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**Research Article**

An abnormal fibrinogen (fibrinogen Cleveland II) was detected in the plasma of a 23-yr-old white man with a mild bleeding diathesis. The one-stage prothrombin time, thrombin time, and Reptilase time were all prolonged. 16 of 24 tested relatives had the defect, which appeared to be transmitted as an autosomal dominant characteristic. The thrombin time of normal plasma was slightly inhibited by the proband's plasma. The abnormally long thrombin time of fibrinogen Cleveland II was partially corrected by addition of calcium ions. Fibrinogen Cleveland II was indistinguishable from normal fibrinogen by immunoelectrophoresis, DEAE-cellulose column chromatography, or polyacrylamide gel electrophoresis of reduced fibrinogen in sodium dodecyl sulfate. The major defect detected appeared to be impaired release of fibrinopeptide A when fibrinogen Cleveland II was incubated with thrombin. This defect was localized to the $NH_2$-terminal disulfide knot portion of the molecule. An abnormality of polymerization of fibrin monomers was also present, but the abnormal fibrin demonstrated relatively normal crosslinking. Despite these defects, fibrinogen Cleveland II achieved a degree of coagulability similar to normal fibrinogen and appeared to incorporate some molecules of fibrin with intact fibrinopeptide A into the clot. The fibrin clot that was formed appeared to be abnormal by electron microscopy. These functional defects and other descriptive characteristics appear to distinguish fibrinogen Cleveland II from other inherited abnormal fibrinogens.



# Fibrinogen Cleveland II

## AN ABNORMAL FIBRINOGEN WITH DEFECTIVE RELEASE OF FIBRINOPEPTIDE A

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ABSTRACT An abnormal fibrinogen (fibrinogen Cleveland II) was detected in the plasma of a 23-yr-old white man with a mild bleeding diathesis. The one-stage prothrombin time, thrombin time, and Reptilase time were all prolonged. 16 of 24 tested relatives had the defect, which appeared to be transmitted as an autosomal dominant characteristic. The thrombin time of normal plasma was slightly inhibited by the proband's plasma. The abnormally long thrombin time of fibrinogen Cleveland II was partially corrected by addition of calcium ions. Fibrinogen Cleveland II was indistinguishable from normal fibrinogen by immunoelectrophoresis, DEAE-cellulose column chromatography, or polyacrylamide gel electrophoresis of reduced fibrinogen in sodium dodecyl sulfate. The major defect detected appeared to be impaired release of fibrinopeptide A when fibrinogen Cleveland II was incubated with thrombin. This defect was localized to the $NH_2$-terminal disulfide knot portion of the molecule. An abnormality of polymerization of fibrin monomers was also present, but the abnormal fibrin demonstrated relatively normal cross-linking. Despite these defects, fibrinogen Cleveland II achieved a degree of coagulability similar to normal fibrinogen and appeared to incorporate some molecules of fibrin with intact fibrinopeptide A into the clot. The fibrin clot that was formed appeared to be abnormal by electron microscopy. These functional defects and other descriptive characteristics appear to distinguish fibrinogen Cleveland II from other inherited abnormal fibrinogens.

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## INTRODUCTION

A number of hereditary abnormalities of fibrinogen (factor I) have been described in which the plasma of an individual contains normal amounts of qualitatively defective fibrinogen (1).

Fibrinogen consists of three pairs of polypeptide chains, designated $A\alpha$, $B\beta$, and $\gamma$, joined at their $NH_2$-terminal ends by disulfide bonds (2). The conversion of plasma fibrinogen to fibrin clot takes place in three steps. First, thrombin selectively hydrolyzes an arginylglycine bond in each $A\alpha$ and $B\beta$ chain, releasing fibrinopeptides A and B, respectively (2). What remains, a so-called fibrin monomer, undergoes polymerization, forming a visible fibrin clot (3). Finally, fibrin undergoes covalent cross-linking under the influence of fibrin-stabilizing factor (factor XIII), resulting in a more stable clot, insoluble in dispersing agents (4).

Qualitative abnormalities of fibrinogen of each of the three steps of fibrin formation have been described. In the majority of cases studied, the site of the defect was localized to abnormal polymerization of fibrin monomers (1). In at least five other families, the release of fibrinopeptides was abnormal (5–9). One family has been described in which the fibrin of affected individuals was thought to respond defectively to the cross-linking action of fibrin-stabilizing factor (10).

The present report describes an apparently new abnormal fibrinogen found in a 23-yr-old white man and in

16 of his relatives, characterized by defective release of fibrinopeptide A and impaired aggregation of fibrin.

## METHODS

*Plasma* was prepared from venous blood, drawn in silicone-coated syringes from the proband, his relatives, and normal controls, mixed with 1/50 vol sodium citrate buffer (0.5 M, pH 5.0) and centrifuged at 2,200*g* for 15 min at 2°C. The plasma was used fresh or stored in silicone-coated containers at −20°C or −70°C.

*Fibrinogen fractions* I-0, I-2, or I-4, prepared by techniques described by Blombäck and Blombäck (11), were separated from normal or abnormal fresh or frozen plasmas.

*Thrombin.* Crude bovine thrombin (Topical Thrombin, Parke, Davis & Co., Detroit, Mich.) was dissolved in barbital-saline buffer at suitable concentrations. Purified thrombin was prepared by a method described earlier and dissolved in 0.05 M potassium phosphate buffer (pH 7.0) (12). The preparation used had a specific activity of 1,150 NIH U/mg protein and was known to be contaminated with traces of Hageman factor (Factor XII) (13).

*Venoms.* Reptilase, an extract of *Bothrops jararaca* venom, was a product of Pentapharm, Basel, Switzerland. Ancrod, the coagulant fraction of *Ancistrodon rhodostoma* (Venacil), was a gift of Abbott Laboratories, North Chicago, Ill. The procoagulant fraction of the venom of *Ancistrodon contortrix contortrix,* the southern copperhead snake, was separated from a crude preparation (Sigma Chemical Co., Inc., St. Louis, Mo.) as described previously (14).

*Other materials and procedures.* Rabbit antiserum to human fibrin was obtained from Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif. and used undiluted. Barbital-saline buffer was composed of 0.025 M sodium barbital and 0.125 M sodium chloride, at pH 7.4.

The whole blood clotting time was measured in 13 × 100-mm glass or 12 × 75-mm polystyrene tubes at 25°C, with fresh whole blood collected in silicone-coated syringes (15). Clot retraction (16) and platelet counts (17) were performed on fresh whole blood; and the activated partial thromboplastin time, one-stage prothrombin time, serum prothrombin activity (prothrombin consumption), and plasma proaccelerin (factor V) and antihemophilic factor (factor VIII) activities were measured by previously referred-to methods (18).

*The concentration of plasma fibrinogen* was measured on fresh or frozen plasmas by a modification (19) of an earlier method (20). The coagulable fraction of fibrinogen preparations was determined by dividing the amount of fibrin that could be formed upon incubation with thrombin for 30 min at 37°C by the total protein content, determined in the same manner on the fibrinogen solutions (20).

*Factor XIII activity* was measured by incubating 0.2 ml plasma with 0.2 ml 0.025 M calcium chloride for 1 h at 37°C. 3 ml of 1% monochloroacetic acid was added, and the clots were incubated at room temperature for 24 h. The protein content of the remaining fibrin clot was measured (19, 20) and compared with the plasma fibrinogen concentration.

The *thrombin time* was determined by measuring the clotting time at 37°C of a mixture of 0.2 ml of plasma or fibrinogen solution, pre-incubated for 1 min at 37°C, and 0.2 ml of crude bovine thrombin, diluted appropriately in barbital-saline buffer. In one experiment, 0.1 ml of 0.025 M calcium chloride solution was added just before the addition of thrombin. The effect of abnormal plasma upon the thrombin time was measured by adding 0.1 ml of abnormal or normal plasma to 0.1 ml of normal plasma. The mixtures were incubated for 1 min at 37°C; 0.2 ml crude bovine thrombin (1.25 NIH U/ml) was added, and the clotting time measured at 37°C. The thrombin time of such mixtures was also measured in the presence of 0.1 ml of 0.025 M calcium chloride solution, added just before the addition of thrombin.

*The effect of thrombinlike snake venoms* was tested by an adaption of the thrombin time. The Reptilase time of whole plasma was assayed by measuring the clotting time at 37°C of a mixture of 0.2 ml of plasma, incubated at 37°C for 1 min, and 0.2 ml of Reptilase, diluted with an equal part of 0.025 M calcium chloride solution. The effect of venoms on purified fibrinogen (fraction I-2) was tested by incubating 0.2 ml of fibrinogen solution for 1 min at 37°C, after which 0.1 ml of barbital-saline buffer or 0.025 M calcium chloride was added, followed immediately by 0.2 ml of ancrod, the procoagulant fraction of the southern copperhead snake venom, or Reptilase, and clotting time was measured at 37°C. The fibrinogen solution contained 2.4 mg coagulable protein/ml; that prepared from normal plasma was 95% coagulable, while that prepared from the proband was 92% coagulable.

*All clotting times* were measured in 10 × 75-mm disposable glass tubes.

*Aggregation of platelets* was measured at 37°C in 1-ml samples of platelet-rich plasma (approximately 250,000 platelets/mm$^3$), with a Chrono-log platelet aggregometer (Chrono-log Corp., Broomall, Pa.) to measure the light transmittance through the mixture after the addition of 0.05 ml ADP, (Sigma Chemical Co.), 10 μg/ml, of barbital-saline buffer or 0.1 ml bovine collagen, 0.7 mg protein/ml of 0.1 N acetic acid, the gift of Dr. Howard Bensusan, Case Western Reserve University.

Serum prepared from fresh plasma was tested for the presence of *fibrinogen-related antigens* by crossed-immunoelectrophoresis (21).

*Immunodiffusion* was carried out on 1 × 3-in microscope slides in 0.9% agarose gel in 0.05 M sodium barbital buffer (pH 8.4) (22). The central well contained 0.01 ml undiluted rabbit anti-human fibrin antiserum, and the peripheral wells contained 0.01 ml of normal or abnormal plasma diluted with 2 vol of barbital-saline buffer, or 0.01 ml normal or abnormal fibrinogen (0.6 mg/ml of the same buffer). The gels were kept at room temperature for 24 h and then photographed.

*Immunoelectrophoresis* (23) of 0.004 ml of normal or abnormal plasma was carried out on a 1 × 3-in microscope slide in 0.9% agarose in 0.05 M sodium barbital buffer (pH 8.4) or 0.05 M citrate-phosphate buffer (pH 5.3). After electrophoresis at room temperature at 100 V (constant voltage) for 1–14 h, lines of precipitation were developed by addition of 0.2 ml undiluted rabbit anti-fibrin antiserum to the central trough. The gels were kept at room temperature for 24 h and then photographed.

*Column chromatography of purified fibrinogen* was performed by the method of Mosesson, Alkjaersig, Sweet, and Sherry (24, 25) with DEAE cellulose (Whatman DE52, Scientific Div., H. Reeve Angel & Co., Inc., Clifton, N. J.), equilibrated with 0.005 M sodium phosphate, 0.039 M Tris buffer (Trizma, Sigma Chemical Co., pH 8.6) in a 1 × 30-cm column. 10-18 mg of abnormal or normal fibrinogen fraction I-4 (abnormal 95% coagulable, normal 98% coagulable) dissolved in and dialyzed for 12 h at 4°C against

TABLE I

*Coagulation Studies in the Proband with Fibrinogen Cleveland II*

| Studies | Proband | Normal or control |
|---|---|---|
| Clotting time, Glass | 21 min | 20–30 min |
| Polystyrene | 145 min | 75–235 min |
| Bleeding time (modified Duke test) | 5½ min | <9 min |
| Clot retraction | 70% | >60% |
| Platelet aggregation by ADP and collagen | Normal | |
| One-stage prothrombin time | 16.8 s | 13.4 s |
| Activated partial thromboplastin time | 53.6 s | 52.7 s |
| Serum prothrombin activity | <10% | <10% |
| Plasma factor V activity | 100% | |
| Plasma factor VIII activity | 100% | |
| Plasma fibrinogen | 289 mg/ml | 177–415 mg/ml |
| Thrombin time | 68 s | 30 s |
| Reptilase time (with calcium ion) | 50 s | 21 s |
| Plasma factor XIII activity | Normal | |
| Serum fibrin-related antigen | Negative | |

100 vol of the same buffer was applied to the column. The column was developed at 4°C with a concave gradient of 500 ml 0.005 M sodium phosphate, 0.039 M Tris (pH 8.6) as starting buffer and 500 ml 0.5 M sodium phosphate, 0.5 M Tris (pH 4.1) as the final buffer, collecting 5-ml fractions. The elution of protein was monitored by measuring optical density at 280 nm. The fractions were tested for conductivity and pH, while the presence of fibrinogen in protein-rich fractions was tested by addition of crude bovine thrombin to samples of the fractions and observing clot formation and by immunodiffusion of 0.01 ml of protein-rich fractions against 0.01 ml rabbit anti-fibrin antiserum.

*The molecular weights of the polypeptide chains of the abnormal fibrinogen* were estimated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) [1] of fibrinogen fraction I-4, reduced with 2-mercaptoethanol (26).

*Electron microscopy of fibrin* was carried out by incubating normal or abnormal fibrinogen fraction I-2 (2.0 mg/ml barbital-saline buffer) with one equal volume of crude bovine thrombin (200 NIH U/ml) for 1 min for normal or 2 min for abnormal fibrinogen, at room temperature, directly on carbon-coated Formvar membranes (Belden Mfg. Co., Chicago, Ill.). At the end of the incubation time, the membranes were blotted with filter paper, washed twice with barbital-saline buffer, and then stained with 0.5% uranyl acetate, as described by Kay and Cuddigan (27). The membranes were examined in an AEI EM-6B electron microscope (AEI Scientific Apparatus, Inc., Elmsford, N. Y.) at original magnifications of 10,000 and 80,000.

*Total fibrinopeptide release* was measured by a method adapted from that described by Sherman, Gaston, Kaplan, and Spivack (28), in which the guanidino group of arginine is measured fluorometrically after the addition of aqueous ninhydrin at alkaline pH (29). Arginine is COOH-terminal amino acid in both fibrinopeptides A and B; thus, its appearance in the supernatant fluid after clotting is a measure of release of the fibrinopeptides. Normal and abnormal fibrinogen fractions I-0 (both 85–90% coagulable) were dissolved in barbital-saline buffer at a concentration of 3.3 mg coagulable protein/ml. Disodium EDTA and epsilon aminocaprois acid (EACA) were added at concentrations of 0.004 M and 0.005 M, respectively. 0.8 ml fibrinogen solution was placed in a series of 12 × 75-mm polystyrene tubes, and 0.1 ml distilled water was added to all except the internal standard tubes. At zero time, 0.1 ml purified bovine thrombin (2.5 NIH U/ml of barbital-saline buffer) was added to each tube except to the 0-min and internal standard samples. Samples were then incubated at 37°C for 1–64 min, after which the reaction was stopped by the addition of 1.0 ml 15% trichloroacetic acid (TCA), and by shaking to disperse the clot. 1 ml of 15% TCA was added to the 0-min sample and internal standard samples before the addition of thrombin. Finally, 0.1 ml of arginine (200 μmol/liter and 400 μmol/liter) was added to the internal standard aliquot tubes to give a final concentration of 10 μmol/liter and 20 μmol/liter, respectively. All tubes were placed in an ice bath for 20–30 min and then centrifuged at 2,200*g* and 2°C for 20 min. The supernatant fluids were filtered through Millipore filters (13 mm, 0.45 μm, HAWPO 1,300, Millipore Corp., Bedford, Mass.). 1.5 ml of the filtered supernatant fluid was added to 1.5 ml 1.0 N sodium hydroxide in 13 × 100 mm disposable glass tubes. 1.5 ml of 0.5% aqueous ninhydrin was added to each, and the tubes were incubated at room temperature in the dark for 17 min. The fluorescence of each solution was measured in a Turner Fluorometer, Model 110 (G. K. Turner Associates, Palo Alto, Calif.) with a #7-60 excitation filter (365 nm), a #8 emission filter (485 nm) and × 1 sensitivity by reading the values of each tube against a blank of the 0-min aliquot, adjusted to zero fluorometric units.

A standard curve, constructed by measuring the fluorescence of known amounts of arginine in 7.5% TCA reacted with equal volumes of 1.0 N sodium hydroxide and 0.5% aqueous ninhydrin, revealed a linear relationship existed between the degree of fluorescence observed and the concentrations of arginine between 1 and 40 μmol/liter. Adjusted fluorometric readings of the internal standard aliquots containing fibrinogen, thrombin, and 10 or 20 μmol

[1] *Abbreviations used in this paper:* BAMe, benzoyl arginine methyl ester; EACA, epsilon aminocaproic acid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

arginine/liter were the same as the fluorometric readings of 10 and 20 μmol arginine/liter on the standard curve. Thus, an estimation of the quantity of fibrinopeptide present in solution could be made by this method.

A comparison of the total fibrinopeptides released and fibrin clot formed was performed by a variation of this technique. Solutions of abnormal and normal fibrinogen fraction I-0 in barbital-saline buffer containing 0.005 M EACA and 0.025 M calcium chloride were incubated at 37°C with purified bovine thrombin, at final concentrations of 1.25 or 10 NIH U/ml for various times, and total fibrinopeptide release was determined as described above. Duplicate tubes with 0.5 ml of the same mixtures were incubated at 37°C, the clot was removed at times corresponding to the incubation times of the fibrinopeptide release assay, and the protein content of the clot was measured (20). Total fibrinopeptides released and the protein concentration of the fibrin clot were converted to moles (assuming fibrin mol wt = 330,000) and the ratio was determined.

*Individual fibrinopeptide release* was measured by incubating 2 ml (2 mg) of normal or abnormal fibrinogen in 0.008 M Tris buffer in 0.142 M sodium chloride solution (pH 7.4) and 0.5 mM disodium EDTA with 20 μl purified bovine thrombin of varying concentrations and for varying lengths of time. At the end of the desired incubation time, the clot was removed and dissolved in 1% monochloroacetic acid, and the protein content measured by spectrophotometry at 380 nm. 0.2 ml 30% TCA was added to the supernatant fluid to precipitate any remaining protein. The supernatant fluids were processed to remove the TCA and salt (30) and subjected to electrophoresis, and the fibrinopeptides were quantified as described previously (31). This electrophoretic analysis also detected a fraction with the mobility of the tripeptide Gly-Pro-Arg with a precision no better than 30% (30).

*Fibrin monomer* was prepared by incubating abnormal and normal fractions I-2 (92% and 95% coagulable, respectively), dissolved in 0.3 M HCl-Tris buffer (pH 7.4) with purified bovine thrombin (5.0 NIH U/ml) for 1 h at 35°C. A gel formed in tubes containing normal fibrinogen after 4 min incubation; at this point both fibrinopeptides A and B had been released. The abnormal fibrinogen did not form a gel under these conditions, although other experiments suggested that maximum fibrinopeptide release had been achieved. The pH was then decreased to 5.30 in each by the addition of 0.75 vol of a buffer (5 parts 0.3 M sodium acetate, 1 part 0.3 M benzoyl arginine methyl ester (BAMe), pH 4.7) to 1 vol of fibrin solution. Under these conditions, the normal fibrin gel dissolved. The fibrin solutions, now at pH 5.3, were cooled to 0°C, and 1/3 vol of a solution of ammonium sulfate (neutralized and saturated at room temperature) was added to each to precipitate the protein. The precipitate was washed twice with 0.055 M sodium citrate buffer containing 1 M glycine, 6.5% ethanol, and 0.015 M BAMe (pH 6.0) and dissolved and dialyzed in a buffer (9 parts 0.42 M Tris HCl, 1 part 0.3 M Tris acetate, pH 5.3). The fibrin solution was reduced with 2-mercaptoethanol and subjected to polyacrylamide gel electrophoresis in SDS and examined for evidence of cross-linking (32).

*Aggregation of the abnormal and normal fibrin* was compared in the following manner. 0.8 ml of fibrin solution (0.45 mg/ml Tris-HCl-acetate buffer, 0.3 M, pH 5.30) was added to 0.4 ml 0.1 M Tris buffer (pH 10.5) to increase the pH to 7.4. The change in optical density at room temperature, reflecting aggregation of fibrin (33), was measured in quartz cuvettes in a Beckman spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.), recording the optical density at 350 nm with a Gilford Recorder (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

TABLE II

*The Thrombin Times of Fibrinogen Cleveland II in the Presence of Calcium Ions*

| Mixture | Clotting time |
|---|---|
| | *s* |
| Control fibrinogen | 27.4 |
| Control fibrinogen plus calcium ions | 15.8 |
| Fibrinogen Cleveland II | 77.0 |
| Fibrinogen Cleveland II plus calcium ions | 25.7 |

0.2 ml fibrinogen fractions I-0 dissolved in barbital-saline buffer at a concentration of 2.0 mg/ml were mixed with 0.2 ml bovine thrombin (2.5 NIH U/ml): 0.1 ml calcium chloride (0.025 M) was added to one set.

*The progression of cross-linking of fibrin* was performed as described by McKee, Schwartz, Pizzo, and Hill (32). Abnormal and normal fibrinogen fractions I-2 (96% and 97% coagulable, respectively) in barbital-saline buffer (pH 7.4) containing 0.005 M EACA, 0.025 M calcium chloride, and 1/50 vol of fresh normal plasma were incubated with purified bovine thrombin (2.0 NIH U/ml) at 37°C for 1, 15, 30, 60, and 120 min. Noncross-linked fibrin was formed by substituting 0.0025 M disodium EDTA for 0.025 M calcium chloride and incubating at 37°C for 30 min. At the end of the incubation time, 0.5 ml of a solution containing 8 M urea, 0.15% SDS, 0.15% 2-mercaptoethanol (by volume), and 0.01 M sodium phosphate (pH 7.0) was added to 0.25 ml of the fibrin solution (1.8 mg protein/ml), and this mixture was incubated at 37°C for 2 h. The resulting reduced protein solutions were prepared in the described manner (26), and 25 μl (0.0075 mg protein) was applied to 5% polyacrylamide gels and subjected to electrophoresis in SDS at pH 7.4 for 4 h at 8 mA per tube. Gels were stained with 0.25% Coomassie Brilliant Blue in 7% acetic acid and destained electrophoretically in 7% acetic acid at 10 mA per tube (26).

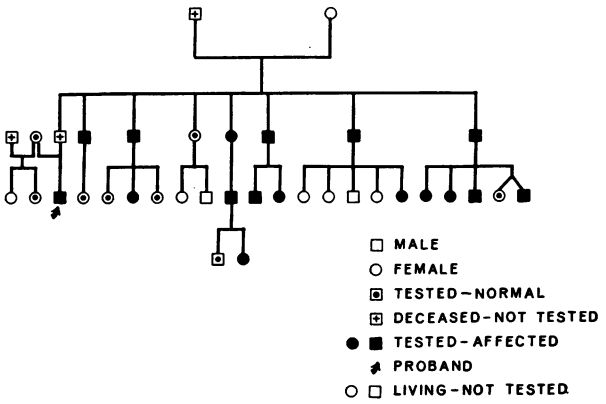


FIGURE 1 Pedigree of fibrinogen Cleveland II.

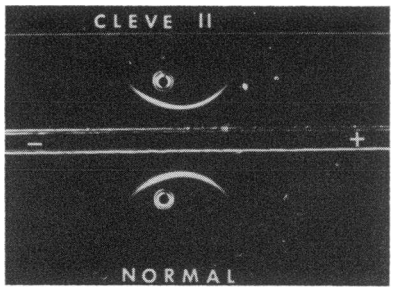


FIGURE 2 Immunoelectrophoresis of fibrinogen Cleveland II. 0.04 ml of the proband's plasma and normal plasma electrophoresed for 21 h at 100 V (constant voltage) in 0.9% agarose in sodium barbital buffer (0.05 M, pH 8.4). The center well contained 0.2 ml rabbit anti-fibrin antiserum. The anode is to the right.

The $NH_2$-terminal disulfide knot was prepared from 160 mg fibrinogen Cleveland II (fraction I-2, 96% coagulable) and 160 mg normal fibrinogen (fraction I-4, 98% coagulable) by treatment with CNBr in 70% formic acid as described by Blombäck, Hessel, Iwanaga, Reuterby, and Blombäck (34). The CNBr-treated fibrinogen was applied to a column (20 $cm^2 \times 70$ cm) of Sephadex G-100 in 10% acetic acid and eluted with 10% acetic acid. The first 2% of the total protein applied, as measured by the Lowry, Rosebrough, Farr, and Randall method (35), was considered "fraction 1" (34) and discarded. The next 18% of the protein eluate ("fraction 2"), which was believed to be rich in the dimeric $NH_2$-terminal disulfide knot (34) was examined by electrophoresis in 7.5% polyacrylamide gel in 5% acetic acid (2, 36) and in 5% polyacrylamide gel in SDS (2, 26). To release fibrinopeptides for measurement, this fraction was first neutralized either by adding 1 M sodium carbonate or by lyophilizing and dissolving in 0.2 M ammonium bicarbonate at pH 8.2, and then was incubated in heavy-walled glass centrifuge tubes with purified thrombin at either 360 or 30 U/ml for periods of 2 and 18 h, respectively. The residual protein was precipitated by adding ⅓ vol of 50% TCA, and fibrinopeptides in the supernatant fluid were determined as described above (30, 31).

TABLE III
*Inhibitory Effect of Fibrinogen Cleveland II upon the Thrombin Time of Normal Fibrinogen*

| Mixture | Clotting time |
|---|---|
| | *s* |
| Normal fibrinogen | 44.0 |
| Normal fibrinogen + fibrinogen Cleveland II | 57.0 |
| Normal fibrinogen + calcium ions | 21.0 |
| Normal fibrinogen + fibrinogen Cleveland II + calcium ions | 21.0 |

0.2 ml of normal plasma and a mixture of 0.1 ml fibrinogen Cleveland II plasma and 0.1 ml normal plasma were mixed with 0.2 ml bovine thrombin (1.25 NIH U/ml); 0.1 ml calcium chloride (0.025 M) was added to one set.

TABLE IV
*The Clotting Times of Fibrinogen upon Addition of Thrombinlike Snake Venoms*

| Enzyme | Clotting times | |
|---|---|---|
| | Cleveland II | Normal control |
| | *s* | |
| Ancrod, 2 U/ml | >1,200 | 33.3 |
| Ancrod, 2 U/ml plus calcium ions | 39.4 | 18.4 |
| Ancrod, 40 U/ml | >1,200 | 7.0 |
| Copperhead procoagulant | >1,200 | 78.4 |
| Copperhead procoagulant plus calcium ions | >1,200 | — |
| Reptilase, 1 U/ml | 196.0 | 84.0 |

0.2 ml fibrinogen fraction I-2 dissolved in barbital-saline buffer at a concentration of 2.4 mg/ml was mixed with 0.1 ml barbital-saline buffer or 0.1 ml calcium chloride (0.025 M) and 0.2 ml enzyme. The clotting times were measured at 37°C.

## RESULTS

*Case history.* A 23-yr-old white man was referred to University Hospitals of Cleveland for evaluation of a mild bleeding tendency, characterized by bleeding after tonsillectomy and recurrent minor bleeding for several days from lacerations. Studies of his clotting system demonstrated that the one-stage prothrombin time, thrombin time, and Reptilase time were abnormally long (Table I). The concentration of fibrinogen in the patient's plasma was normal, and fibrin-related antigens were not detected in his serum.

*Family studies.* In 16 of 24 tested relatives, the thrombin time and Reptilase time were prolonged and the levels of fibrinogen in their plasmas were normal. In all affected individuals, the prolonged thrombin times fell within 1 or 2 s of each other. Except for an uncle of the proband who bled after prostatectomy, all affected relatives were asymptomatic. The defect appeared to be inherited as an autosomal dominant trait; no consanguinity was known to the family (Fig. 1).

*Localization of the patient's defect to fibrinogen.* The thrombin time of the patient's fibrinogen (fraction I-0) was prolonged, an abnormality partially corrected by the addition of calcium ions (Table II). Similar results were obtained with the patient's fibrinogen fractions I-2 and I-4. When the patient's plasma was added to normal plasma, the thrombin time of the normal plasma became slightly prolonged. This inhibitory effect increased when an increased amount of patient's plasma was added, but was totally obliterated when calcium ions were added (Table III).

The effect of snake venoms upon the coagulation of the abnormal fibrinogen was tested in an attempt to localize the abnormality. Ancrod and Reptilase selectively separate fibrinopeptide A from fibrinogen (37, 38), while the procoagulant fraction of the venom of the

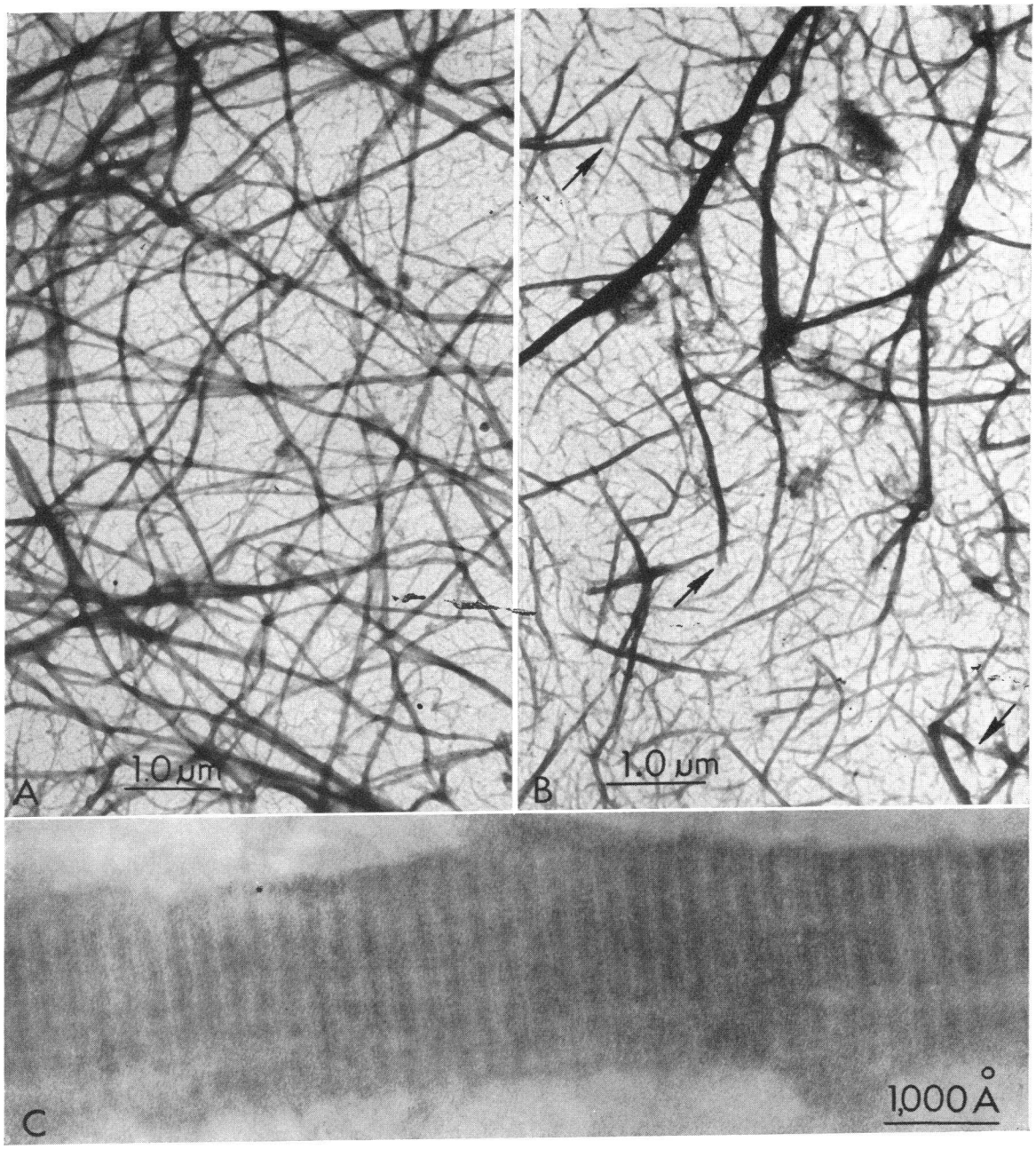


FIGURE 3 Electron micrographs of fibrin films negatively stained with 0.5% aqueous uranyl acetate. A. Fibrin formed from normal human fibrinogen. Fibrin strands are long and tapered, and rarely end abruptly. × 15,000. B. Fibrin formed from fibrinogen Cleveland II. Abrupt termination of fibrin strands (arrows) is encountered commonly. × 15,000. C. Higher magnification of fibrin strand formed from fibrinogen Cleveland II. The cross striations are not detectably different from those of normal fibrin, and have a similar periodicity of 220–230 Å. × 165,000.

southern copperhead snake separates fibrinopeptide B from fibrinogen at a faster rate than it separates fibrinopeptide A (14). In the absence of calcium ions, the copperhead procoagulant fraction and ancrod did not clot the abnormal fibrinogen (Table IV). In the presence of calcium ions, ancrod clotted the abnormal fibrinogen,

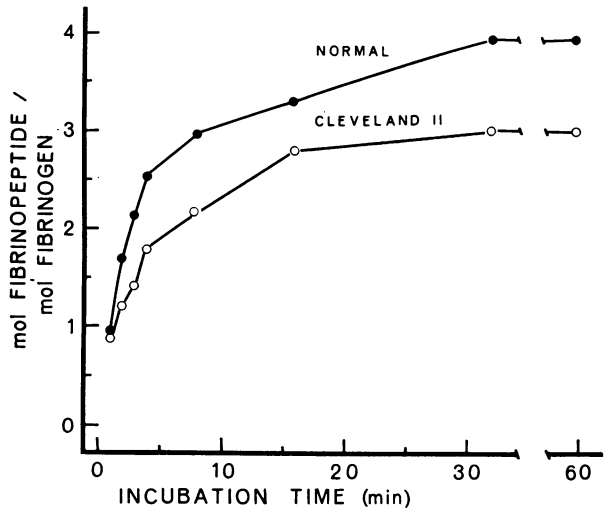


FIGURE 4 Total fibrinopeptide release from fibrinogen Cleveland II and normal fibrinogen. 2.6 ml of fibrinogen were incubated at 37°C with thrombin (0.25 NIH U/ml). The total fibrinopeptides released at various incubation times were measured by fluorescence of a mixture of the fibrinopeptides and alkaline ninhydrin, converted to micromoles and divided by 0.0076 μmol (0.0076 μmol = 2.6 mg fibrinogen) to determine moles fibrinopeptides per mole fibrinogen.

although more slowly than normal fibrinogen, but the copperhead procoagulant fraction was without discernible effect.

*Immunologic studies of the abnormal fibrinogen.* Abnormal plasma and purified fibrinogen formed a line of identity upon immunodiffusion against anti-fibrin antiserum. Upon immunoelectrophoresis, the abnormal fibrinogen migrated identically with normal fibrinogen toward the anode at pH 8.4 (Fig. 2) and toward the cathode at pH 5.3. This identical migration was observed regardless of the length of time of electrophoresis.

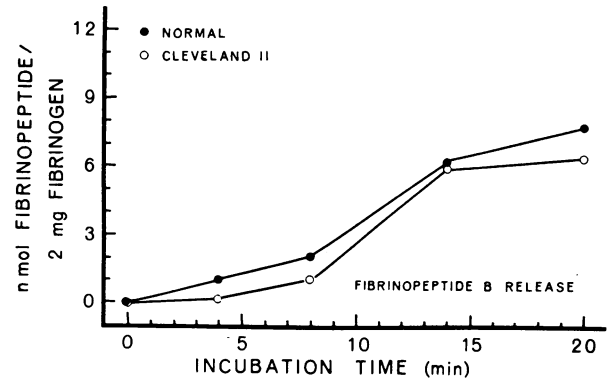


FIGURE 5 Fibrinopeptide B release from fibrinogen Cleveland II and normal fibrinogen. 2 mg of fibrinogen were incubated at 37°C with thrombin (0.25 NIH U/ml). Fibrinopeptide B was measured in nanomoles by an electrophoretic technique.

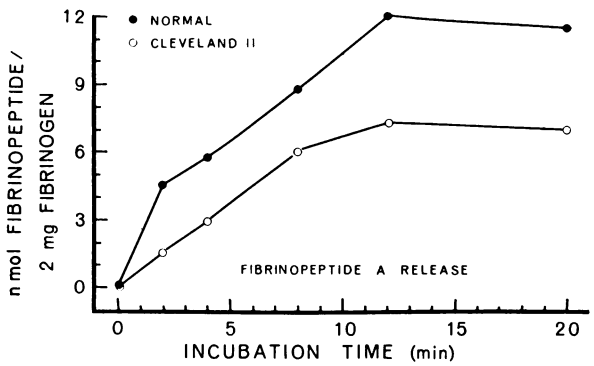


FIGURE 6 Fibrinopeptide A release from fibrinogen Cleveland II and normal fibrinogen. (See Fig. 5).

*Chromatographic behavior of the abnormal fibrinogen.* Upon chromatography on a column of DEAE cellulose, normal fibrinogen elutes in two major peaks (25). The abnormal fibrinogen (fraction I-4) was eluted in two peaks in elution fractions with identical conductivity and pH as those elution fractions containing the same respective peaks of normal fibrinogen (fraction I-4). The coagulable protein was found in those fractions of eluate that contained antigens related to fibrin, as measured by gel diffusion.

TABLE V

*Observed Molar Ratios of Total Fibrinopeptides Released to Fibrin Present in the Formed Clot*

| Incubation time | Cleveland II molar ratios | Normal control molar ratios |
|---|---|---|
| *min* | | |
| Final thrombin concentration 1.25 NIH U/ml | | |
| 1 | 2.7 | 2.1 |
| 4 | 2.4 | 3.1 |
| 16 | 3.0 | 3.9 |
| 64 | 2.9 | 3.8 |
| Final thrombin concentration 10.0 NIH U/ml | | |
| 1 | 3.2 | 4.0 |
| 4 | 3.0 | 3.8 |
| 60 | 3.0 | 4.5 |
| 120 | 3.3 | 4.5 |

Moles of total fibrinopeptides released at varying incubation times were divided by the moles of fibrin, measured as protein present in the clot at the corresponding incubation times; experiments were performed in the presence of calcium ions. Theoretic value should be 4 mol of fibrinopeptides released for 1 mol of fibrin formed.

TABLE VI

*Yields of Fibrinopeptides after Prolonged Reaction with Thrombin*

| | Normal | | | Cleveland II | | |
|---|---|---|---|---|---|---|
| Thrombin concentration | 3 U/ml | 3 U/ml | 30 U/ml | 3 U/ml | 3 U/ml | 30 U/ml |
| Incubation time | 25 min | 60 min | 25 min | 25 min | 60 min | 25 min |
| nmol A/mg fibrinogen | 5.0 | 5.6 | 4.9 | 3.2 | 2.8 | 2.1 |
| nmol B/mg fibrinogen | 5.0 | 4.6 | 5.1 | 4.8 | 4.5 | 4.6 |
| nmol fibrin formed/mg fibrinogen | 2.9 | 3.0 | 2.8 | 1.9 | 2.3 | 2.2 |

Statistical analyses: the ratio of peptides (A/B) from eight determinations on normal fibrinogen averaged 1.16±0.073 (SEM) which differed only slightly ($0.05 < P < 0.1$) from the theoretical value of 1.0. A much smaller ratio of A/B, 0.63±0.055 (SEM) was obtained from fibrinogen Cleveland II. The average yield of B (4.60±0.12 SEM nmol/mg) from fibrinogen Cleveland II did not differ significantly from the yield (4.63±0.028) obtained from the control.

*Molecular weights of the polypeptide chains of the abnormal fibrinogen.* The molecular weights of the polypeptide chains of the abnormal fibrinogen, as estimated by SDS gel electrophoresis, were 75,000 for the Aα, 59,000 for the Bβ, and 51,000 for the γ chains, not appreciably different from those obtained for the molecular weights of normal fibrinogen polypeptide chains, 76,000±1,500, 61,000±1,500, and 51,500±1,000 for the Aα, Bβ, and γ chains, respectively, as determined in our laboratory.[2]

*Electron microscopy of the abnormal fibrinogen.* Fibrin clots from the abnormal fibrinogen examined by electron microscopy were found to have some unconnected strands that appeared to end abruptly, while in the normal fibrin network, almost all strands tapered gradually and were interconnected (Fig. 3, A and B). Under greater magnification, the abnormal and normal fibrin strands had similar cross-banding of the same periodicity (220–230 Å) (Fig. 3 C).

*Locus of the functional defect in the abnormal fibrinogen.* The locus of the functional defect in the abnormal fibrinogen was determined by examining each step of the fibrinogen-to-fibrin conversion process separately. In repeated experiments, the release of total fibrinopeptides from the abnormal fibrinogen was slower than from normal fibrinogen when both were incubated with thrombin (0.25 NIH U/ml) (Fig. 4). At this concentration of thrombin, complete release of the expected amount of fibrinopeptides from the abnormal fibrinogen was not observed. When the abnormal fibrinogen was incubated with thrombin (1.25 or 10.0 NIH U/ml) in the presence of calcium ions, approximately 3 mol of fibrinopeptide were released per mol of fibrin, compared to the expected 4 ml fibrinopeptide per mol of fibrin, observed in the study of normal fibrinogen (Table V).

The defect in the release of fibrinopeptides was localized to the separation of fibrinopeptide A. When the abnormal fibrinogen was incubated with thrombin, the rate of release of fibrinopeptide B fell only slightly below normal (Fig. 5), whereas the release of fibrinopeptide A was significantly retarded (Fig. 6). After prolonged incubation, yields of fibrinopeptide B reached the same level as normal, but fibrinopeptide A consistently fell short of normal by 35–55%, regardless of thrombin concentration, spanning 3–30 U/ml (Table VI). These experiments were carried out in the presence of EDTA, and there was incomplete incorporation of abnormal fibrinogen into fibrin clot. In most experiments, however, less than the expected 2 nmol of fibrinopeptide A were released for each nmol of fibrin clot formed from the abnormal fibrinogen.

The method of preparation of fibrin monomers (soluble fibrin) allowed maximum release of those fibrinopeptides that could be separated from the normal or abnormal fibrinogen. The fibrin monomers showed no evidence of cross-linking by SDS gel electrophoresis of the reduced proteins (32). When solutions of abnormal and

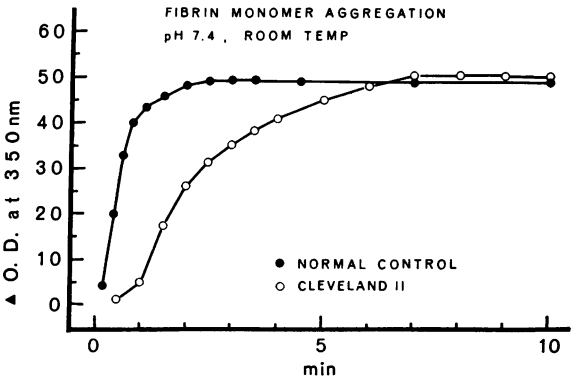


FIGURE 7 Aggregation of fibrin monomers from fibrinogen Cleveland II or normal fibrinogen. Normal and Cleveland II fibrin monomer solutions of identical protein concentration (0.45 mg/ml) were adjusted to pH 7.4 at room temperature and the optical density at 350 nm recorded in arbitrary units.

[2] These observations were made by Dr. W. B. Forman, Cleveland Veterans Administration Hospital, Cleveland, Ohio.

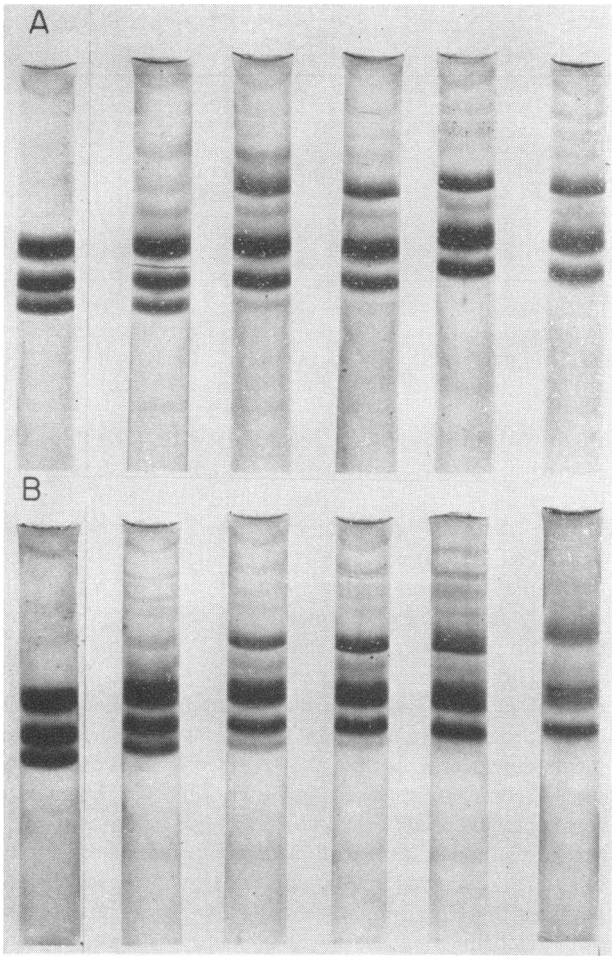


FIGURE 8 Progression of cross-linking in fibrin from normal fibrinogen (A) and fibrinogen Cleveland II (B). Fibrinogen (fraction I-2) incubated with thrombin (2.0 NIH U/ml) in the presence of 0.025 M calcium chloride and 1/50 vol fresh plasma at 37°C. Fibrin formed after varying incubation times was reduced with 2-mercaptoethanol in the presence of SDS, then subjected to electrophoresis in 5% polyacrylamide gels and SDS buffer at 8 mA/tube for 4 h. Gels were stained with Coomassie Brilliant Blue in 7% acetic acid. A. Normal fibrin, B. Cleveland II fibrin; 1. noncross-linked fibrin; 2. 1-min incubation; 3. 15-min incubation; 4. 30-min incubation; 5. 60-min incubation; 6. 120-min incubation. $\alpha$ polymers, observed in both normal and abnormal samples at 15 min and increasingly apparent as incubation continued, cannot be discerned in the photographic reproduction; these did not penetrate the gels.

normal fibrin monomers at identical protein concentrations were adjusted to pH 7.4, the rate of aggregation, measured by the change in optical density at 350 nm (33), was slower in the abnormal fibrin preparation, but the degree of aggregation ultimately achieved was similar to that of normal fibrin (Fig. 7).

The progression of cross-linking was evaluated by polyacrylamide gel electrophoresis of reduced fibrin in SDS. The relative migration of the polypeptide chains has been published elsewhere (32). After incubation of both the normal and abnormal fibrinogen with thrombin (2.0 NIH U/ml) for 1 min some $\gamma$-$\gamma$ dimers had formed; after 15 min incubation, $\gamma$-$\gamma$ dimer formation was almost complete in both. No free $\gamma$ chains were apparent after incubation for 30 min in the normal mixture. Not until 120 min incubation, however, was the abnormal mixture free of detectable $\gamma$ monomer. $\alpha$ polymers were present in both after incubation for 15 min, and increased progressively as incubation continued. The samples of the normal and abnormal fibrinogen, incubated for 120 min, were indistinguishable from one another (Fig. 8). Thus, the abnormal fibrin was cross-linked at a slower rate than normal fibrin but eventually it appeared to be cross-linked to the same degree as normal fibrin.

Fraction 2 of CNBr-treated normal fibrinogen and fibrinogen Cleveland II were characterized by electrophoresis in polyacrylamide gel in 5% acetic acid (36) and in SDS (26), and both were found to be in close agreement with a previous description of this fraction (2), supporting the notion that fraction 2 contained dimeric NH-terminal disulfide knot. Upon incubation of fraction 2 with thrombin, the yield of fibrinopeptide A from the fibrinogen Cleveland II $NH_2$-terminal disulfide knot was approximately one-half that of fibrinopeptide B, in contrast to the normal $NH_2$-terminal disulfide knot, which yielded approximately equimolar amounts of the two fibrinopeptides (Table VII). Notably, the total yield of fibrinopeptides from the $NH_2$-terminal disulfide knot was less than predicted, assuming a mol wt for the dimeric knot of approximately 60,000 (2), presumably because fraction 2 is not completely pure dimeric $NH_2$-terminal disulfide knot. The tripeptide, Gly-Pro-Arg, which is adjacent to the fibrinopeptide A in the A$\alpha$ chain and is released from the $NH_2$-terminal disulfide knot by thrombin (2) was released from fraction 2 after 18 h incubation with thrombin, in amounts similar to the amount of fibrinopeptide A released in each case, within the limits of an admittedly imprecise technique (30). Thus, it appeared that the defect of impaired fibrinopeptide A release from fibrinogen Cleveland II persisted in the $NH_2$-terminal disulfide knot portion of the molecule.

## DISCUSSION

A family has been described in which affected individuals have within their plasmas a species of fibrinogen that clotted abnormally slowly upon the addition of thrombin. This defect appeared to be inherited as an autosomal dominant trait and was associated with a mild bleeding tendency. Using a convention proposed by Beck (39), we have designated this abnormal protein as fibrinogen Cleveland II.

TABLE VII

*Release of Fibrinopeptides from the Disulfide Knot of Fibrinogen Cleveland*

| | Concn of disulfide knot | Concn of thrombin | Period of incubation | Fibrinopeptide released | | |
|---|---|---|---|---|---|---|
| | | | | A | B | Gly-Pro-Arg |
| | *mg/ml* | *U/ml* | *h* | *nmol/mg* | *nmol/mg* | *nmol/mg* |
| Liquid sample | | | | | | |
| Normal | 0.3 | 360 | 2 | 12.0 | 11.6 | — |
| Patient | 0.3 | 360 | 2 | 5.4 | 12.3 | — |
| Lyophilized sample | | | | | | |
| Normal | 0.9 | 30 | 18 | 13.9 | 12.5 | 9.7 |
| Patient | 0.5 | 30 | 18 | 6.5 | 14.1 | 7.3 |

The abnormal fibrinogen demonstrated a partial correction of the thrombin time by the addition of calcium ions and the property of being mildly inhibitory to clotting of normal fibrinogen by thrombin. Fibrinogen Cleveland II, however, was indistinguishable from normal fibrinogen by immunoelectrophoresis, DEAE-cellulose column chromatography, or polyacrylamide gel electrophoresis of reduced fibrinogen in SDS.

Functionally, the major defect discerned in the abnormal fibrinogen was impaired release of fibrinopeptide A upon incubation with thrombin. The release of fibrinopeptide A occurred more slowly than normally, and the total amount of this peptide released was less than the expected amount. Under the experimental conditions used, the total release of fibrinopeptide A was not observed.

In addition to, and perhaps as a result of, the defect in fibrinopeptide release, the abnormal fibrinogen demonstrated a slower than normal polymerization of its fibrin monomers at pH 7.4.

Finally, the abnormal fibrin demonstrated a decrease in the rate of progress of cross-linking, but the degree of cross-linking eventually achieved appeared to be the same as normal fibrin.

Despite these defective steps in the fibrinogen-to-fibrin clot conversion, under some conditions the abnormal fibrinogen demonstrated a degree of coagulability similar to that of normal fibrinogen. This was shown by three separate observations. In the absence of EDTA, purified preparations of abnormal and normal fibrinogens exhibited a similar degree of coagulability, while the abnormal fibrinogen retained its prolonged thrombin time. Polyacrylamide gel electrophoresis of reduced fibrin, which demonstrated the progress of cross-linking, showed no free $\gamma$ chain after prolonged incubation. One would anticipate that free $\gamma$ chains would be observed if some fibrinogen molecules remained disassociated from the fibrin clot. Thirdly, there was no fibrin-related antigen in the proband's serum. Thus, after clotting, it appeared that all the fibrinogen-related antigen had been incorporated into the clot.

Since fibrinogen Cleveland II demonstrated a degree of coagulability similar to normal fibrinogen, yet under no conditions was it observed to release all of its fibrinopeptide A, it seemed likely that some fibrin molecules with fibrinopeptide A still intact were incorporated into the fibrin clot. This notion was further supported by the consistent observation that, upon incubation of fibrinogen Cleveland II with thrombin in the presence of calcium ion, about 3 mol of fibrinopeptides was released per 1 mol of fibrin formed. Under the same conditions, normal fibrinogen released about 4 mol of fibrinopeptides per 1 mol of fibrin formed. In experiments in which individual fibrinopeptides were measured, incomplete coagulability of fibrinogen Cleveland II was noted. These experiments were performed in the presence of EDTA, which may account for this observation, since EDTA makes conditions for polymerization of fibrin monomers less favorable (40). Nevertheless, in most of these experiments, less than 2 nmol of fibrinopeptide A were released for each nmol of fibrin clot formed, implying that some fibrin molecules in the formed clot had not released all of its fibrinopeptide A.

Thus it appeared that in this abnormal fibrinogen, complete release of fibrinopeptide A from the fibrinogen was not necessary for its conversion to cross-linked fibrin by thrombin. The process was slower than normal, however, and electron microscopic study of the fibrin suggested that it was not entirely structurally normal.

The exact site of the defect in the patient's fibrinogen was no determined. Studies of the NH-terminal disulfide knot (i.e., the portion of the fibrinogen molecule containing the fibrinopeptides) however, demonstrated that the defect was within this region.

Since the affected individuals with fibrinogen Cleveland II released about one-half the expected amount of fibrinopeptide A and since this defect appeared to be transmitted as an autosomal dominant trait, it was probable that the affected individuals were heterozygous for defective fibrinopeptide A release. Whether this was a result of two species of one of the polypeptide chains, one normal and one abnormal, making up the fibrinogen

molecule or two species of fibrinogen, one normal and one abnormal, could not be determined. However, two species of fibrinogen, presumably one normal and one abnormal, have been detected in the plasmas of affected individuals with some other abnormal fibrinogens (fibrinogen Baltimore [41], Cleveland I [18], Bethesda I [6], Zurich I [42] and Los Angeles [43]).

Fibrinogen Cleveland II is most likely a new form of inherited abnormal fibrinogen. At least five families have been described in which an abnormal fibrinogen demonstrated an abnormality in fibrinopeptide release. In fibrinogen Baltimore there was a slower than normal release of fibrinopeptide A, but upon prolonged incubation all of the fibrinopeptide A was released (5). In fibrinogen Detroit the release of fibrinopeptide A was apparently normal, but the fibrinopeptide B was not released (7). In fibrinogen Bethesda I both fibrinopeptides A and B were released abnormally slowly, but if incubation with thrombin was prolonged, total release finally occurred (6). Functionally, fibrinogen Cleveland II closely resembled fibrinogens Geissen (9) and Metz (8). Fibrinogen Geissen did not release its fibrinopeptide A (44), while fibrinogen Metz had a defect in total fibrinopeptide release; presumably this defect represented failure to release the fibrinopeptide A since there was no fibrinopeptide release with Reptilase (8). Both fibrinogens Geissen (45) and Metz (8) as well as fibrinogens Baltimore (46) and Bethesda I (6) had a mobility on immunoelectrophoresis that was different from normal fibrinogen, unlike fibrinogen Cleveland II.

By virtue of the demonstration of abnormal fibrinopeptide release, fibrinogen Cleveland II differed from those abnormal fibrinogens shown to have relatively normal fibrinopeptide release: fibrinogens Cleveland I (18), St. Louis (28), Troyes (8), Nancy (47), Zurich II (48), and probably Paris II (49). Fibrinogen Cleveland II had to be distinguished from those abnormal fibrinogens in which the fibrinopeptide release was unknown or unreported. Unlike fibrinogen Cleveland II, fibrinogens Amsterdam (50), Paris I, and Vancouver (46) migrated differently on immunoelectrophoresis from normal fibrinogen. Fibrinogens Parma (51) and Vancouver (52) did not inhibit normal clotting, unlike fibrinogen Cleveland II. Fibrinogens Leuven (53), Wiesbaden (54), and Iowa City (55) have been reported to have disordered fibrin monomer aggregation. The descriptions of these abnormal fibrinogens did not include characteristics that definitely distinguish these from fibrinogen Cleveland II. Patients with fibrinogen Leuven, however, had decreased plasma fibrinogen levels by all methods, whereas patients with fibrinogen Cleveland II had normal levels. The patient with fibrinogen Wiesbaden had repeated thrombotic episodes, whereas this was not a clinical feature of patients with fibrinogen Cleveland II.

Thus, it is probable that fibrinogen Cleveland II is a new abnormal fibrinogen that has as its unique property failure to release all of its fibrinopeptide A upon incubation with thrombin. Despite this defect, fibrinogen Cleveland II appears to undergo complete conversion from fibrinogen to cross-linked fibrin, although at a slower rate than normal.

## ACKNOWLEDGMENTS

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