.05 M Tris phosphate buffer, pH 6.8 (46). After dialysis, 2-ml portions were clotted with 0.8 unit of human thrombin, and clottabilities were determined by a modification of the spectrophotometric method of Laki (49). The elution pattern of clottable protein generally paralleled that of total protein; however, the clottabilities were relatively low even when the clotting was done in the presence of a constant amount of normal fibrinogen (method B of reference 48). In the absence of added normal fibrinogen the clotting times of the chromatographic fractions were 3–4 times as long as those of normal, unchromatographed fibrinogen.

[18] Amicon Corp., Lexington, Mass.

The tube of eluate representing the apex of peak 1 was dialyzed, and several 2-ml portions were clotted. The clot was removed from the first tube after 1.5 hr; clots were removed from the remaining tubes after 4, 7, and 16 hr, respectively. The apparent clottabilities, determined from the A_{280} values of the supernates, were as follows: 77.8%, 88.1%, 92.2%, and 96.7%.

Clotting times of the two major chromatographic peaks of fibrinogen Bethesda were compared with those of the corresponding fractions of normal fibrinogen. In addition, the effect of the thrombin level on the clotting

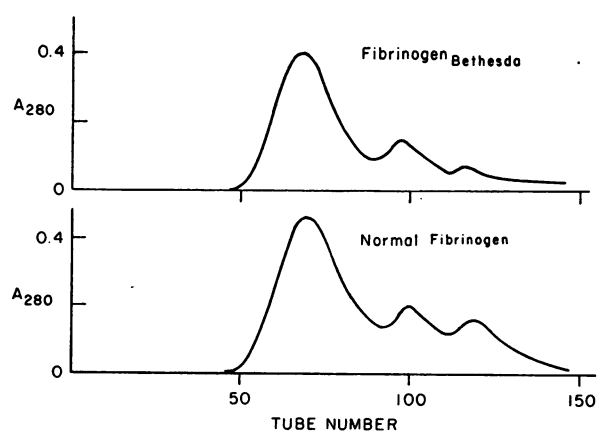

FIGURE 7 Gradient elution chromatography on DEAE-cellulose. The sample of fibrinogen Bethesda (165 mg) was an ammonium sulfate preparation from the propositus' mother; that of normal fibrinogen (200 mg) was Blombäck fraction I-2 from a large pool of donors. A concave gradient from 0.005 M Tris phosphate, pH 8.6, to 0.5 M Tris phosphate, pH 4.2, was employed. Each tube contained 12 ml of eluate.

TABLE IV

Clotting of Chromatographic Fractions at Various Thrombin Concentrations

Chromatographic peak	Fibrinogen	Thrombin concentration	Clotting time	Clottability		
				1 hr	2 hr	3 hr
		U/ml	*sec*		%	
1*	Bethesda	0.4	167	68	82	85
	Bethesda	2.0	67	88	91	95
	Bethesda	4.0	56	92	96	96
	Normal	0.4	74	96		
2*	Bethesda	0.4	251			
	Normal	0.4	108			

* Fibrinogen concentrations: peak 1, 0.27 mg/ml; peak 2, 0.10 mg/ml. Tubes of eluate at or adjacent to the apexes of the respective peaks (Fig. 7) were dialyzed against 0.05 M Tris phosphate, pH 6.8, and 2-ml portions were clotted with human thrombin at 25°C.

time and apparent clottability of peak 1 from fibrinogen Bethesda was determined. The results of these comparisons are presented in Table IV. At a constant thrombin concentration, the ratio of clotting times (fibrinogen Bethesda: normal fibrinogen) was the same for both chromatographic peaks. Raising the thrombin concentration by a factor of 5 brought the clotting time of the abnormal peak 1 slightly below that of the normal, but even under these conditions 3 hr were required for the yield of fibrin from fibrinogen Bethesda peak 1 to approach its maximum value.

This obviously low reactivity to thrombin, coupled with the lack of any demonstrable aggregation defect (Fig. 6), suggested that the clotting abnormality might lie in fibrinopeptide release. Accordingly, the remaining tubes of eluate from peak 1 were pooled, made 33% saturated with $(NH_4)_2SO_4$, and allowed to stand at 5°C for 2 hr. The precipitate was harvested by centrifugation, dissolved, and dialyzed against 0.3 M NaCl. Peak 1 of normal fibrinogen was prepared in a similar manner. Fibrinopeptide release was then measured as described under Methods. The rate of release as determined by the modified Folin-Lowry procedure was strikingly lower in the case of abnormal peak 1 from the propositus' mother though the amount of peptide eventually reached that released from normal peak 1.

Unchromatographed fibrinogen from the propositus, his mother, and normal donors (individual or pooled) were then compared with respect to the rate of fibrinopeptide release (Fig. 8). No differences between the various normal preparations were seen regardless of whether Blombäck fraction I-2 or $(NH_4)_2SO_4$ fractionated fibrinogen from individual or pooled plasma was examined. The difference between normal fibrinogen and fibrinogen Bethesda confirmed that observed in the case of chromatographic peak 1. The rates of fibrinopeptide release from the propositus' and the mother's fibrinogen were similarly depressed (Fig. 8). This defect appeared to be specific for fibrinogen Bethesda; the peptide release curve obtained for the fibrinogen of a patient with dysfibrinogenemia secondary to hepatoma[14] was identical with the curve from normal fibrinogen.

In order to study the release of the individual fibrinopeptides, a fibrinopeptide release experiment, involving fibrinogen from a normal pool and from the propositus' mother, was carried out at a somewhat higher protein

[14] Gralnick, H. R., H. Givelber, and J. S. Finlayson. Unpublished observations.

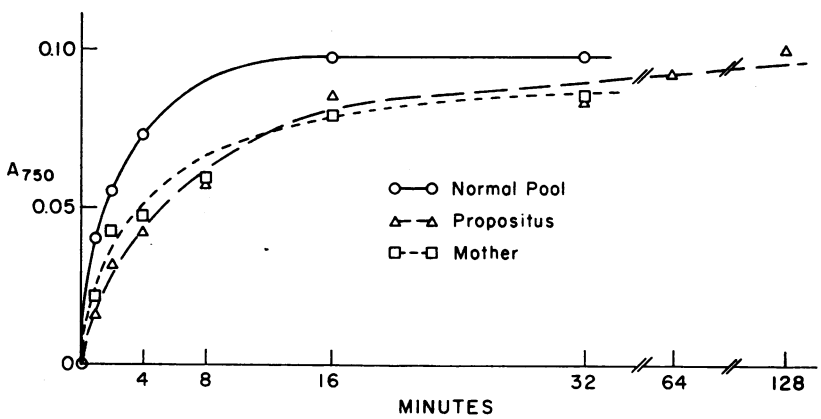

FIGURE 8 Fibrinopeptide release from normal fibrinogen and fibrinogen Bethesda studied by a modified Folin-Lowry method. The abnormal fibrinogens were prepared by $(NH_4)_2SO_4$ fractionation; the normal was Blombäck fraction I-2. Ordinate shows absorbance of the Folin-Lowry chromophore at 750 nm; abscissa shows minutes after treatment with human thrombin. Final concentrations in the clotting mixture: fibrinogen, 4.4 mg/ml; thrombin, 1.8 U/ml. Temperature 25°C.

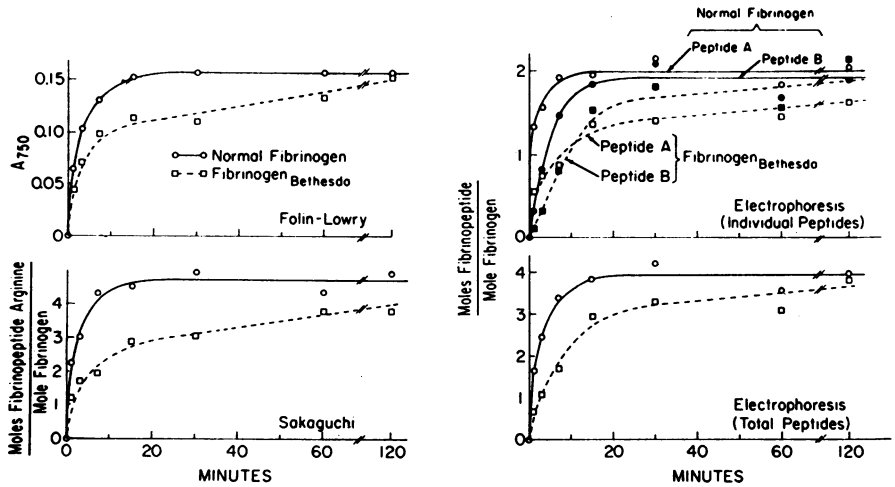

FIGURE 9 Fibrinopeptide release from normal fibrinogen and fibrinogen Bethesda studied by various methods. Upper left, modified Folin-Lowry method; lower left, Sakaguchi method; upper right, sum of fibrinopeptides A (i.e. A+AY+AP), and B (i.e., B+BX) from densitometry after paper electrophoresis; lower right, sum of appropriate values (A+AY+AP+B+BX) from upper right. Ordinates are as indicated (fibrinogen mol wt, 340,000); abscissae are minutes after addition of human thrombin. Fibrinogen Bethesda was an ammonium sulfate preparation from the propositus' mother; normal fibrinogen was Blombäck fraction I-2 from a large pool of donors. Final concentrations in the clotting mixture: fibrinogen, 8.9 mg/ml; thrombin, 3.6 U/ml. Temperature: 25°C. Clotting of normal fibrinogen occurred at 35 sec; clotting of fibrinogen Bethesda, at 105 sec.

concentration. One set of portions from the TCA supernates was analyzed by the Folin-Lowry method, a second set was then analyzed by the Sakaguchi technique, and a third set was subjected to paper electrophoresis to separate the individual fibrinopeptides.

The difference in the rates of total fibrinopeptide release demonstrated by the Folin-Lowry procedure was confirmed by the Sakaguchi method, which yielded values for the actual number of moles released (Fig. 9). After 15–20 min the curve for normal fibrinogen had reached a plateau at slightly more than 4 moles of fibrinopeptide per mole of fibrinogen (based on a fibrinogen mol wt of 340,000); the curve for fibrinogen Bethesda approached this value only after 1 hr (Fig. 9, lower left).

Densitometry of the paper electrophoresis strips permitted computation of values for fibrinopeptides A, AY, AP, B, and BX. Fig. 9 (upper right) shows the A peptides (A + AY + AP) and B peptides (B + BX) released as a function of time; fibrinogen Bethesda exhibited slower release of both the A and the B peptides. There was no difference between fibrinogen Bethesda and normal fibrinogen with respect to the ratios of individual peptides (e.g., A:AY:AP). However, after 10–12 min the ratio of total peptide B to total peptide A appeared slightly greater than 1 (viz. 1.2 ±0.12) in the case of fibrinogen Bethesda whereas it closely approached 1 (0.94 ±0.03) for normal fibrinogen. The quantities of peptide A and peptide B released approached 2 moles per mole of fibrinogen, but fibrinogen Bethesda required more than 1 hr to reach this level. The curves for total peptide release measured by electrophoresis closely resembled those obtained by the other two methods (Fig. 9, lower right).

DISCUSSION

Despite the fact that fibrinogen Bethesda showed an inheritance pattern like that of the other congenital dysfibrinogenemias reported (viz., autosomal dominance) and had certain characteristics common to all of them (e.g. prolonged thrombin time, reaction with anti-human fibrinogen, normal electrophoretic mobility), a number of features served to distinguish it from these other abnormal fibrinogens. Unlike fibrinogen Baltimore (7), its ultracentrifugal behavior was normal (Fig. 4) as was that of fibrinogen Detroit (5), fibrinogen St. Louis (50), and, apparently, fibrinogen Paris I (2). Chromatographic behavior of fibrinogen Bethesda on DEAE-cellulose was also normal (Fig. 7), a respect in which it differed from fibrinogens Baltimore (7) and Paris I (51) but was similar to fibrinogen St. Louis (50) and fibrinogen Oklahoma (52, 53). Fibrinogen Paris II was reported to show normal chromatographic behavior, but recent experiments by Mosesson

as described by Samama, Soria, Soria, and Bousser, have suggested a slight displacement in the elution pattern (9). Fibrinogen Bethesda, like fibrinogen Baltimore (7), had a sialic acid content equal to that of normal fibrinogen. In contrast, fibrinogen Paris II was found to have appreciably more sialic acid than normal (54), whereas fibrinogen Detroit was reported to contain somewhat less (5).

Abnormal fibrinogens have shown various types of behavior on immunoelectrophoresis. Fibrinogen Zurich (6), fibrinogen Paris I (2), fibrinogen Paris II (9), and fibrinogen St. Louis (50) gave a normal immunoelectrophoretic pattern. On the other hand, fibrinogen Detroit (5), fibrinogen Cleveland (8), and fibrinogen Baltimore (55) all showed differences in immunoelectrophoretic mobility from normal fibrinogen as well as from each other (5, 8). A direct comparison of the immunoelectrophoretic characteristics of five of these abnormal fibrinogens as well as fibrinogen Vancouver has been reported (56). Fibrinogen Bethesda presented a complex immunoelectrophoretic situation; the pattern given by plasma was consistently abnormal, whereas in purified fibrinogen preparations the difference from normal fibrinogen was not as great. A discrepancy of this nature has not been reported previously inasmuch as the studies cited above were carried out with unfractionated plasma.

In clotting tests, fibrinogens Paris I (2), Paris II (9), Zurich (6), Cleveland (8), Detroit (5), Los Angeles (57), and now fibrinogen Bethesda (Fig. 4) exerted an inhibitory effect on the coagulation of normal plasma or fibrinogen. Only the fibrinogen described by Imperato and Dettori (1) and fibrinogen Baltimore (55) failed to show such an inhibition.

The functional defect in a number of dysfibrinogenemias is a delayed aggregation of fibrin monomers. This delay has been demonstrated in fibrinogen Zurich (6), fibrinogen Detroit (5), fibrinogen Cleveland (8), fibrinogen St. Louis (50), and fibrinogen Los Angeles (57). Fibrinogen Paris II (9) and the fibrinogen reported by Verstraete (58) exhibited a slight delay in fibrin monomer aggregation. No such delay could be detected in the case of fibrinogen Bethesda (Fig. 6). The defect in fibrinogen Oklahoma was originally thought to involve clot stabilization (59), but the fibrinogen of this kindred has recently been recharacterized (53).

Paper electrophoretic analyses have been carried out on the fibrinopeptides released by thrombin from fibrinogens Detroit (60), Cleveland (8), Paris I (61), and Paris II (9). Like the peptides from fibrinogen Bethesda, these had normal migration rates. Moreover, in those fibrinogens studied (fibrinogen Detroit and fibrinogen Baltimore), the amino acid sequence of fibrinopeptide A was found to be normal (60, 62). Apparently only fibrinogens Cleveland (8) and Detroit (60) had hitherto been investigated with regard to the *rate* of fibrinopeptide release, which was normal in both cases despite the fact that fibrinogen Detroit has undergone an amino acid substitution very near the site of thrombin cleavage (60). Thus, as pointed out by Beck in his review (63), a decreased[15] rate of cleavage of peptide A or B had not been shown by direct means. Fibrinogen Bethesda must therefore be considered the first example of a congenitally abnormal fibrinogen manifesting this defect (10). Recent work has indicated that fibrinogen Baltimore may show a delayed release of fibrinopeptides A and AP (65).

Chromatography has provided evidence for the coexistence of normal and abnormal fibrinogens in patients with fibrinogen Baltimore (7); immunoelectrophoresis has suggested it in the case of fibrinogens Cleveland (8) and Detroit (5) even though the latter apparently contains no normal α(A)-chain (60). Studies with Reptilase were used to obtain good evidence for the coexistence of normal fibrinogen and fibrinogen Zurich (66). The copresence of normal fibrinogen and fibrinogen Bethesda is implied by certain observations reported above. First, the immunoelectrophoretic pattern was considerably more abnormal when whole plasma was analyzed than when purified fibrinogen was examined. Second, whereas the curves for release of fibrinopeptides from normal fibrinogen consisted of a smooth rise followed by a plateau, those for fibrinogen Bethesda could apparently be resolved into a "faster" and a "slower" component (Fig. 9). This agrees with the finding that, at moderate thrombin levels, a considerable length of time was required for the last 20% of fibrinogen Bethesda to clot regardless of whether purified fibrinogen or whole plasma was used.

A quantitative difference in the coexistence of normal and abnormal fibrinogen might account for the fact that the propositus shows a bleeding tendency, whereas his mother and siblings do not. That is, if the assumption is made that fibrinogen measured by the Clauss method reflects readily clottable ("normal") fibrinogen levels, the ratio of "normal" to total fibrinogen (as measured by fibrin tyrosine content) in the propositus' plasma is only about half that in the plasma of the other family members (Table III). This is true even though the difference between total and readily clottable fibrinogen ("abnormal fibrinogen") is approximately the same in each case and may account for the propositus' prolonged prothrombin and partial thromboplastin times.

[15] The same could be said for an increased rate of fibrinopeptide release since the abnormal fibrinogen reported by Egeberg (64) manifested a marked shortening of the thrombin time compared with normal fibrinogen.

Inasmuch as fibrinogen Bethesda is the first dysfibrinogenemia associated with a decreased rate of fibrinopeptide release, some consideration of the mechanism by which this decrease occurs is in order. If the alteration represents a single amino acid replacement (cf. references 53 and 60), it must be at a sufficiently critical point in the molecule that the resultant conformational change renders it less susceptible to attack by thrombin. The curves for individual peptide release show that, compared with normal fibrinogen, the appearance of both peptides A and B is delayed. Since the curve for peptide B passed that of A (Fig. 9), one may speculate that the release of peptide A was the more severely affected —a conclusion consistent with, but not required by, the prolonged Reptilase time.

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