Functional Evaluation of an Inherited Abnormal Fibrinogen: Fibrinogen "Baltimore"

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ABSTRACT The rate of clotting and the rate of development and degree of turbidity after addition of thrombin to plasma or purified fibrinogen from a patient with fibrinogen Baltimore was delayed when compared with normal, especially in the presence of low concentrations of thrombin. Optimal coagulation and development of translucent, rather than opaque, clots occurred at a lower pH with the abnormal fibrinogen than with normal. Development of turbidity during clotting of the abnormal plasma or fibrinogen was less than normal at each pH tested, but was maximal in both at approximately pH 6.4. The physical quality of clots formed from fibrinogen Baltimore was abnormal, as demonstrated by a decreased amplitude on thromboelastography. The morphologic appearance of fibrin strands formed from fibrinogen Baltimore by thrombin at pH 7.4 was abnormal when examined by phase contrast or electron microscopy, but those formed by thrombin at pH 6.4 or by thrombin and calcium chloride were similar to, though less compact, than normal fibrin. The periodicity of fibrin formed from fibrinogen Baltimore was similar to normal and was 231–233 Å.

A study of the release of the fibrinopeptides from the patient's fibrinogen and its chromatographic subfractions verified the existence of both a normally behaving and a defective form of fibrinogen in the patient's plasma. The defective form differed from normal in three functionally different ways: (*a*) the rate of release of fibrinopeptides A and AP was slower than normal; (*b*) no visible clot formation accompanied either partial or complete release of the fibrinopeptides from the defective form in 0.3 M NaCl at pH 7.4; and (*c*) the defective component possessed a high proportion of phosphorylated, relative to nonphosphorylated, fibrinopeptide A, while the coagulable component contained very little of the phosphorylated peptide (AP). The high phosphate content of the defective component did not appear to be the cause of the abnormality, but may be the result of an associated metabolic or genetic phenomenon.

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INTRODUCTION

In 1958 Imperato and Dettori (1) described a functionally defective fibrinogen in the plasma of a young girl with a congenital bleeding disorder, but no other affected individuals were encountered in a study of her family. Abnormal fibrinogens occurring in successive generations have been recognized in at least seven families (2–8). Abnormal fibrinogens inherited as dominants have been demonstrated (9, 10) in two additional families originally reported as having congenital hypofibrinogenemia (11) and factor XIII deficiency (12). The precise molecular defects of these abnormal fibrinogens remain unknown, although an amino acid substitution (arginine to serine) in the N-terminal portion of the α-(A)-chain (13) and a decreased content of carbohydrate (7) have been reported in one. A comparative study (9) of six of the abnormal fibrinogens (2–4, 6, 7, 11) suggested that at least five (2–4, 6, 7) are distinct. A nomenclature similar to that used to designate the abnormal hemoglobins has been suggested (3) for the tentative identification of inherited abnormal fibrinogens.

We have reported (3, 14) preliminary studies of an abnormal fibrinogen which has been designated fibrinogen Baltimore (3). These studies were performed on

the blood of a young woman with a history of recurrent thrombosis, pulmonary emboli, and mild bleeding. The amount of fibrinogen detectable in her plasma by immunologic techniques was normal. The rate of coagulation of her plasma or purified fibrinogen by thrombin was delayed. Addition of calcium ions accelerated, but did not normalize the rate of clotting by thrombin. No clot formed after addition of an extract of the venom of *Bothrops atrox* (Reptilase) to prothrombin-deficient plasma. Clots of recalcified plasma were insoluble in 5 M urea. There was no evidence of increased fibrinogenolysis or fibrinolysis. The present investigations provide additional information on the functional abnormalities and biochemical properties of fibrinogen Baltimore.

METHODS

Venous blood (10–90 ml) was obtained from the patient with fibrinogen Baltimore (3) or normal subjects using 18-gauge needles, silicone[1]-coated syringes, and 1/9 volume of 3.8% sodium citrate as anticoagulant. Plasma was separated by centrifugation at 1110 *g* for 20 min and used immediately or stored at −20°C. Larger volumes of plasma for preparation of purified fibrinogen were obtained from the patient and normal subjects by plasmapheresis using plastic bags containing acid citrate dextrose (ACD),[2] USP formula A as anticoagulant. Acetate-barbital buffer (15), tris-buffered saline (16) or phosphate buffer were used for stabilization of pH of plasma, fibrinogen or reagents, as indicated.

Bovine thrombin[3] (1000 NIH U per ml) was dissolved in barbital buffer, pH 7.4, stored at −20°C, thawed immediately before use, and diluted to the desired concentration with barbital buffer. Activity of the diluted thrombin was tested using normal plasma or purified fibrinogen during the course of each experiment. The rate of clotting of plasma or fibrinogen after addition of thrombin (i.e. thrombin clotting time) was determined at 37°C using a wire loop to determine the end point (16). The first appearance of fibrin threads on the wire loop was taken as the end point. Thrombin used for studies on fibrinopeptide release consisted of both bovine and human[4] material.

Fibrinogen was prepared from citrated or ACD plasma obtained from the patient or individual, normal subjects. Fraction I-2 was prepared by the method of Blombäck and Blombäck (17) and processed further (18) to remove cold-insoluble protein. Clottability of the preparations of purified fibrinogen was at least 95%, as determined by the spectrophotometric procedure of Laki (18). Purified fibrinogen was measured by the method of Ratnoff and Menzie (19) modified to allow maximal polymerization by adjusting the pH to 6.4 with 0.15 M phosphate buffer and incubation for at least 12 hr at 4°C.

Aggregation of preformed, dissolved fibrin was achieved by a shift of pH from 5.4 to neutrality, as originally suggested by Mommaerts (20). In some studies coagulation of preformed fibrin was evaluated by a modification of this method, as described by Forman, Ratnoff, and Boyer (6). In these studies, 0.5 ml of plasma was acidified with 1 ml of Michaelis buffer to obtain a final pH of 5.4. Thrombin (100 NIH U) was added and the mixtures were incubated at 37°C for various intervals. Portions (0.1 ml) of the mixture then were added to buffered hirudin to inhibit the enzymatic action of thrombin and neutralize the pH.

Changes in turbidity during coagulation of plasma or purified fibrinogen after addition of thrombin were measured at room temperature in a Beckman DBG spectrophotometer at 350 nm and recorded graphically. Mixing of the samples in the cuvette and closing of the shutter was achieved within 3–5 sec. The corresponding part of curves on graphs was extrapolated towards zero or, if initial turbidity was recorded, following the base line.

Fibrin was prepared for electron microscopy directly on carbon-coated collodion membranes, using a modification (21) of the method of Kay and Cuddigan (22).

The rate of release of fibrinopeptides was studied by treating samples (1 ml containing 1.8 mg fibrinogen) with thrombin (20 μl containing 0.024 U) at 25°C and then stopping the reaction after alloted times by precipitating protein with 5% trichloroacetic acid (TCA). The supernatant fluids were processed (23) to remove the TCA and salt, and then subjected to paper electrophoresis to separate the fibrinopeptides. Electrophoresis was performed as described previously (23), except that the buffer consisted of 0.045 M potassium orthoborate at pH 8.6. Quantitation was based on arginine content as determined with a modification (24) of Sakaguchi's reaction. Densitometry was performed with a Joyce Loebl (Gateshead, England) "Roboscan" fitted with a 500 nm wave-length filter.

RESULTS

The rate of clotting of the patient's plasma or purified fibrinogen after addition of thrombin (i.e. thrombin clotting time) was delayed when compared to normal plasma or fibrinogen in all of the test systems employed. Differences in the rates of clotting of the patient's and normal plasma by thrombin were accentuated by de-

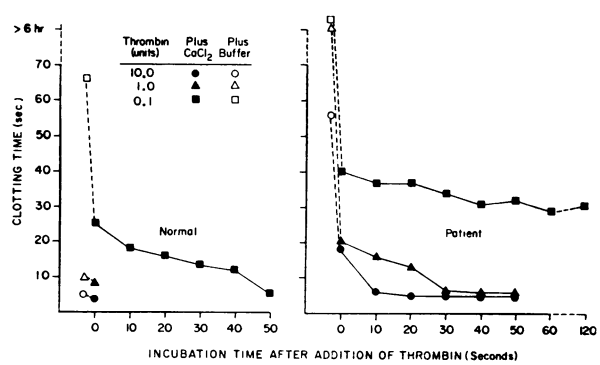

FIGURE 1 Effect of calcium on rate of clotting of plasma preincubated with thrombin. Thrombin (0.1 ml) was added to 0.1 ml of normal or patient's citrated plasma. After incubation for the indicated intervals, 0.1 ml of 0.025 M calcium chloride was added, and the clotting times determined. The open symbols indicate the clotting times after addition of 0.1 ml of barbital buffer, pH 7.5, rather than calcium chloride.

[1] G. E. Silicone SC-87 dri-film.

[2] *Abbreviations used in this paper:* AP, phosphorylated peptide; ACD, acid citrate dextrose; TCA, tricholoracetic acid.

[3] Thrombin, topical. Parke, Davis & Company, Detroit, Mich.

[4] Fibrindex, Ortho Diagnostics, Raritan, N. J.

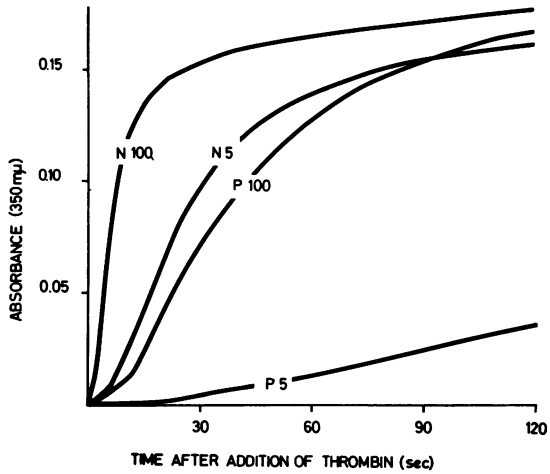

FIGURE 2 Effect of thrombin concentration on aggregation of fibrin. Increase in absorbance was recorded after addition of 0.1 ml of thrombin (5 or 100 U) to 0.8 mg of normal (N) or patient's (P) fibrinogen in 0.9 ml of 0.15 M barbital buffer, pH 7.4.

creasing the concentration of thrombin used to induce clotting (Fig. 1). Addition of calcium chloride simultaneously with the addition of thrombin accelerated the rate of clotting of the patient's plasma and normal plasma. However, the rate of clotting of the patient's

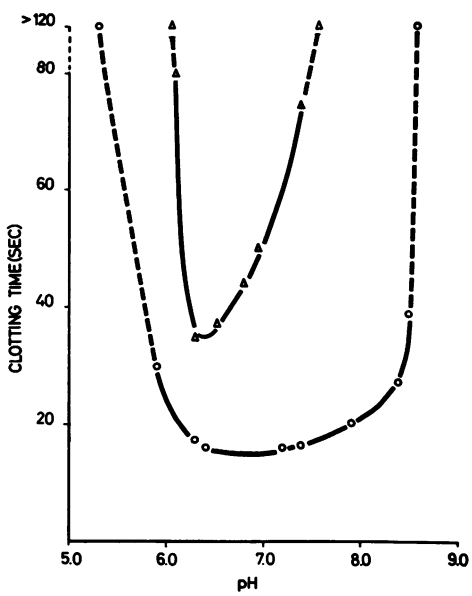

FIGURE 3 Effect of pH on rate of clotting of plasma by thrombin. Barbital buffer (0.3 ml, 0.1 M) at various pH's was added to 0.1 ml of citrated plasma from the patient (△—△) and a normal control (○—○). Clotting times were measured after addition of 0.1 ml of thrombin (1.0 U in 0.15 M NaCl). The pH values indicated were obtained after clotting had occurred or within 60 min after addition of thrombin.

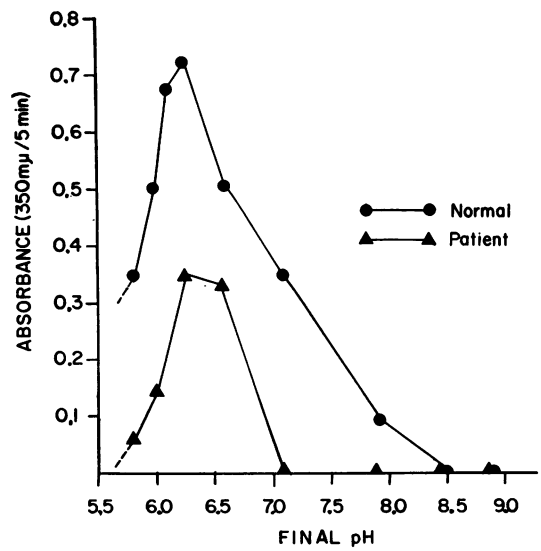

FIGURE 4 Effect of pH on turbidity of plasma clots. Plasma (1.0 ml) was diluted with 2.0 ml of acetate-barbital buffer to obtain the final pH values indicated. Formation of turbidity was measured 5 min after addition of thrombin (0.1 ml, 100 U).

plasma by thrombin was delayed as compared with normal plasma, even in the presence of calcium chloride (Fig. 1). Addition of calcium chloride to plasma preincubated with thrombin accelerated the rate of clotting of the patient's and normal plasma, but the results were dependent upon the concentration of thrombin employed (Fig. 1). Coagulation of plasma or fibrinogen also was observed by measurement of increase in turbidity which reflects formation and aggregation of fibrin under appropriate conditions. The rate of development and degree of turbidity after addition of thrombin to the patient's plasma or purified fibrinogen was markedly impaired as compared with normal (Fig. 2). Addition of calcium chloride enhanced, but did not completely normalize, the development of turbidity of the patient's fibrin in this test system.

Optimal coagulation of the patient's plasma occurred over a narrow range of pH around 6.3, whereas normal plasma clotted optimally over a wider range of pH (Fig. 3). Under these conditions, solid though translucent clots formed from normal plasma at pH 8.5 and at lower pH values the clots were opaque. The patient's plasma did not clot at pH values above 7.9 and translucent clots were noted at pH 7.2. The rate of development and degree of turbidity during clotting of the patient's plasma or purified fibrinogen also occurred over a narrower range of pH and was less than that of normal at each pH tested. Maximum turbidity was reached, both in normal and patient's plasma, between pH 6.3 and 6.5 (Fig. 4).

Coagulation of preformed, unpolymerized fibrin derived from normal or patient's fibrinogen was measured by the method of Forman et al. (6). Normal plasma that was preincubated with thrombin for 2 min clotted within 30 sec after neutralization and after preincubation for 16 min, within 22 sec. No visible clot formed after neutralization of the patient's plasma, which had been preincubated with thrombin for as long as 60 min, unless calcium chloride (0.1 ml, 0.05 M) was added at pH 7.4.

The rate of clotting of normal plasma by thrombin was not significantly inhibited by the patient's plasma (Fig. 5). The thrombin clotting time of normal plasma was prolonged by addition of greater than 25% by volume of the patient's plasma, but the degree of prolongation was less than that produced by addition of comparable amounts of buffer at each of the concentrations tested (Fig. 5).

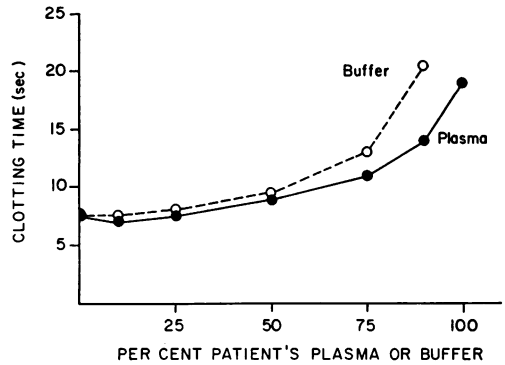

FIGURE 5 Effect of patient's plasma and buffer on rate of clotting of normal plasma by thrombin. Citrated normal plasma was mixed with either citrated patient's plasma or barbital buffer, pH 7.4 in the proportions indicated. Clotting times of 0.2 ml portions of the mixtures were determined after addition of 0.1 ml (1.0 U) of thrombin.

The physical quality of clots formed was evaluated by thromboelastography (Fig. 6). The amplitude obtained after recalcification of the patient's plasma, with or without the addition of platelets, was markedly decreased. The clots formed from mixtures of equal

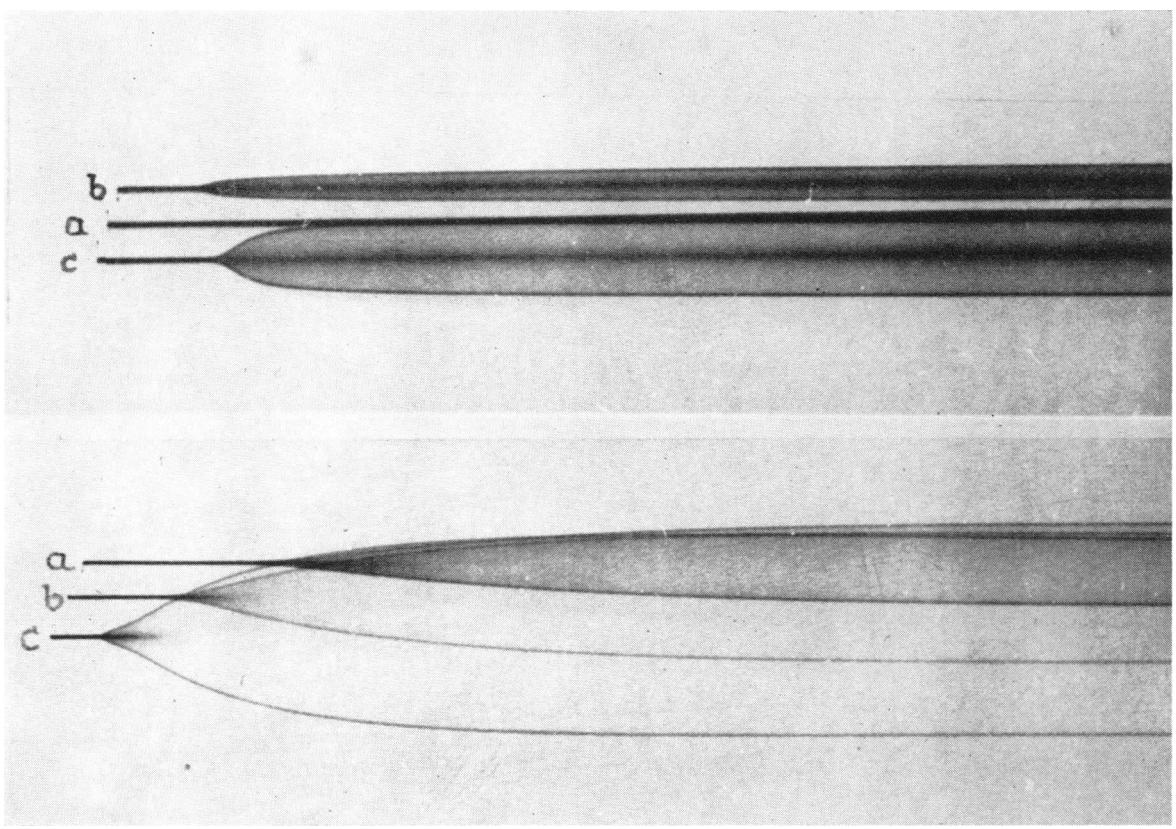

FIGURE 6 Thromboelastograms on: (a) patient's plasma, (b) mixture of equal portions of patient's and normal plasma, and (c) normal plasma. The incubation mixtures consisted of: 0.11 ml plasma + 0.11 ml barbital buffer (pH 7.4) containing 1% EDTA [top] *or* 0.11-ml washed normal platelets (400,000 per mm^3) in barbital buffer (pH 7.4) containing 1% EDTA [bottom] + 0.11 ml of 0.05 M calcium chloride.

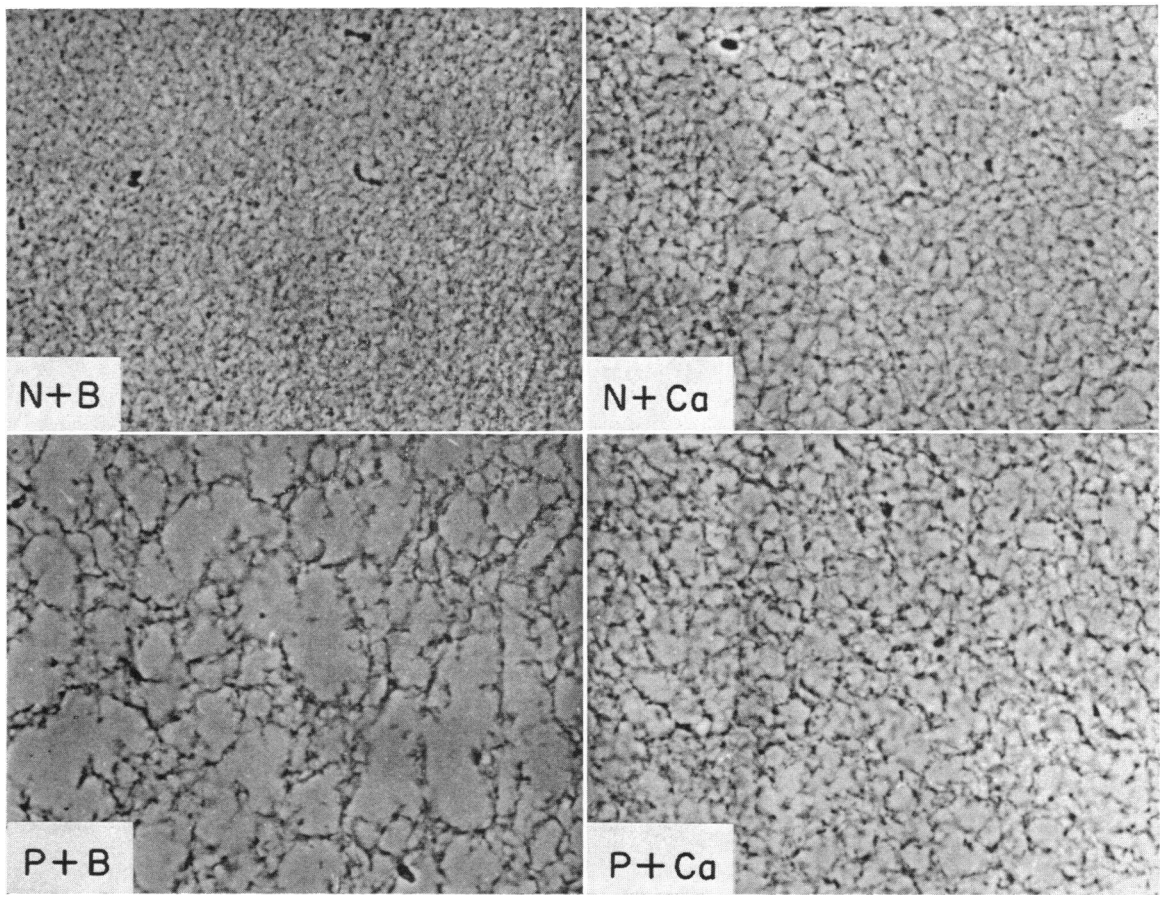

FIGURE 7 Morphologic appearance of clots of normal (N) and patient's (P) platelet-free plasma. Citrated platelet-free plasma (0.1 ml) was clotted with 1.0 U of thrombin in 0.1 ml of barbital buffer alone (B), pH 7.4, or in barbital buffer containing 0.05 M calcium chloride (Ca). 5 min after addition of thrombin, the clots were washed twice and fixed in 5% formol. Sections were stained with hematoxylin-eosin and photographed, using a phase contrast microscope. Original magnification × 600.

amounts of the patient's and normal plasma resulted in intermediate amplitudes. Addition of thrombin (10 NIH U), together with calcium chloride, to the patient's plasma failed to normalize the amplitude obtained on thromboelastography, even though conversion of fibrinogen into fibrin was complete under these conditions.

Morphologic studies by light and phase contrast microscopy of sections of clots formed after addition of thrombin alone to the patient's plasma revealed an irregular, fragmented appearance of the fibrin strands in contrast to the more regular, granular appearance of the normal (Fig. 7). The fibrin strands, formed by addition of thrombin and calcium chloride to the patient's and normal plasma, appeared relatively coarse and similar to each other (Fig. 7). Observations by electron microscopy demonstrated that the fibrin strands which formed from the patient's plasma after addition of thrombin alone at pH 7.4 appeared thinner and more fragmented than normal fibrin strands (Fig. 8). In other studies, the average diameter of the patient's and normal fibrin were similar and the abnormal fibrin appeared as a regular network after addition of either thrombin at pH 6.4 or thrombin and calcium chloride. However, at higher magnification, the fibrin strands derived from the abnormal fibrinogen appeared less compact than normal fibrin. The periodicity of the patient's fibrin and normal fibrin were similar and averaged 231–233 Å for both (Fig. 9).

Proteolytic attack of thrombin upon the patient's fibrinogen was demonstrated by the release of TCA-soluble peptide fragments. The peptides had the same electrophoretic mobilities as the fibrinopeptides from normal fibrinogen (Fig. 10). The rate of release of fibrinopeptide A and its congeners AY and AP (25)

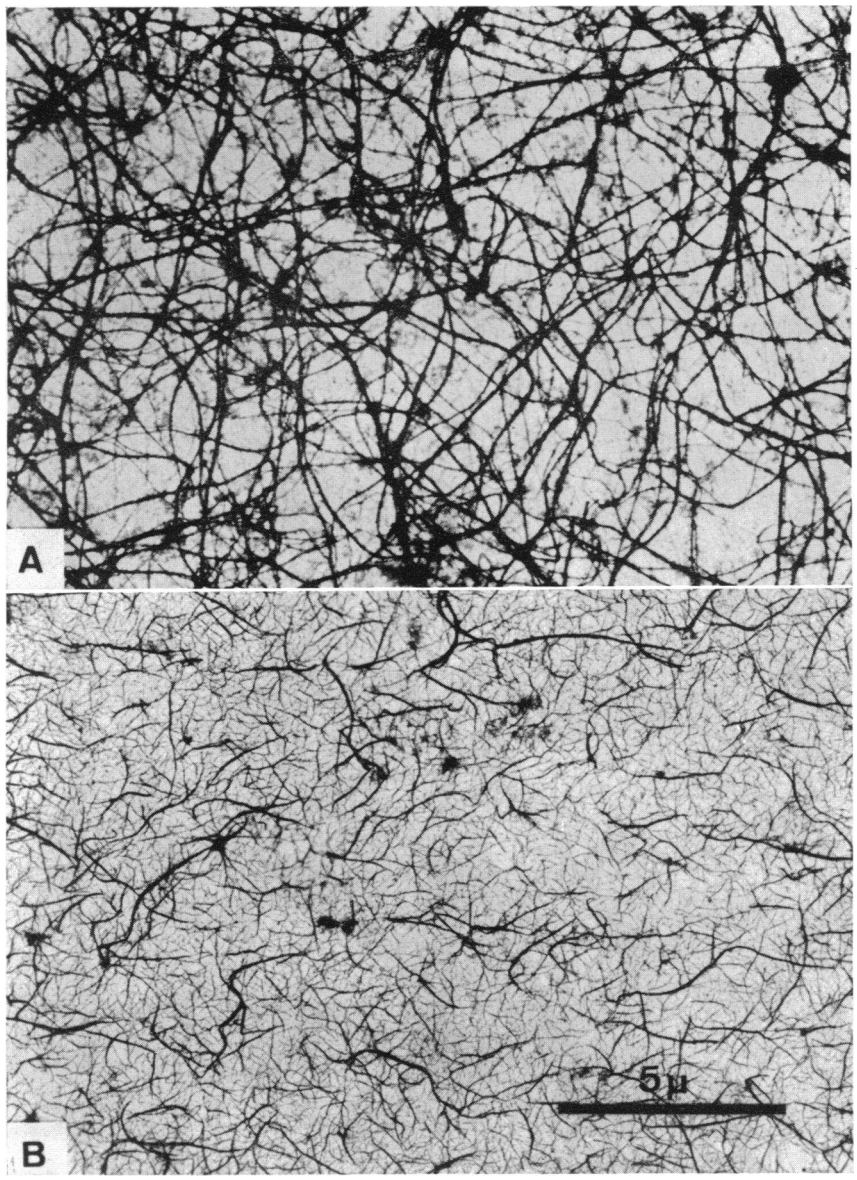

FIGURE 8 Electron microscopic appearance of normal (A) and patient's (B) fibrin. Films of plasma were immersed in thrombin (10.0 NIH U per ml) in barbital buffer pH 7.4 and incubated at room temperature for 2 min before fixation. Original magnification × 6500.

appeared somewhat slower than normal. A statistically significant difference was indicated by the fact that the A-fibrinopeptides (A + AY + AP) lagged behind normal in each of six comparisons made before the end of clot formation (< 120 min, Fig. 11). The ultimate yield of peptide was the same as normal, the sum total of fibrinopeptide A and its congeners equaling B and falling in the range of 1 μmole per 0.18–0.2 g equivalents of protein.

Another indication of a difference in rate of fibrinopeptide release was the observation that the release of fibrinopeptide AP from the patient's fibrinogen consistently lagged behind fibrinopeptide A (Fig. 10, mid-patterns). No such difference in release of fibrinopeptides A and AP from normal fibrinogen has been observed in our laboratory (Fig. 10, lower patterns). These observations suggested that there were two components in the patient's fibrinogen—one that was reacting with

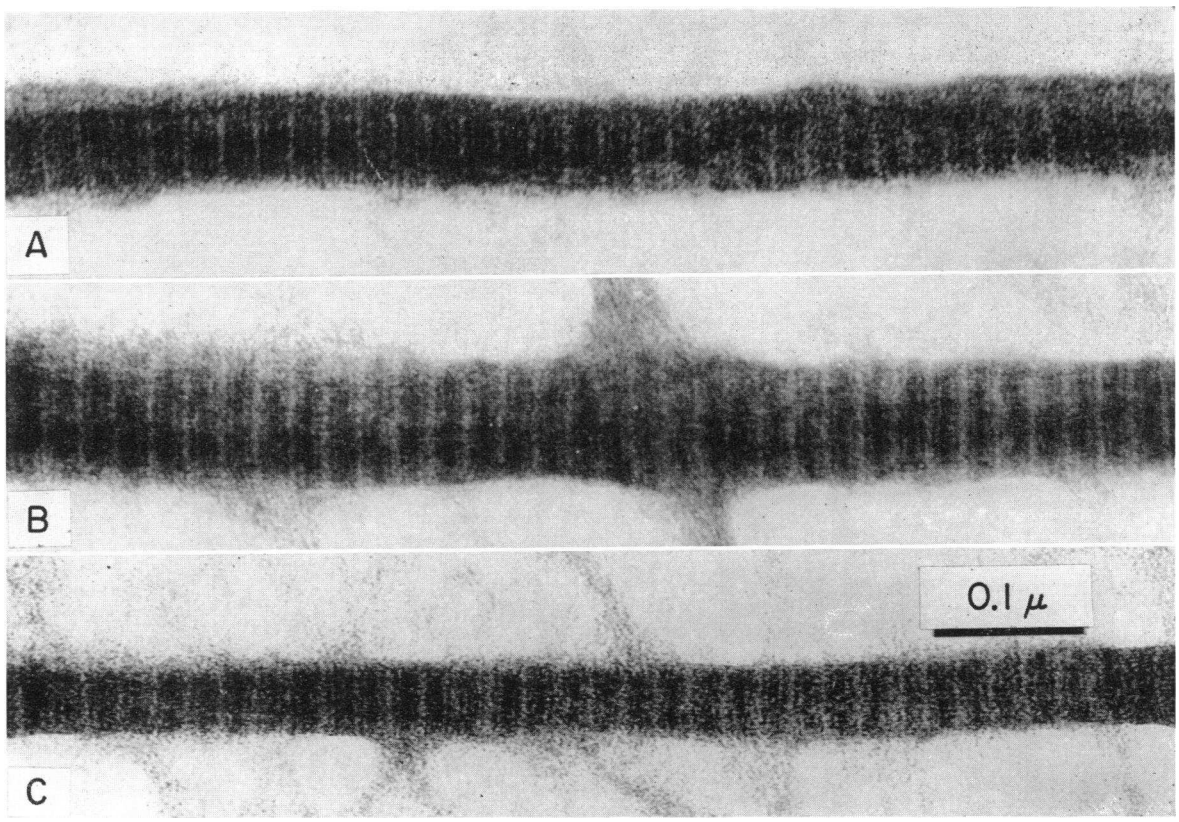

FIGURE 9 Periodicity of fibrin strands derived from normal and patient's plasma. Films of plasma were immersed in thrombin (10.0 NIH U per ml) in barbital buffer at pH 7.4 or 6.4 and incubated at room temperature for 2 min before fixation. Original magnification × 160,000. A, Fibrin from normal plasma clotted at pH 7.4; B, Fibrin from normal plasma clotted at pH 6.4; C, Fibrin from the patient's plasma clotted at pH 6.4.

thrombin at a normal rate and giving little fibrinopeptide AP, and a second that was reacting at a slow rate and giving the bulk of the fibrinopeptide AP.

Previous work (26) had shown that a defective form of fibrinogen was chromatographically separable from a normally behaving form of fibrinogen occurring in the patient's plasma. A separation of these two forms was made and their fibrinopeptide contents determined. As expected (26), the defective component was eluted in a boundary just behind the normally behaving component. The defective component gave no clot when treated with thrombin in 0.3 M NaCl, as in Fig. 11, while the normally behaving component clotted in the expected (26) way. Electrophoretic analysis of fibrinopeptides (Fig. 10, upper patterns) from the components verified that the defective component had reacted with thrombin. Aside from its poor clottability, the defective component was found to differ from the normal in a second respect, in that it had a fairly high proportion of phosphorylated, relative to nonphosphorylated, fibrinopeptide A (Fig. 10, upper patterns). The normally behaving component contained virtually no phosphorylated fibrinopeptide. Total yields of fibrinopeptide were the same for both subfractions of the fibrinogen, being of the order of 1 μmole of both the A group and the B fibrinopeptides per 0.2 g equivalents protein.

Enough material was available to make a single measurement of the relative rates of release of fibrinopeptides A and AP from the defective chromatographed fibrinogen. This was done by stopping the reaction after only 45 min incubation which, according to conditions of reaction, corresponded to the time at which two-thirds of fibrinopeptide A would be released from normal fibrinogen, as in Fig. 11. No preferential release of A, relative to AP, occurred with the defective fibrinogen itself, as demonstrated by the fact that the proportion of A to AP was near 1:1, just as it was after prolonged incubation (Fig. 10, upper patterns). Evidence that this particular reaction had indeed been stopped before completion was given by the fact that the amount of fibrinopeptide B was only one-fourth of the sum total of A and AP. Nonpreferential release of fibrinopeptide A

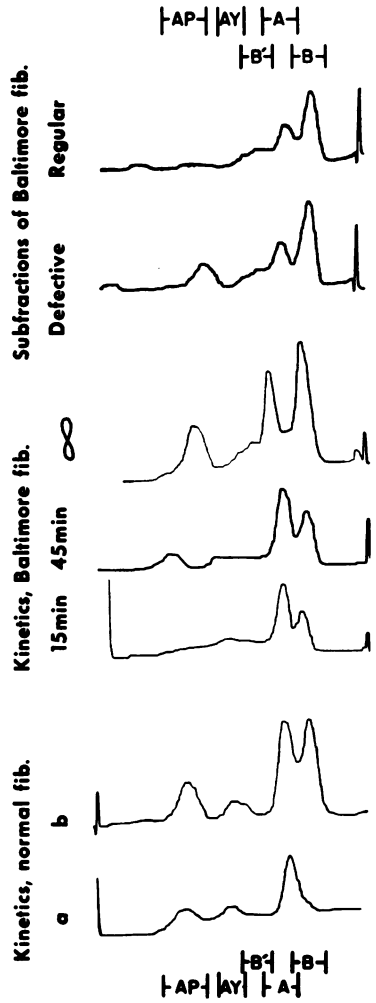

FIGURE 10 Electrophoretic patterns of the fibrinopeptides. The uppermost patterns show ultimate yields of peptides from the chromatographic subfractions of the patient's fibrinogen. Amounts of fibrinogen: 0.9 mg for the regular (normally) behaving subfraction and 1.2 mg for the defective subfraction. Thrombin: 2.4 U. Incubation: 120 min. The three tracings in the center illustrate the relative rates of release of the fibrinopeptides from the patient's unchromatographed fibrinogen, from which the release of fibrinopeptide AP lagged behind fibrinopeptide A. Amounts of fibrinogen and thrombin were 1.8 mg and 0.024 U, respectively. The measurements plotted in Fig. 11 were taken, in part, from these tracings. The last two tracings illustrate the concomitant release of fibrinopeptides A and AP from normal fibrinogen. Pattern A: yield of peptide from a large amount of fibrinogen (9.0 mg) and a proportionately large amount of thrombin (0.12 U) after only 10 min of reaction. Pattern B: yield from regular quantity (1.8 mg) after 120 min incubation with 0.024 U thrombin.

was demonstrated in still another way by alkylating (30) TCA-precipitates of the partially reacted protein and treating with thrombin again to complete the release of peptides. Such treatment gave small amounts of both AP and A in equal proportions. Thus, AP and A were released together, just as they are released together from normal fibrinogen, but both were released at a slower rate than normal. The reaction rate appeared to be on the order of one-half, or possibly an even smaller fraction of normal, as evident from the measurements on unchromatographed fibrinogen (Figs. 10 and 11) in which fibrinopeptide AP (stemming predominantly from the defective component) was released at only a fraction of the rate of A (which came from both the normal and defective components).

No consistent differences from normal were evident in the rate of release of fibrinopeptide B from the patient's fibrinogen (Fig. 11). Two forms of fibrinopep-

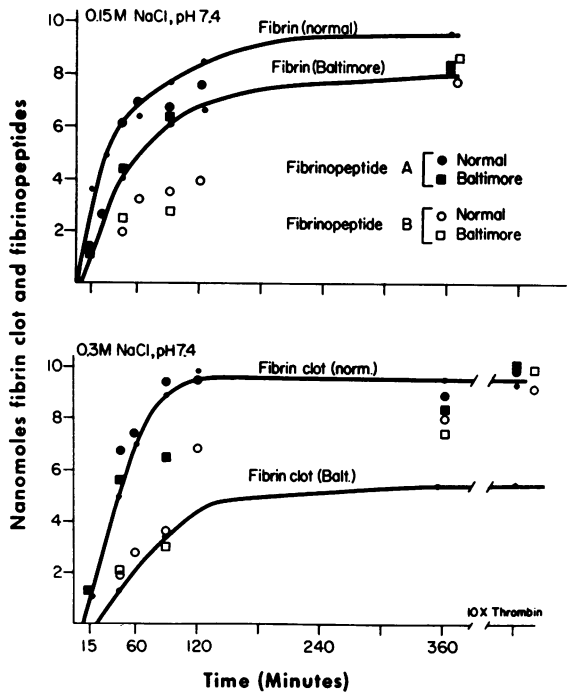

FIGURE 11 Comparison of clot formation and fibrinopeptide release from patient's and normal fibrinogen during incubation (25°C) with thrombin. Yields of fibrin clots are graphically portrayed by the solid lines, with actual measurements shown by the small sized plotted points. A chemical weight of 180,000 g/mole was assumed to express measurements in molecular terms. The plotted yields of fibrinopeptide A represent the summation of all of the A-group of fibrinopeptides (A + AY + AP), as determined from planimetry of the densitometric tracings of electrophoretic strips (*cf.* Fig. 10). Plotted yields of fibrinopeptide B likewise represent a summation of two portions which are shown and described as B and B' in Fig. 10. Frequent overlapping of boundaries of AY and B' often made it necessary to approximate the yield of each of these two minor components by assuming that relative amounts (AY/B') of each in the overlapping boundaries (AY + B') were the same as the relative amounts of the other A and B peptides, e.g. AY/B' = (A + AP)/B.

tide B occurred in both the normal and the patient's fibrinogen, a major component that was labeled B in Fig. 10, and a minor one labeled B′. The two were identified as being related because neither contained terminal amino groups and were accordingly presumed (*27*) to contain the pyroglutamic end group of fibrinopeptide B. The fast anodic migration of B, relative to B′, has been suggested, but not as yet proven to be due to a deficit of amide ammonia (*28*).

Since the release of fibrinopeptide A is essential to clotting (*23, 29,* 30), the slow release of the A fibrinopeptides provided a partial explanation for the slow rate of clot formation by the patient's fibrinogen (Fig. 11). This, however, did not comprise the only abnormality affecting coagulability. The incomplete yield of fibrin, despite ultimate release of fibrinopeptides in amounts equivalent to normal, appeared due to the poor coagulability of the defective component of the patient's fibrinogen. As already noted, no clot formation was observed when the chromatographically separated defective component was treated by itself with thrombin in 0.3 M NaCl at pH 7.4. As shown by the data of Fig. 11, only half of the patient's total (unchromatographed) fibrinogen coagulated after prolonged reaction with excess thrombin in 0.3 M NaCl. A somewhat greater, but still incomplete, ultimate yield of fibrin was obtained in 0.15 M NaCl.

DISCUSSION

Alterations of physicochemical properties of fibrinogen caused by structural aberration may influence any stage of conversion of fibrinogen to fibrin by thrombin, i.e. cleavage of peptides A and B, aggregation of fibrin molecules or clot stabilization. The exact structural defects of the known inherited abnormal fibrinogens (1–10) have not been fully characterized, but studies of the functional abnormalities have provided indirect evidence of the stage involved in some instances. An abnormality in the stage of peptide cleavage as the sole cause of abnormal coagulation has not been described. Abnormal aggregation of fibrin appears to be the predominant, functional defect in at least two instances (4, 6); and abnormal clot stabilization has been described in one (10).

The present studies have demonstrated that the rate of clotting of our patient's plasma or purified fibrinogen by thrombin was abnormal in all of the test systems employed, especially in the presence of low concentrations of thrombin. Development of turbidity, after addition of thrombin to fibrinogen, reflects primarily lateral aggregation of fibrin strands which increases at low pH and ionic strength (31). The rate of development of turbidity, after addition of thrombin to fibrinogen Baltimore, was delayed. In the presence of low concentrations of thrombin, a prolonged lag period was observed before turbidity increased. These observations were attributable to both a decreased rate of peptide cleavage and incomplete aggregation of fibrin. Calcium ions accelerate the aggregation of preformed normal fibrin, but have little effect on peptide cleavage (32). Addition of calcium ions accelerated the rate of clotting and increased the formation of turbidity, after coagulation of fibrinogen Baltimore by thrombin. The acceleration by calcium ions of coagulation of the patient's plasma that had been preincubated with thrombin (Fig. 1) suggests that some form of incompletely converted fibrinogen was present for significant periods of time after addition of thrombin. These results were consistent with the studies on purified fibrinogen, which indicated a partial defect in the cleavage of peptides by thrombin. The decreased cathodal mobility of fibrinogen Baltimore on immunoelectrophoresis (3, 14) and its delayed elution from anion exchange columns (*26*) suggest that this abnormal fibrinogen has an increased negative charge. The partial correction of the abnormal coagulation of fibrinogen Baltimore by calcium ions could be explained by a neutralizing effect on the abnormal charge.

A defect involving cleavage of peptide A of fibrinogen Baltimore was suggested previously (3), since Reptilase, which predominantly splits peptide A from fibrinogen (33), did not clot the patient's prothrombin-free plasma. The present results substantiated this possibility. The reason for the lag is not readily apparent, because peptide analysis of the known N-terminal portion of the α-(A)-chain (34), prepared from fibrinogen Baltimore, has not revealed any difference from that of normal fibrinogen by fingerprint of tryptic digests.[5]

Ferry and Morrison (31) noted that coarse clots formed when clotting of normal fibrinogen occurred below neutral pH, but that fine (i.e. translucent) clots formed when clotting occurred at higher pH values. The high degree of opacity of coarse clots has been correlated with a heterogenous distribution of fibers due to lateral interfibrillar aggregation (31). Formation of fine, translucent clots has been correlated with predominant end-to-end aggregation under conditions less favorable for syneresis (31) and may occur after cleavage of peptides A alone (33). Thus, conditions that favor predominant end-to-end aggregation of fibrin (i.e. high pH, low concentrations of thrombin or Reptilase) accentuate the coagulation defect of fibrinogen Baltimore. The transition from coarse to translucent clots occurred at about pH 7.2, after clotting of our patient's plasma, whereas this transition occurred at pH 8.5, after clotting of normal plasma. The effect of pH on the appearance of clots of fibrinogen Baltimore may be related to the increased negative charge of this abnormal fibrinogen,

[5] Blombäck, M., and B. Blombäck. Personal communication.

since Edsall and Lever (35) have demonstrated that binding of anions to normal fibrinogen produced translucent clots, even at pH 6.3.

The abnormal functional properties of fibrinogen Baltimore not only affected the rate of fibrin formation, but also resulted in clots which appeared more fragile than normal. Thromboelastography clearly demonstrated the abnormal physical properties (i.e. decreased amplitude) of clots derived from the patient's plasma, even in the presence of calcium ions or of additional thrombin. The addition of 50% of normal plasma to the patient's plasma did not completely normalize the physical properties of the clots as measured by thromboelastography. These results could be interpreted as indicating inhibition of formation of a clot with normal mechanical properties by the abnormal fibrinogen. However, only minimal inhibition of the rate of clotting of normal plasma by fibrinogen Baltimore was demonstrated by the other techniques employed. Indeed, mixtures of patient's and normal plasma, containing as little as 10% of the normal, clotted more rapidly than the patient's plasma alone, after addition of thrombin.

The patient's plasma contained two types of fibrinogen that were separable by chromatography (26)—a seemingly normal component that was fully coagulable, and a defective component that clotted poorly. The abnormal mechanical properties of clots derived from the patient's plasma or from mixtures of normal and abnormal plasma, may be the result of interaction of the defective with the normal monomers.

Morphologic studies of fibrin by light and electron microscopy revealed that the structure of fibrin formed by the patient's fibrinogen after addition of thrombin alone at pH 7.4, was irregular and single fibrin strands appeared less continuous, relatively thin and more transparent than normal fibrin strands. The appearance of fibrin formed by addition of thrombin and calcium chloride to the patient's plasma resembled normal in light microscopy. Slight, and as yet unexplained, differences were still suggested by electron microscopy when clotting occurred in the presence of calcium or at pH 6.4 instead of pH 7.4.

The relationship of these in vitro observations of the functional properties of fibrinogen Baltimore to some of the clinical symptoms, particularly thrombosis, observed in our patient remains unknown. Procedures for the preparation of purified fibrinogen for in vitro studies usually involve removal of cold-insoluble fibrinogen or other forms of altered fibrinogen, which (23, 24) may have biologic activity. Even though the defective component of the patient's fibrinogen appeared virtually incoagulable under simulated physiologic conditions, the possibility exists that thrombic derivatives of the fibrinogen might still form cryoprecipitates analogous to cryoprofibrin (24).

Further studies of the various abnormal fibrinogen variants, including identification of specific molecular defects and their associated functional and biologic effects, should provide a better understanding of the role of normal fibrinogen in hemostasis, thrombosis and wound healing. The studies may also be of use in detecting genetically determined differences in normal fibrinogen. Normal fibrinogen is known to vary in content of phosphorylated fibrinopeptide A, but for undetermined reasons. The high degree of phosphorylation associated only with the defective component of our patient's fibrinogen raises the possibility that the phosphate content may, in part, be genetically determined. Family studies (3, 9) indicate that our patient probably is heterozygous for the gene controlling the abnormal fibrinogen. Further study of the family, particularly with regard to fibrinopeptide AP, may prove useful in clarifying this question. The fact that normal fibrinogen may occasionally possess high content of phosphorylated fibrinopeptide, while exhibiting no defect in coagulation, indicates that the high content of phosphate in the patient's fibrinogen is not responsible for its defective coagulation. Yet, the converse possibility has to be considered, namely, that the defective qualities of the fibrinogen were, in part, responsible for its phosphate content. Perhaps, the defective fibrinogen was unable to undergo dephosphorylation as well as normal. Current studies in one of our laboratories [6] indicate that normal fibrinogen can be chromatographically separated into AP-rich and AP-poor species. Such separability will provide a means of assessing phosphorylation and dephosphorylation.

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