PRODUCTS OF FIBRINOLYSIS

by

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ABSTRACT

Many haemorrhagic complications have been attributed to increased intravascular fibrinolytic activity. The degradation products resulting from fibrinolysis have been shown to interfere in fibrin polymerization and have been observed in defective clot formation. Although considerable knowledge has accrued concerning the mechanism of fibrinolysis and the resulting products, areas of controversy and intermittent obscurity remain. The study presented herein was conducted to resolve, if possible, existing discrepancies and characterize with certainty intermediate and end proteolytic products. An effort was made to correlate experimental findings with clinical disease states.

The products of serial digestion of fibrinogen by plasmin were studied by immunodiffusion, immunoelectrophoresis and gel exclusion. Two products were observed. These products were identified as the D and E fragments, originally reported by Nussenweig et al. (1960). The D fragment was represented by a band located in the beta area of the electrophoretic field. This product was formed early in the digestion process and underwent no further degradation. The second product, a migratory band, probably representing a plasmin susceptible precursor of the E fragment, was initially observed within the locus of the D fragment. However, as digestion progressed the band migrated across the electrophoretic field to the prealbumin area, remaining in this position with exhaustive proteolysis. This

band represented the plasmin resistant E fragment end product. Under certain experimental conditions the hydrolysis of fibrinogen into the E fragment may apparently be reversed.

Serum samples from 196 hospitalized patients were studied for the presence of fibrinolytic intermediates and end products. Pathologic products were found in 10 of the 196 samples. Of these 10 samples, 8 contained D fragments, whereas 2 samples contained both the D and E fragments. Clinical-pathologic correlation indicates that demonstrable fibrinolytic products were associated with injury to the hepatic, renal and vascular systems. The small molecular weight E fragment was encountered only in instances of renal failure.

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INTRODUCTION

Bleeding diathesis secondary to intravascular thrombosis have recently been attributed to increased fibrinolytic activity. Fibrinolysis has been defined as the dissolution of the human blood clot or fibrin by a proteolytic enzyme; whereas, fibrinogenolysis refers to the enzymatic digestion of human fibrinogen.

Accumulating knowledge concerning a group of naturally occuring blood components primarily functioning in fibrinolysis or fibrinogenolysis has greatly facilitated the understanding of the fibrinolytic mechanism. The basic component of this mechanism is plasminogen, a proenzyme normally circulating as part of the globulin moiety of the plasma proteins. Plasminogen activated to plasmin, a proteolytic enzyme, is capable of degrading both fibrinogen and fibrin.

The products resulting from the degradation of fibrinogen and fibrin and the relationship of these products to disease states have stimulated considerable interest. Fibrinolytic products and fibrinogenolytic products appear to be immunologically indistinguishable and are, therefore, often referred to synonymously as fibrinolytic products. The end products of fibrinolysis have been defined as: (1) small polypeptide fragments accounting for approximately 30% of the parent fibrinogen molecule and, (2) two large molecular weight fragments accounting for the remaining 70%. Recent studies have focused upon the characterization of the two

larger molecular weight fragments labelled "D" and "E". These products have been implicated in the inhibition of the enzymatic conversion of fibrinogen to fibrin and appear to account for the abnormal clot formation often demonstrated in persons exhibiting increased fibrinolytic activity.

A well defined correlation between the products of fibrinolysis and the bleeding diathesis often encountered as a result of increased fibrinolytic activity has not been clearly delineated. Therefore, further studies are needed to elucidate fibrinolysis, the products of fibrinolysis and the relationship of fibrinolytic products to disease. Immunological procedures and other methods have been employed to study (1) the products of fibrinolysis resulting from the degradation of fibrinogen by commercial plasmin and, (2) the fibrinolytic products appearing spontaneously in the serum of hospitalized patients. The results of these studies, together with additional findings are presented.

REVIEW OF LITERATURE

I CLOTTING MECHANISM

In recent years, it has become apparent that the fibrinolytic process in man is in a more dynamic state than previously recognized and that aberrations in its mechanisms may contribute significantly to the pathogenesis of disease. Such consideration has stimulated investigative interest that has resulted in an increased number of publications. In as much as fibrin formation is the end product of coagulation process, consideration should be given to the clotting mechanism.

According to Miale (1967) the clotting process, leading to the fibrin clot, is represented by four interdependent phases (see Figure 1): Phase I, in which platelet factor 3 is released following the breakdown of platelets during the initiator or contact reaction; Phase II, in which plasma thromboplastin is formed; Phase III, in which thrombin is formed from prothrombin; and Phase IV, in which fibrin is formed from fibrinogen. Many workers prefer to combine Phase I and Phase II, since both phases result in the generation of thromboplastin. Potential resources for thromboplastin are the blood (intrinsic) and the tissues (extrinsic) (Seegers, 1962).

Phase I and II involve the generation of intrinsic thromboplastin from factors readily available in the blood. Following contact with a foreign surface inactive Hageman factor (factor XII)

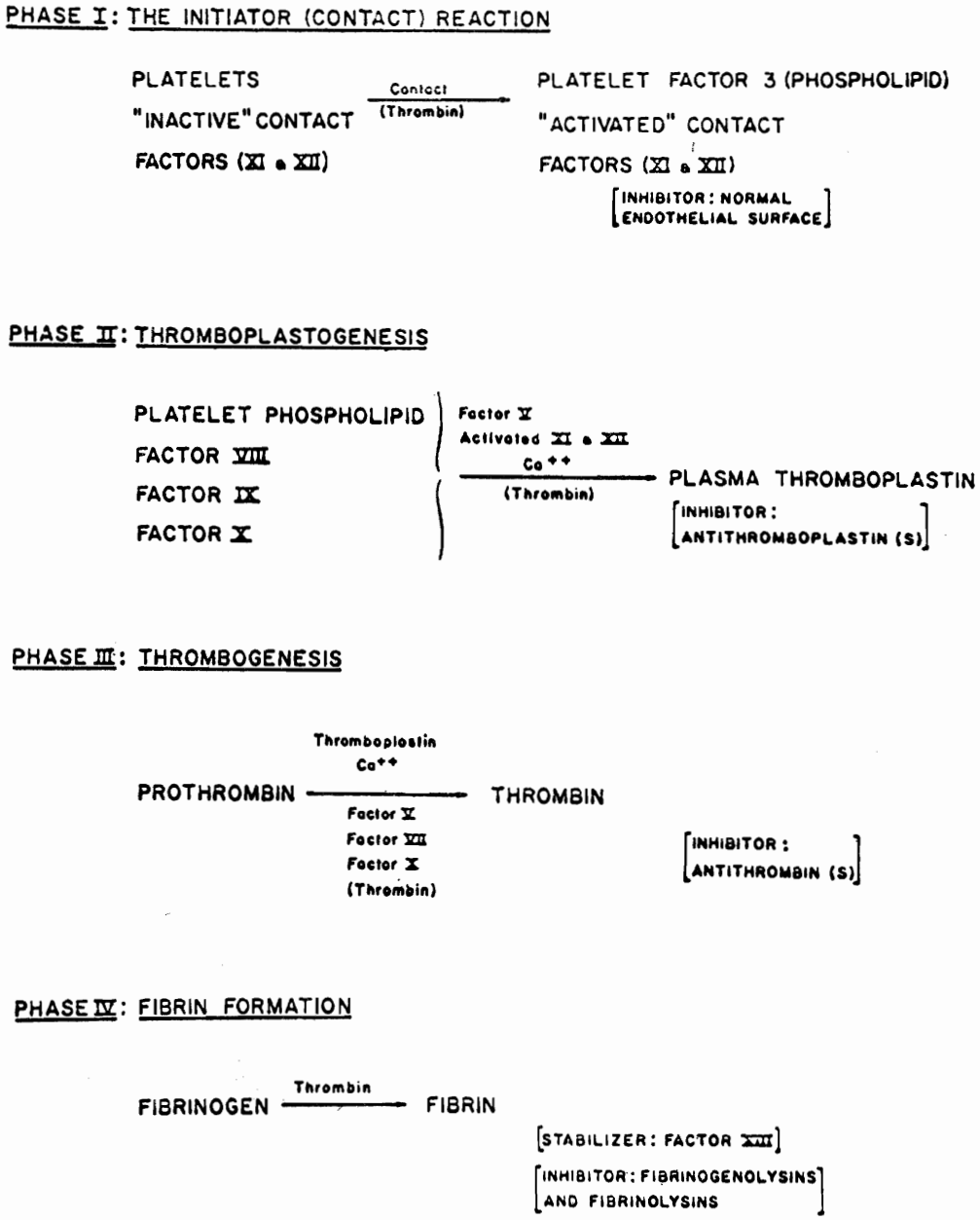


Fig. 1. Schematic representation of the process of blood coagulation. The division into 4 phases is arbitrary and is used only as a convenient scheme for classifying the hemorrhagic disorders and the tests used to study them. (Miale, 1967)

is converted to its active form. The activation of factor XII is followed by the activation of plasma thromboplastin antecedent (PTA or factor XI). In the presence of this product, plasma thromboplastin component (PTC or factor IX) is changed to its active form. Activated factor IX reacts with antihemophiliac factor (AHF or factor VIII), Stuart-Prower factor (factor X) and ionized calcium (factor IV) to form a substance called intermediate product I. Intermediate product I reacts with platelet phospholipid to form intermediate product II. Finally, intermediate product II combines with proaccelerin (labile factor or factor V) to form intrinsic blood thromboplastin (Rodman, 1968). The generation of intrinsic thromboplastin is complex, time consuming and usually involves about 60% of the total time required for coagulation. An alternate, more rapid route to clot formation occurs following injury to tissues whereby blood is exposed to body tissues rich in intrinsic thromboplastin.

Prothrombin is an alpha globulin produced in the liver, requiring vitamin K for its synthesis, and is consumed during the clotting process. One molecule of prothrombin probably yields two molecules of thrombin (Rodman, 1968). Activated, purified prothrombin yields thrombin at a concentration of about 2,700 units/mg of dry material. Estimates indicate the concentration of prothrombin in normal plasma is about 12 mg/100 ml (Seegers, 1967).

The activation of prothrombin, then, results in the formation

of thrombin, a highly specific esterase (McKay, 1965). This enzyme acts upon the fibrinogen molecule to produce the resultant fibrin monomer. Fibrinogen (factor I) is a plasma protein normally present at concentrations of 300 to 500 mg/100 ml plasma (Quick, 1966). The molecular weight of this protein in man has been estimated at 350,000 (Schultze, 1966). Electron micrographs have been obtained of the shadow cast bovine fibrinogen molecule establishing the general morphology and dimensions in the dry state (see Figure 2). The molecule consists of a linear array of three nodules held together by a very thin thread. The two end nodules are alike, but the center one is slightly smaller. When polymerization occurs, the fibrinogen molecule is altered (see Figure 3). Electron micrographs of fibrin indicate the fibrinogen molecule shrinks in length during the fibrinogen-fibrin conversion. The cross bands binding the fibrinogen molecules together are also apparent (Hall and Slayter, 1959).

Using fluorescent antibody techniques, Forman and Barhard (1964) have shown that fibrinogen is synthesized in the liver and have identified the liver parenchymal cells as the exact site of production. Fibrinogen is formed in the liver at a rate governed by its concentration in plasma. When circulating fibrinogen levels are depressed, synthesis can be enhanced by as much as eight times the normal rate. Fibrinogen and fibrin are composed of six peptide chains (see Figure 4). During the conversion of fibrinogen to the

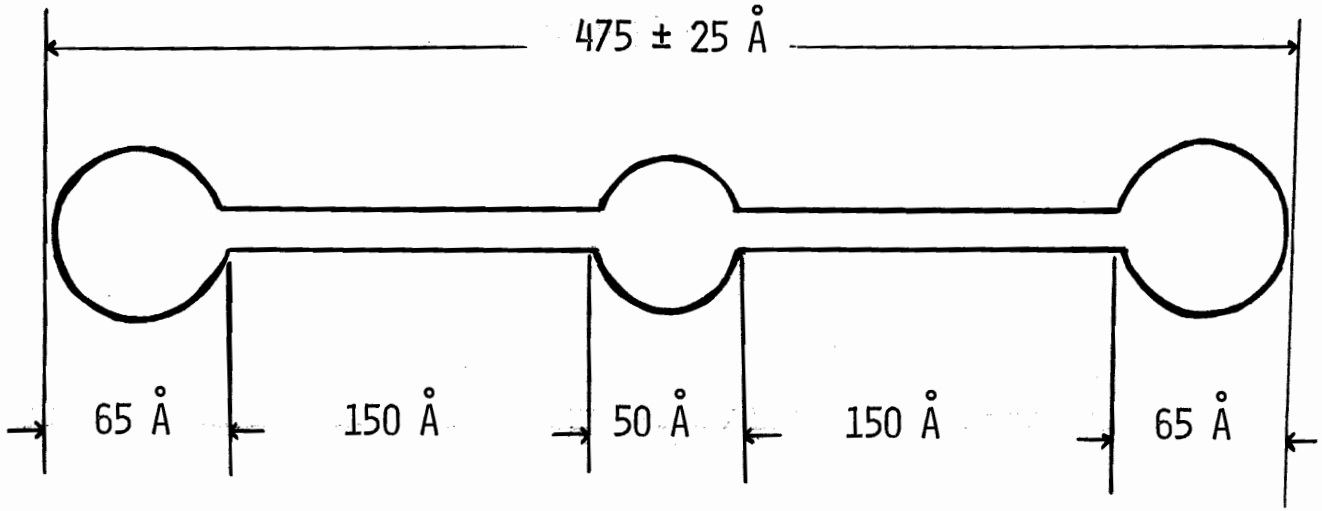


Fig. 2. A structural model of the fibrinogen molecule based on data derived from electron microscopic and other physicochemical observations. (Hall and Slayter, 1959)

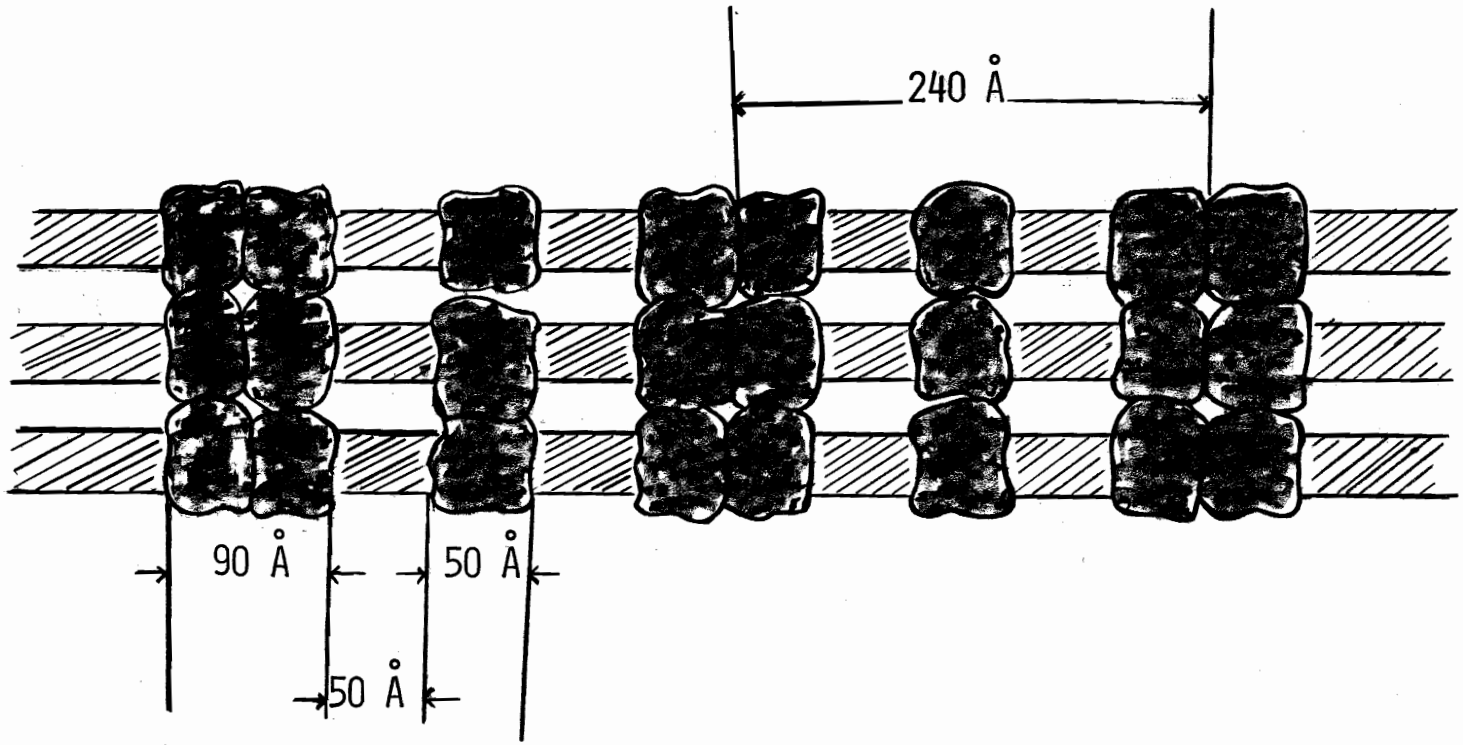


Fig. 3. Model of fully polymerized fibrin molecule. (Hall and Slayter, 1959)

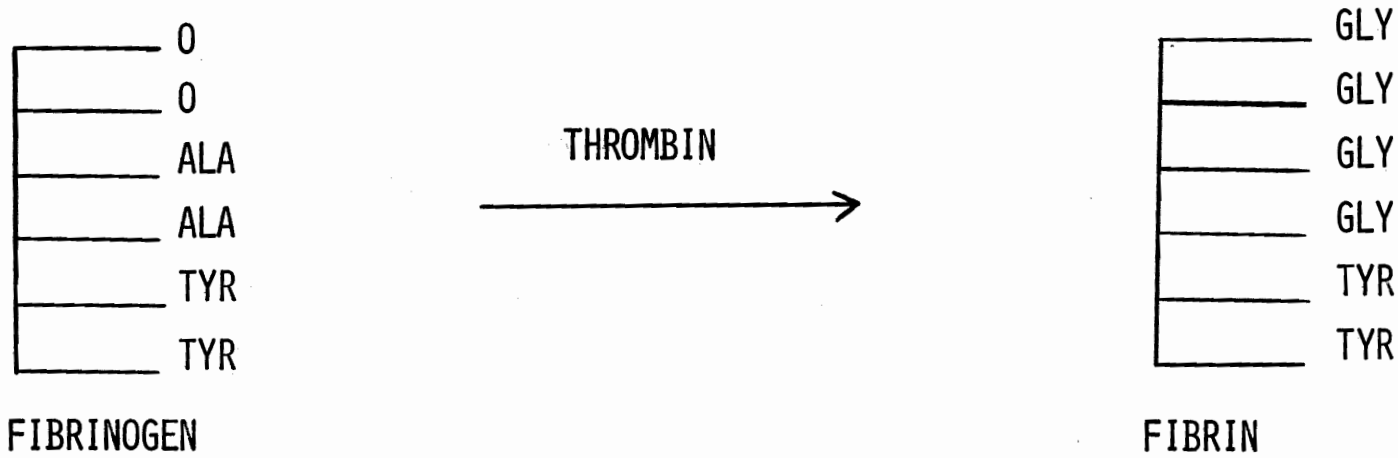


Fig. 4. A schematic drawing of the terminal amino acid residues on the peptide chains of fibrinogen fractions isolated from human plasma. The action of thrombin produces fibrin with identical terminal amino acid residues. The end groups found on the polypeptide chains include alanine (Ala), tyrosine (Tyr), glycine (Gly), and unidentified amino acid groups (O). (Adapted from Searcy, 1969)

fibrin monomer, four of the terminal amino acid residues in fibrinogen are replaced with glycine (Searcy, 1969). During the action of thrombin, arginyl-glycine bonds are disrupted in the fibrinogen molecule and peptides are separated from the molecule (Gladner et al., 1964). This action causes a loss of negative charges from the remaining fibrin monomers. Polymerization of the molecules occurs by electrostatic attraction. Fibrinogen-fibrin conversion takes place in stages and depends on environmental conditions such as pH and ionic strength. The fibrin monomers first form an intermediate polymer by lining up end to end. These polymers then line up side to side to form the subsequent course fibrin strands (Latallo et al., 1962a).

In 1960, Laki et al., suggested that the thrombin-induced conversion of fibrinogen to fibrin clot is a two phase process: first, an enzyme action modifying fibrinogen to fibrin; and second, the cross linkage of fibrin molecules.

Lorand and Jacobsen (1958) have described a factor known as the fibrin stabilizing factor (FSF). These investigators contend that the naturally occurring insoluble fibrin clot is a co-polymer of fibrin and the fibrin stabilizing factor. These workers demonstrated that when fibrinogen is clotted in vitro by thrombin in the presence of calcium ions and fibrin stabilizing factor a clot is formed resembling the fibrin clot formed in vivo. In the absence of fibrin stabilizing factor, a mechanically weaker clot is formed that can be

dissolved in 5M urea or monochloracetic acid.

Upon further study, Lorand (1965) suggested that the enzyme, thrombin, has a dual role in catalyzing two overlapping reactions. The first reaction induces the polymerization of fibrinogen, and the second activates the fibrin stabilizing factor causing cross-linkage of the fibrin molecule, thus supplying the stabilizing effect.

Nussbaum and Morse (1964) determined the fibrin stabilizing factor (FSF) activity in the plasma of patients with various disease states. These investigators found diminished fibrin stabilizing factor activity in 28 of 86 patients with liver disease, 16 of which had severe cirrhosis in the terminal phase of the disease. Diminished FSF activity was also found in 21 of 28 patients with metastases to the liver.

Other workers (Alimi et al., 1968) have shown that fibrin clots formed in the presence of fibrin stabilizing factor, Factor XIII, are more resistant to plasmin and other lytic enzymes than clots formed in the absence of Factor XIII. These workers also noted reduced fibrin stabilizing factor activity in patients with liver disease, suggesting that Factor XIII may be synthesized in the liver.

II FIBRINOLYSIS

The process of fibrin clot digestion is called fibrinolysis. Fibrinolysis undoubtedly represents an important mechanism in the

maintenance of the integrity of the vascular lumen (McKay, 1965). The dissolution of human blood clots has been reported in the literature as early as 1794, but it was not until 1905 that Nolf, one of the great pioneers in blood coagulation, introduced the view that fibrinolysis was due to a proteolytic enzyme in plasma, and that indeed the coagulation process itself was of a proteolytic origin with fibrinolysis representing the end stages of the process. Later, Ratnoff (1955) modified Nolf's concept by suggesting that the fibrin proteolytic activity of plasma is activated simultaneously with the clotting process, thus permitting the destruction of fibrin. It is now, however, generally agreed that the processes involved in clotting are distinct from those involved in fibrinolysis (Sherry and Alkjaersig, 1957). A review of current concepts concerning fibrinolytic mechanisms follows.

Fibrinolysis is controlled *in vivo* by an enzymatic process. This process involves the conversion of the proenzyme, plasminogen, (143,000 molecular weight) into a proteolytic enzyme, plasmin (molecular weight 108,000). Plasminogen normally circulates in an inactive form as part of the globulin moiety of the plasma proteins (Rodman, 1968). When plasminogen is converted to the proteolytic enzyme, plasmin, soluble peptide fragments are released (Alkjaersig *et al.*, 1958a). Activated plasminogen or plasmin was originally called fibrinolysin, but it has been demonstrated to be a potent proteolytic enzyme resembling pepsin. This enzyme can digest a host

of protein substances including fibrinogen, fibrin, Factor V, Factor VIII, some components of complement, corticotropin, growth hormone and glucagon. Additional protein substrates hydrolyzed by plasmin and used for assaying plasmin are: casein, gelatin, beta-lactoglobulin, azocal, hide power and protamine complexes (Fletcher et al., 1962a; Sherry et al., 1966). Although generated plasmin can participate in at least three distinct reactions, physiologically the lytic action upon fibrin is favored (see Figure 5). Fibrin is capable of competing with antiplasmins for plasmin. The latter attacks proteins other than fibrin as previously described, but only when the lytic factor is produced in great excess (Searcy, 1969).

The conversion of plasminogen to plasmin is an enzymatic reaction mediated by activator substances called kinases. Many tissues of the body, particularly vascular intima, lung, pancreas, and prostate contain activator substances capable of splitting the inactive plasminogen from the globulin molecule and converting it to its active form, plasmin (Rodman, 1968). Activators, as described today, were originally considered to be fibrinolysins. These fibrinolysins were first demonstrated in certain strains of hemolytic streptococci and later correlated with the activation of the proenzyme plasminogen.

A substance from filtrates of broth cultures of certain strains of hemolytic streptococci capable of inducing rapid fibrinolysis of human blood clots was first observed by Tillett and Garner in 1933.

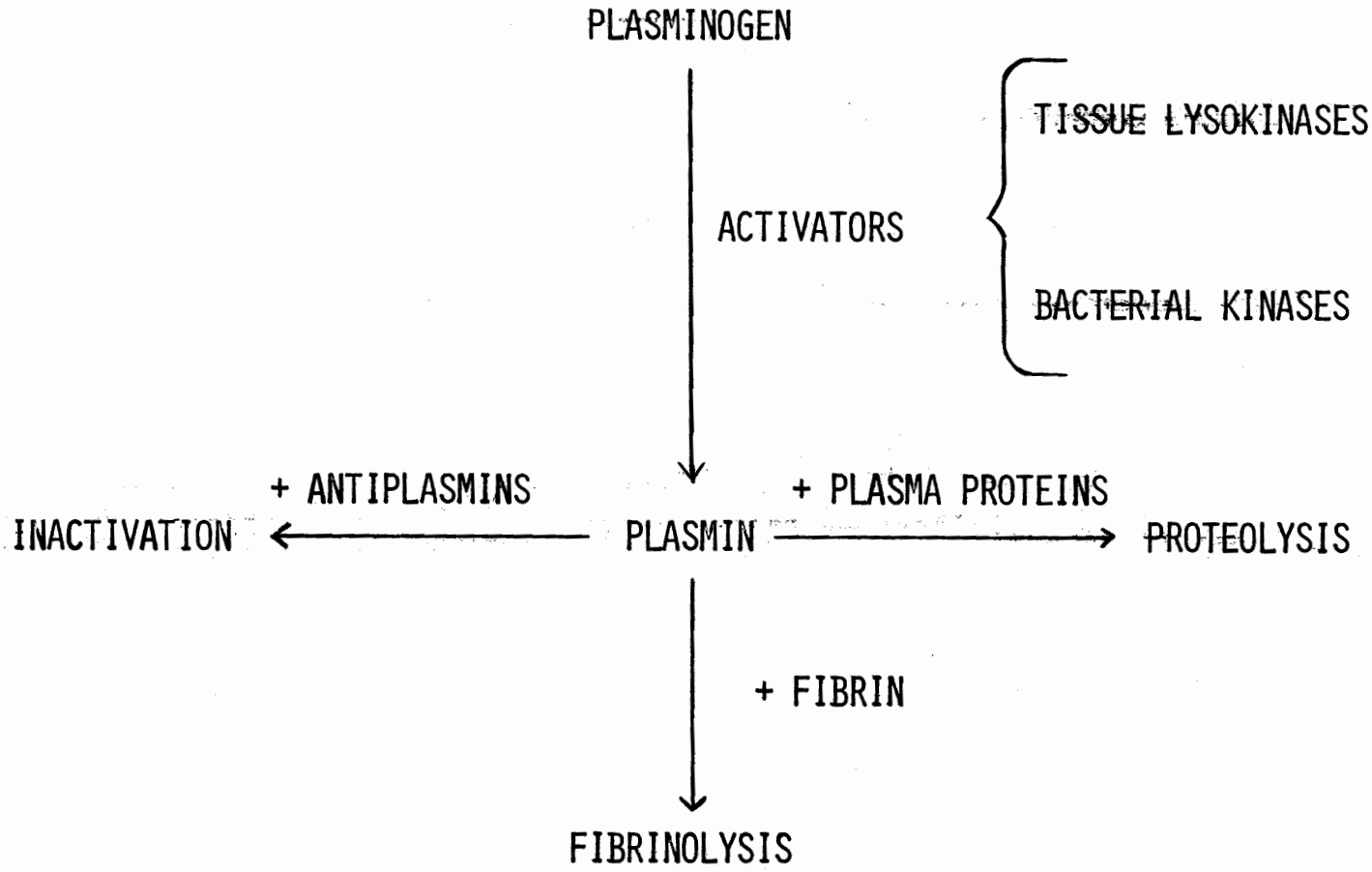


Fig. 5. A simplified scheme of the multiple actions of plasmin. (Searcy, 1969)

This substance was named streptococcal fibrinolysin. Later, Milstone (1941) demonstrated that the streptococcal fibrinolysin would not lyse thrombin induced fibrin clots when the substrate was highly purified human fibrinogen. Milstone noted, however, that if a small amount of euglobulin was added from human serum, rapid lysis of the clot resulted. Milstone named this substance the plasma lysing factor and reported that it interacted with streptococcal fibrinolysin to form an active lysing system. Later, other workers (Kaplan, 1944; Christensen, 1945) showed that the plasma euglobulin which Milstone had described was an inactive precursor (plasminogen) of a proteolytic enzyme (plasmin) which was rapidly activated by streptococcal fibrinolysin. Christensen (1945) suggested that since the streptococcal substance was not a fibrinolysin, but instead an activator, that streptococcal fibrinolysin be renamed streptokinase.

To date, plasminogen activator has been found in the plasma (plasma activator) of various tissues, as well as in the urine (urokinase). Alkjaersig et al., (1958b) demonstrated that plasminogen is activated by urokinase, streptokinase, trypsin and autocatalytically by plasmin. Although the possible identity or the relationship of these activators is presently unknown, Fletcher et al., (1962a) suggested that urokinase may represent excreted plasma activator and that a similarity exists between tissue activator and urokinase. This observation led to the theory that tissue activator is the primary source, that plasma activator is its product

and that urokinase is its excretion form. Mullertz (1955) maintains there is evidence that streptokinase does not react directly with plasminogen, instead a chain reaction is initiated. Plasminogen interacts with a "proactivator", which is converted to an activator, which in turn catalyzes the plasminogen-plasmin reaction.

Numerous studies have been performed to characterize the plasmin activator. Troll and Sherry (1955) have shown that the activation of human plasminogen by streptokinase involves two steps: first, the stoichiometric interaction of streptokinase with a plasma activator; and second, the enzymatic activation of plasminogen by this activator. Alkjaersig et al. (1959b) investigated the respective roles of plasminogen activators and plasmin by means of test systems using ^{131}I labeled clots containing various concentrations of plasminogen. Other studies on plasminogen activators in human plasma clots indicate that the quantity of plasminogen activators varies in response to stress, drug administration, and disease (Alkjaersig et al., 1959a; Sawyer et al., 1960a; Sherry, 1959b). Further studies, Sawyer et al., 1960b; Beck and Jackson, 1966, have compared the effects of plasminogen activators and proteolytic enzymes on fibrinolysis. These investigators discovered that plasminogen activators added to a fibrin clot would initiate the lysis of more fibrin per unit fibrinogen than the addition of either plasmin or trypsin.

A counter effect to proteolysis is evident in the competition for plasmin by antiplasmin (see Figure 5). Inhibitors of plasmin are found

in the circulating blood as well as in other tissues. McFarlane and Biggs (1948) found plasmin inhibitors in saline extracts of human liver, kidney, spleen, adrenal, thyroid, muscle, lung, heart, and brain. Fletcher et al. (1959a) showed that the concentration of plasma antiplasmin averages approximately five casin inhibitory units per milliliter and exceeds the plasminogen content of plasma, which is 3.8 casein units per milliliter. Gallimore and Shaw (1967) demonstrated plasmin inhibitors in serum by studying the lysis of fibrin clots prepared from plasminogen-deficient fibrinogen. In a system containing no plasminogen, the clot lysis time was inversely proportional to the concentration of added plasmin. When serum was included, a reduction of reciprocal lysis time resulted, the extent of which varied linearly with the amount of serum added.

Recently, Jacobson (1968a) demonstrated other controls of plasmin inactivators in his description of a modified caseinolytic method to measure the proteolytic activity in plasma. His studies indicated that in normal populations there was a large variation between individual plasma samples, probably due to plasminogen-plasmin levels. Of significance was the variation produced as a result of hormone influence. Increased proteolytic activity was found in pregnant women and women receiving oral contraceptives.

Further studies (Jacobsen, 1968b) were made concerning "proteolytic capacity" (i.e. the maximal proteolytic activity that can be generated in plasma with the inhibitors present) in several persons

of the same family. These studies suggested that the property of "low proteolytic capacity" was inherited as an autosomal dominant trait. However, no clear cut inheritance could be found concerning the property of "high proteolytic capacity".

III DEGRADATION PRODUCTS OF FIBRINOGEN

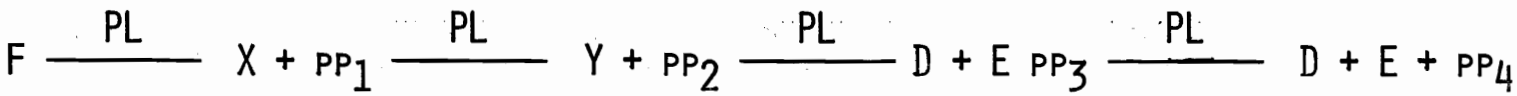
Godal and Helle (1963) compared the influence of fibrinogenolytic and fibrinolytic products in the last stages of coagulation and reported that fibrinogen breakdown products prolong the thrombin time of plasma more effectively than the breakdown products of fibrin. However, this may be an erroneous assumption. Hirsh et al. (1965) compared the proteolysis products of fibrinogen and of fibrin and found no qualitative or quantitative differences in their actions. From these studies it would appear that distinguishing fibrinogen breakdown products from the breakdown products of fibrin is difficult. Later work by Beller and Mak, (1967) indicated that proteolytic products derived from fibrinogen are immunologically indistinguishable from the proteolytic products of fibrin.

During the past decade, a great deal of research has been done in an effort to characterize the proteolytic products of fibrinogen. Nussenwieg and co-workers (1960) described at least four antigenic groups resulting from plasmin proteolysis of fibrinogen. Fletcher et al. (1962) suggested that only two of the four fractions (D and E located in the alpha-beta electrophoretic regions) are antigenically reliable, i.e., demonstrable immunologically. During plasmin

digestion, intermediate breakdown products are formed, but the end result is plasmin-resistant fragments. Some of the plasmin-resistant fragments contained the antigenic Group D and some Group E. Other smaller fragments exhibited no antigenic qualities. Jamieson (1963) suggested that the two larger fragments (D and E) are aggregates of these lower molecular weight pieces.

Other workers have shown by density gradient centrifugation that during proteolysis of fibrinogen molecular components were formed with respective values of 5.6S, 6.27S, 3.0S, and 1.4S (Fischer et al., 1963; and Alkjaersig and Fischer, 1964). The two larger fragments (D and E) contain individual antigenic determinants that are resistant to further action by plasmin. These fragments have been isolated by a number of workers using various techniques: continuous flow paper electrophoresis, (Triantaphyllopoulas and Triantaphyllopoulas, 1962); gel titration chromatography on Sephadex$^{(R)}$(Laursen and Gormsen, 1967; Nilehn, 1967a); simple radial diffusion, turbidimetric assays (Beller and Maki, 1967); immunoelectrophoresis (Lewis and Wilson, 1964; Nilehn and Nilsson, 1964; and Nilehn, 1967b) and plasma electrophoresis in narrow pore acrylamide gel (Fletcher, 1965).

Recently, Kowalski (1968) summarized these studies characterizing the physical and immunologic properties of the degradation products of fibrinogen and proposed a simplified scheme of the reactions representing the sequence of events occurring during plasmin fibrinolysis. (see Figure 6) Two main groups of products were



F = fibrinogen

PL = plasmin

PP = small polypeptides or peptides

X AND Y = intermediate fragments

Fig. 6. Adapted from Kowalski (1968).

recognized: small polypeptide fragments, soluble in trichloroacetic acid (TCA), and high molecular weight fragments. The TCA-soluble fragments, formed in the course of fibrinogen proteolysis by plasmin, were released at a steady rate during the reaction. After exhaustive proteolysis, these fragments contained about 30 per cent of the total nitrogen derived from fibrinogen. The fragments of high molecular weight underwent a number of changes leading to the formation of several intermediates. These intermediates underwent further proteolysis to form the two fragments referred to by Nussenwieg as "D" and "E". (see Table I) Fragment D is thermolabile and has a reported molecular weight of 80,000. Fragment E is thermostabile and has a molecular weight of 30,000. One mole of fibrinogen (molecular weight 350,000) during proteolysis yields two moles each of D and E fragments.

IV EFFECTS OF FIBRINOGEN BREAKDOWN PRODUCTS ON CLOTTING

In 1959b Fletcher et al. demonstrated that purified fibrinogen, partially digested by plasmin, clotted slowly on treatment with thrombin, and when added to normal fibrinogen inhibited clotting by thrombin. These investigators suggested that the accumulation of fibrinogen breakdown products and the development of defective fibrin polymers is a significant abnormality, and that the biochemical lesion of defective fibrin polymerization may be comparatively common and relevant to the hemorrhagic complication encountered in many diseases.

Table 1. Properties of D and E Fractions

DEGRADATION PRODUCTS	MOLECULAR WEIGHT	SEDIMENTATION COEFFICIENT	THERMAL PROPERTIES
D Fraction	80,000	5.6 S	Thermolabile
E Fraction	30,000	5.27 S	Thermostabile

Kopec et al. (1960) associated fibrinogen breakdown products with antithrombin activity and suggested that an increase of degradation products is the intermediate cause of delayed clotting. These investigators proposed that the nature of the inhibitory action of degradation products may be two-fold: (1) antithrombin activity in the strict sense, i.e. inhibition of the activity of thrombin; and (2) inhibition of the polymerization of fibrin monomers. Latallo et al. (1962c) further confirmed that fibrinogen proteolysis products interfere with fibrinogen-fibrin conversion at the stage of fibrin polymerization and gel formation. Alkjaersig et al. (1962) suggested that a single large molecular weight fibrinogen fragment, resistant to enzymatic digestion by plasmin, is predominantly responsible for the inhibition of fibrin polymerization.

Fibrinogen degradation products appear to influence platelet activity as well as fibrin formation. In 1964 Kowalski et al. reported that digestion of platelet rich plasma with streptokinase and plasmin diminished the aggregation of platelets. This treatment also diminished the adhesive properties of platelets to glass and connective tissue fibers (collagen). Platelet aggregation and adhesiveness is elicited by thrombin (Kowalski, 1968). Later studies (Kopec et al., 1966) indicated that platelet aggregation could also be induced by solutions of fibrinogen preparations; however, the addition of fibrinogen degradation products to platelet suspensions inhibited fibrinogen-induced aggregation. Jerushalmy and Zucker

(1966) distinguished inhibitory effects of early and late products of fibrinogen degradation on platelets. These workers have shown that early degradation products produced stronger inhibitory effects on platelet aggregation than late degradation products.

Release of adenine nucleotides from platelets upon addition of thrombin was also found to be decreased in the presence of fibrinogen degradation products. In this instance the early products also appeared to be more effective inhibitors than the late products (Kopec et al., 1966; Wilson et al., 1968).

Kowalski et al. (1946b) worked with dogs infused with streptokinase-plasminogen to induce the formation of fibrinogen breakdown products. The appearance of early fibrinogen breakdown products was found circulating in the blood. These products were correlated with profuse bleeding and pronounced prolongation of the bleeding time. The effect of early fibrinogen breakdown products was more pronounced than the effects of late breakdown products. Later work has shown the fibrinogen degradation products exhibit a maximum of anticoagulant activity at nine minutes (Nanninga, 1966).

Latallo et al. (1964) clarified the role of early and late fibrinogen degradation products. These investigators demonstrated that the early breakdown products of fibrinogen are plasmin susceptible and interfere with the action of thrombin, i.e. the enzymatic conversion of fibrinogen to fibrin monomer. The late fibrinogen breakdown products interfered with the polymerization of the fibrin monomer and

are plasmin resistant. Recent investigation has indicated that the presence of fibrinogen degradation products may be responsible for defective fibrin clot formation.

In 1962, Bang et al. using the electron microscope, studied the effects of fibrinogen proteolysis products on the fibrin clot and demonstrated defective fibrin polymerization. Low concentrations of fibrinogen degradation products produced striking alterations in clot structure. Not only do proteolytic products produce defective clots, but evidence also indicates these products are actually incorporated into the clot. In 1962, Latallo et al. demonstrated that fibrinogen proteolysis products were, in fact, incorporated into structurally abnormal clots. These workers suggested that proteolysis products inhibit polymerization of the fibrin monomer by bonding with monomer units and in this way mask the sites essential for normal polymerization. This theory was later supported by Hirsch et al. (1965), who indicated that large molecular weight products of fibrinogen proteolysis link with fibrin monomers and inhibit fibrin polymerization. This inhibition produced clots with abnormal structural characteristics, including a defect in the tensile strength of the fibrin clot.

V CRYOFIBRINOGEN

Shainoff and Page (1960) isolated a product from the plasma of endotoxin treated rabbits that corresponded, on the basis of its

peptide composition, to an intermediate product of the fibrinogen-fibrin conversion. Since the product described precipitated in the cold at 4°C, these workers suggested the term "cryoprofibrin". Later work (Shainoff and Page, 1962; Sasaki *et al.*, 1966) suggested that the liberation of peptides occurred during the fibrinogen exposure to thrombin. The resultant fibrinogen monomer polymerizes to form fibrin. In certain disease states the fibrinogen altered by the release of peptides, may combine with native fibrinogen to form a cold precipitable complex. Weerdt and Vreeker (1965) concluded that traces of thrombin formed *in vivo* resulted in the formation of intermediate products of cryoprofibrin. Thrombin in trace quantities induces the formation of intermediate products, but not the formation of fibrin itself. Therefore, the presence of intermediate products results in the formation of cold precipitable cryoprofibrin. From the investigators' description, it seems possible that the cold precipitable protein described as cryoprofibrin is analagous to cryofibrinogen.

Cryofibrinogen has been demonstrated in the plasma of patients with carcinoma, leukemia, secondary polycythemia, and abdominal aneurysm in association with surgical procedures. This cold precipitable protein has also been found, in small amounts, in samples from pregnant women, especially in the eighth month of pregnancy. When pregnancy was complicated by phlebitis, the amount of cryofibrinogen was increased. This protein was not evident in

control samples from normal persons (Glueck and Herrman, 1964).

Lipinski et al. (1967) indicated that the cryoprecipitate described by previous workers may be soluble complexes formed in the presence of excess fibrinogen degradation products. These soluble complexes may be precipitated by protamine sulfate or by cooling to 4°C. Lipinski and coworkers described these complexes as a result of investigative studies using ^{131}I labeled fibrinogen. Samples of normal human blood were mixed with ^{131}I fibrinogen and breakdown products of fibrinogen. The mixture was allowed to clot at 37°C for 90 minutes, and the resultant serum tested for radioactivity. The amount of radioactivity in the serum was directly proportional to the amount of fibrinogen breakdown products present. Aliquots of the serum were cooled to 4°C and a cryoprecipitate occurred. From these experiments, Lipinski et al. suggested that the cryoprecipitate termed by other workers as "cryofibrinogen" is a soluble complex formed by fibrinogen, fibrinogen degradation products, and intermediates of the enzymatic conversion of fibrinogen to fibrin.

The electrophoretic and immunoelectrophoretic behavior of cryofibrinogen has been found to resemble fibrinogen (Korst and Kratchvil, 1955). Recently, immunoelectrophoretic studies were done on a cryoprecipitate found in the plasma, but not the serum, of eight patients with cryofibrinogenemia (Zlolneck and Landau, 1966). These studies demonstrated that the cryoprecipitate primarily

contained fibrinogen; however, albumin and alpha, beta, and gamma globulins were also present.

VI INCREASED FIBRINOLYSIS IN DISEASE

As early as 1914, Goodpasture reported that blood specimens from patients with atrophic hepatic cirrhosis exhibited clot digestion within a few hours at body temperature, while a clot of normal blood did not digest for days or weeks. Goodpasture suggested that dissolution of the clot in the blood of cirrhotic patients is due to an enzyme inhibited by normal serum. Later, Ratnoff (1949) confirmed this finding and substantially extended the study of this phenomenon. He noted that rapid plasma clot lysis was a frequent accompaniment of Laennec's cirrhosis and was sometimes observed in patients who experienced hepatic damage during the course of some other illness. However, rapid plasma clot lysis was not observed in either acute hepatitis or obstructive jaundice, unless hepatic injury was associated.

In recent years, several authors (Finkbinner et al., 1959; Grossi et al., 1961, 1962; Kwaan et al., 1956, 1957; and Bergstrom et al., 1960) have reported that a large proportion of patients suffering from advanced hepatic cirrhosis exhibited rapid spontaneous lysis of whole blood, shortened euglobulin lysis times and other evidence of enhanced plasminogen-plasmin activity. Other authors have shown hepatic cirrhosis is often accompanied by an ill-defined

coagulation disorder. Patients suffering from this anomaly may, when subjected to operative stress, develop a severe hemorrhagic diathesis. This diathesis is often associated with pathological plasma proteolysis (Zucker et al., 1957; Ratnoff, 1954; Purcell and Phillips, 1963).

Nocola and Soardi in 1958 associated increased fibrinolytic activity with all liver disease, particularly cirrhosis. Fletcher et al. (1964) showed that patients suffering from liver cirrhosis had an enhanced plasma fibrinolytic activity after the administration of nicotinic acid or after electroshock treatment. The increase in activity of cirrhotic patients was five times that of normal controls.

Menon (1969) conducted a study on subjects randomly admitted to a medical ward with a variety of illnesses. Based on euglobulin lysis time, increased fibrinolytic activity was ascertained. This investigator found that fibrinolytic activity was increased in 15 per cent (two patients) of the instances of coronary thrombosis, 10 per cent of the instances of acquired valvular heart disease (10 patients), and 18 per cent (five patients) of congenital heart disease. In the instances of congenital and valvular heart disease, the liver function tests were normal. Thus, it appears that increased fibrinolytic activity extends beyond liver involvement.

Further substantiation of this involvement may be postulated from the work of Todd (1959) who studied anatomic sites for fibrinolysis in tissues. Zones of fibrinolysis on a fibrin plate were

observed in all tissues except the liver. The liver did, however, manifest fibrinolytic activity when in a diseased state. The zones of fibrinolysis were related exclusively to veins and venules, except in the lung where these were related to pulmonary, arteries and arterioles. It would be expected that if fibrinolytic activity can be demonstrated in the tissues a relationship between increased fibrinolytic products and disease states may be demonstrated with the extension of the study of fibrinolytic products.

MATERIALS AND METHODS

I SERA AND PLASMA

Normal human serum and plasma were used to standardize the various test and experimental systems. Pathologic serum and plasma were studied for abnormal fibrinogen degradation products. The patient sera used in this study were collected from hospitalized patients at the University Hospital and the Veteran's Administration Hospital. Specimen collection followed the routine procedure of each clinical chemistry laboratory. The serum was removed from the cells immediately after clotting, and stored at -20 C until immunological studies were performed.

Samples of serum used as controls were collected from healthy persons previously screened as blood donors by the Blood Bank laboratory at the University Hospital. Specimens of blood were drawn according to the blood bank procedure of this laboratory. The serum was immediately removed from the cells and stored at -20 C.

Samples of plasma were randomly collected from the outpatient laboratory of the University Hospital and from employees of the hospital. Blood specimens were drawn in tubes containing sodium citrate. The plasma was removed, pooled (8 to 10 samples per pool) and stored at -20 C until needed. Pooled normal plasma was used as a fibrinogen control in immunological studies designed to identify fibrinogen and/or fibrinogen products.

II FIBRINOGEN

The fibrinogen used for this study was dried human fibrinogen procured from the American Red Cross (processed by Cutler Laboratories, Berkeley, California). Fifty-five milligrams of the dried preparation was reconstituted in one milliliter of distilled water. The resultant solution contained 10 mg/ml of clottable protein.

III ANTISERA

The antisera used throughout these studies was obtained from Hyland Laboratories, Los Angeles, California. The polyvalent antisera were commercial preparations obtained from animals (goat and rabbit) hyperimmunized with pooled normal human sera. The antifibrinogen (rabbit) or antifibrin (rabbit) antisera were prepared by hyperimmunization with washed fibrin processed from dilute solutions of purified human fibrinogen.

IV PLASMINOGEN ACTIVATORS AND INHIBITORS

Proteolyses of fibrinogen was studied by varying the concentration of plasminogen activators. The proteolytic agents used were as follows: (1) Streptokinase-Streptodornase Varidase(R), a streptokinase preparation obtained commercially from Lederle Laboratories, Pearl River, New York, and (2) Thrombolysin(R), an activated human fibrinolysin obtained commercially from Merck, Sharp, and Dohme, West Point, Pennsylvania.

The plasmin inhibitor used was Amicar (R), a preparation of ε-amino caproic acid obtained commercially from Lederle Laboratories, Pearl River, New York.

V. FIBRINOGEN ASSAY

A modified turbidimetric fibrinogen assay was performed according to the method of Hunter and Allensworth (1965).

A. Sera

Fresh plasma samples were obtained from two patients. Serum samples from these patients previously demonstrated a positive test for fibrinogen and/or fibrinogen derivatives by immuno-diffusion. Plasma samples from normal persons were used as control samples.

B. Reagents

The precipitating reagent contained 14% (w/v) ammonium sulfate, 3% (w/v) sodium citrate and 1% (w/v) sodium chloride in distilled water. Five-hundredths milliliter of 17 per cent Zephiran was added to 100 ml of solution. The blank reagent contained 3% (w/v) sodium citrate, 1% (w/v) sodium chloride and 0.05 ml of Zephiran in 100 ml. distilled water.

C. Procedure

Using Beckman cuvettes, 0.2 ml of patient plasma was added to 1.4 ml of blank reagent and inserted into a well of a Coleman Jr. spectrophotometer (Coleman Instruments Inc., Maywood, Illinois). At exactly three minutes the spectrophotometer was adjusted to 100%

transmission at a wave length of 400 mμ.

Using a matching cuvette, 0.2 ml of plasma was added to 1.4 ml of precipitating reagent. At exactly three minutes the per cent transmission was recorded. Each test was performed in duplicate. The fibrinogen level in milligrams per cent was determined from a previously prepared curve.

VI CRYOFIBRINOGEN DETERMINATION

The cryofibrinogen method used was proposed by Kalbfleisch and Bird (1960).

A. Sera

Fresh plasma samples were obtained from two patients. Serum samples from these patients previously demonstrated a positive test for fibrinogen and/or fibrinogen derivatives by immunodiffusion. Plasma samples from normal persons were used as control samples.

B. Procedure

A fresh sample of plasma was refrigerated for 48 hours at 4 C. The cryoprecipitate was washed in cold saline and resuspended in saline to the original plasma volume. A fibrinogen assay was performed on the original plasma sample and the resuspended cryoprecipitate.

VII EUGLOBULIN LYSIS TIME

Euglobulin lysis time was performed according to the method proposed by Biggs and McFarlane (1963).

A. Plasma Samples

The following plasma samples were obtained from the Clinical Laboratories: three samples with a prolonged prothrombin time and two samples showing a positive serum test for fibrinogen and/or fibrinogen derivatives by immunodiffusion. Plasma samples from normal persons were obtained from the outpatient department of the hospital and used for controls.

B. Reagents

The reagents used for the test were 0.025 M calcium chloride, 1% acetic acid, and 0.1% borate solution. The borate solution was prepared by dissolving 9 g sodium chloride and 1 g sodium borate in one liter of distilled water. The solution was adjusted to pH 9.0.

C. Procedure

The blood was collected in sodium citrate, kept on melting ice, and the test carried out within 20 minutes after obtaining the specimen. Five-tenths milliliter of the plasma was added to centrifuge tubes containing 9 ml of distilled water. The pH was adjusted to 5.3 by adding 0.1 ml of 1% acetic acid. The tubes were allowed to stand at 4 C for 30 minutes to permit the plasma euglobulin fraction to precipitate. The tubes were then centrifuged for five minutes and the supernatant decanted. The euglobulin fraction was resuspended by adding to each tube 0.5 ml of the borate solution. After the tubes were placed in a 37 C water bath,

0.5 ml of 0.025 M calcium chloride was added and the clotting time recorded. After clotting, the tubes were inspected at intervals and the lysis time recorded. Lysis time longer than two hours was considered normal.

VIII IMMUNODIFFUSION

A. Controls

Samples of serum obtained from normal blood donors screened by the University Hospital Blood Bank Laboratories were used as controls. Samples of normal human plasma were obtained from the outpatient department of the University Hospital and were pooled (eight to ten samples per pool) and used as a plasma control.

B. Patient Sera

A total of 196 serum samples from hospitalized patients were collected from the Clinical Chemistry Laboratories of the University Hospital and the Veteran's Administration Hospital. The specimens were frozen at -20 C until immunological studies were performed.

C. Procedure

Patient sera were screened immunologically on immunodiffusion plates commercially prepared by Hyland Laboratories (Los Angeles, California). Ten microliters of the test samples were loaded in the wells on the immunodiffusion plates. Each sample was run in triplicate. A control sample of normal human plasma was used on each plate. Ten microliters of fibrinogen was placed in the center

well. After loading, the plates were allowed to incubate for 48 hours at room temperature in a moist chamber. The results were read and recorded at 24 and 72 hours. Since there was no significant difference in these readings, only the 24-hour reading was recorded. The presence of fibrinogen and/or fibrinogen degradation products was determined by the presence of a precipitin band. (see Figure 7)

IX IMMUNOELECTROPHORESIS ON CELLULOSE ACETATE

A. Fibrinolytic Agent

Three dilutions of commercial plasmin were prepared in the following manner: (1) 100 mg of plasmin (10,000 units) was reconstituted in 0.1 ml distilled water; (2) 10 mg of plasmin (1,000 units) was reconstituted in 0.1 ml of distilled water; and (3) 1.0 mg of plasmin (100 units) was reconstituted in 0.1 ml of distilled water.

B. Buffer

The buffer solution (pH 8.6, ionic strength, 0.075) was prepared by dissolving 2.76 g of 5,5-diethylbarbituric acid and 15.40 g sodium 5,5-diethylbarbituric acid in distilled water to make one liter.

C. Protein Stain

Ponceau-S fixative dye solution was used as the protein stain. The dye solution contained 0.2% (w/v) Ponceau-S stain, 3.0% (w/v) trichloroacetic acid, and 3.0% (w/v) sulfosalicylic acid in water.

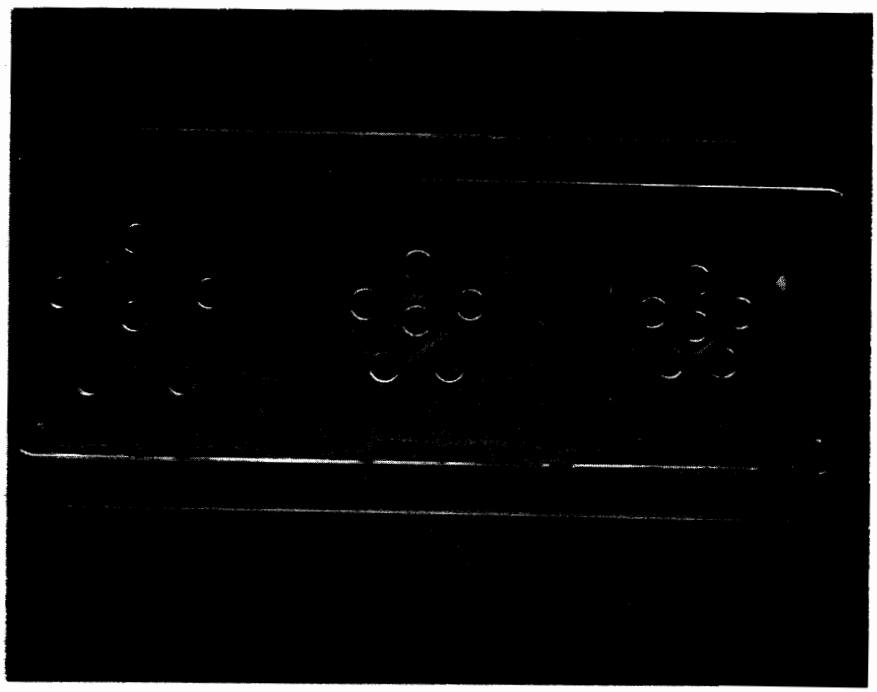


Fig. 7. Immunodiffusion plate. The double diffusion test was carried out in buffered agar. Anti-fibrinogen was applied in the center. Applied in the five different spots were: normal human plasma (starting from top, counterclockwise) and four serum samples from hospitalized patients. The precipitin bands represents the presence of fibrinogen breakdown products.

D. Procedure

Cross-reactivity of plasmin with respect to commercial antifibrinogen was studied using cellulose acetate strips and Microzone equipment (Beckman Instruments, Fullerton, California) in accordance with prescribed protocol (Byers, 1966). The power supply used was a Spinco Duostat (Beckman Instruments, Fullerton, California). Normal human plasma was used as a control.

Cellulose acetate membranes were presaturated with buffer and placed in an embosser that embossed the membrane with two wells for the sample and three troughs for the antisera. The membrane was then mounted on the Microzone (R) electrophoretic cell bridge and 0.3 µl of plasmin dilutions were applied. Electrophoresis was performed at 150 volts for 50 minutes at room temperature. The membrane was then removed from the bridge, antifibrinogen (20 µl) was applied to each of the three troughs, followed by immersion in light mineral oil and incubation for 66 to 72 hours at room temperature. After incubation, the membrane was rinsed two times in petroleum ether and four times in normal saline, stained in the fixative dye solution for seven minutes, rinsed three more times in 5% aqueous acetic acid and dried in a drying frame before a hot-air dryer for 20 minutes.

X IMMUNOELECTROPHORESIS IN AGAR GEL

Immunoelectrophoresis was performed on commercial immuno-

electrophoretic equipment (Gelman Instruments, Ann Arbor, Michigan). The power supply used was a Spinco Duostat (Beckman Instruments, Fullerton, California). The method used was the microtechnique of Scheidegger (1955) as modified by Hirschfield (1960).

A. Agar Buffer

The agar buffer was prepared by dissolving 1.66 g barbituric acid, 10.51 g sodium barbiturate, and 1.54 g calcium lactate in one liter of distilled water. The above solution was adjusted to a pH of 8.6.

B. Tank Buffer

The tank buffer was prepared by dissolving 1.38 g barbituric acid, 8.7 g sodium barbiturate, and 0.399 g of calcium lactate in one liter of distilled water and adjusted to pH 8.6.

C. Agar

The two per cent agar medium was prepared by dissolving 20 g of commercially prepared Ion Agar (Difco Laboratories, Detroit, Michigan) in one liter of distilled water. The agar solution was slowly brought to a boil and allowed to boil until the agar was completely dissolved. The medium was then allowed to solidify in 50 ml aliquots.

D. Protein Stain

The dye used for staining the slides was prepared by dissolving one gram each of thiazine red R, amidoschwarz 10 B, light green SF, mercuric chloride, and 20 g of acetic acid in one liter of distilled

water (Crowle, 1961).

E. Procedure

Three horizontal frames were used, each holding six microscope slides previously cleaned with alcohol and coated with agar by immersion in a boiling 0.2% (w/v) ion agar.

Fifty milliliters of the previously prepared agar was melted by boiling and then allowed to cool to approximately 56 C. While still warm, the agar buffer mixture was applied to each series of six slides in the horizontal frames. (Approximately 20 ml of agar was needed for each frame.) After the agar was solidified, each slide was punched with a die designed to cut two wells for the sample and a center trench between the wells for the antiserum.

The horizontal frames were placed in the electrophoresis cell previously filled with buffer. Cellulose acetate wicks, presaturated in buffer, were used to connect the slides with the buffer. A constant current of 35 ma was applied for about 20 to 30 minutes. The frames were removed from the cell and the agar was aspirated from the wells. In each case, 5 µl of the control sample marked with Bromphenol Blue (Hartman, Leddon Company, Philadelphia, Pennsylvania) was loaded in the top well and the bottom well was filled with 5 µl of the sample to be tested; the frames were returned to the cell and the current (35 ma) applied for approximately one and one-half to two hours.

The cell was again opened and the agar was aspirated from the

center trench, the trench was then filled with 100 µl of antifibrinogen. The slides were allowed to incubate at room temperature in a moist diffusion chamber for 24 hours. After incubation, the slides were washed in three 24-hour rinses of normal saline and one 24-hour rinse of distilled water. The slides were dried in a hot-air dryer and stained for approximately 30 minutes. The excess stain was removed by rinsing briefly several times in cold tap water. A final rinse in 5% aqueous acetic acid was used for differentiation of the stain. After drying, the stained slides were labeled and the results recorded.

XI GEL FILTRATION

A. Preparation of Degradation Products

Dried human fibrinogen was reconstituted to 55 mg/ml in distilled water giving a final concentration of 10 mg/ml of clottable protein. To 5 ml of the reconstituted fibrinogen was added 0.15 ml of plasmin, giving a final dilution of 300 units of plasmin per milliliter of fibrinogen. Proteolysis was allowed to proceed for 48 hours.

B. Preparation of the Column

G-75 and G-200 Sephadex (R) (Pharmacia Fine Chemicals, Incorporated, Piscataway, New Jersey) were allowed to swell in demineralized water for three days with periodic water changes during this time and the fine materials removed by decantation.

The water was then replaced with 0.85% sodium chloride.

The Sephadex was allowed to equilibrate with the saline solution before being packed into a column 2.5 cm in diameter by 45 cm in height, or a column 2.5 cm in diameter by 100 cm in height. Column packing was accomplished by pouring a slurry of Sephadex and saline into the column. The outlet of the column was adjusted to avoid excessive packing of the gel beads. The slurry was continually replaced as packing proceeded until the packed gel bed was the desired height. The column was fitted with upward flow adaptors and connected to a saline reservoir. Saline was allowed to run through the column overnight.

C. Procedure

Five milliliters of the sample were applied to the column using a three-way valve and syringe and the pressure was adjusted to allow a flow rate of 9 ml/hr through the column. A total of three fractions containing three milliliters of effluent were collected each hour at 20 minute intervals with an ISCO(R) automatic fraction collector (Instrumentation Specialties Company, Incorporated, Lincoln, Nebraska). The tubes were analyzed for protein content using a Beckman DU ultraviolet light spectrophotometer (Beckman Instruments, Fullerton, California), with a wavelength setting of 280 mμ. The optical density was graphed as the ordinate and the volume of the effluent was plotted on the abscissa. The effluent from the tubes defined within the absorbance

peaks in the above plot were pooled and concentrated ten-fold using Lyphogell,(R) a polyacrylamide gel acting as a dehydrating agent (Gelman Instruments, Ann Arbor, Michigan).

EXPERIMENTAL RESULTS

I CROSS-REACTIVITY OF PLASMIN

Throughout these studies plasmin was consistently used to effect the proteolysis of fibrinogen. In order to correctly identify the breakdown products resulting from fibrinogen proteolysis, the necessity of ruling out cross-reactivity between plasmin and antifibrinogen became evident.

Agar gel immunoelectrophoresis was performed on the following dilutions of plasmin and expressed in MSD units: 10,000 units/ml, 1,000 units/ml, and 100 units/ml. Table 2 indicates that no cross-reaction occurred using 100 units/ml and 1,000 units/ml of plasmin. However, when 10,000 units/ml of plasmin were tested with anti-fibrinogen, a light precipitin band occurred in the alpha area of the electrophoretic pattern extending into the prealbumin region.

To eliminate the further possibility of cross-reactivity of plasmin with the agar gel, cellulose acetate immunoelectrophoresis was performed on the above dilutions of plasmin, using antifibrinogen as the antisera and normal human plasma as a fibrinogen control. A light band, indicating cross-reactivity, was again seen with this concentration of plasmin (10,000 units), but was not evident at lower concentrations (see Table 2). Therefore, dilutions of plasmin were maintained below 300 units/ml in subsequent studies to avoid observable nonspecific precipitin bands.

Table 2. Immunoelectrophoresis of Plasmin

UNITS OF PLASMIN	AGAR GEL	CELLULOSE ACETATE
100	NEGATIVE	NEGATIVE
1,000	NEGATIVE	NEGATIVE
10,000	LIGHT PRECIPITIN BAND	LIGHT PRECIPITIN BAND

II IMMUNODIFFUSION

Immunodiffusion was used to screen patient sera for the presence of fibrinogen and/or derivatives of fibrinogen. A total of 196 samples of serum from hospitalized patients was collected from the Clinical Chemistry Laboratories of the University Hospital and the Veteran's Administration Hospital. Although most of this population had a history of abnormal liver function, a widespread spectrum of disease was represented. Immunodiffusion was performed in triplicate with all serum samples. From the 196 serum samples screened, ten samples (approximately 5%) gave visible immunological reactions with antifibrinogen, indicating the presence of fibrinogen and/or breakdown products of fibrinogen. Immunodiffusion was also performed on control sera collected from 30 persons previously screened for blood donation by the University Hospital Blood Bank Laboratory. Neither fibrinogen nor fibrinogen breakdown products were demonstrated in the sera of the controls. Using Diagnostic Plasma (Hyland Laboratories, Los Angeles, California) as a fibrinogen reference, studies indicated that fibrinogen in amounts exceeding 10 mg was demonstrable by immunodiffusion.

III IMMUNOELECTROPHORESIS

From the various test systems employed, it appeared that immunoelectrophoretic technique was most satisfactory for identifying and characterizing the products resulting from the proteolysis of human fibrinogen. Preliminary studies were made

using two different agents to induce fibrinolysis. These studies were conducted to determine which agent would be best suited for study of fibrinogen breakdown products. These agents were Thrombolysin (commercial plasmin) and Streptokinase (a plasminogen activator).

When immunoelectrophoresis was performed on normal human plasma after being subjected to the proteolytic activity of plasmin, two distinct bands were observed in the alpha-beta zone (see Figure 8). Both bands differed from the normal immunoelectrophoretic position of fibrinogen. For purposes of this discussion, the band migrating nearest to the origin was labeled Band A, while the band with the faster migration rate was labeled Band B. The following experiments were based on the assumption that these products represent the D and E fractions respectively, as originally reported by Nussenweig and co-workers (1960).

Streptokinase was used in preliminary experiments as a plasminogen activator. This activator was added to samples of pooled normal human plasma, and proteolysis was allowed to proceed for periods of time from zero to three hours. The timing was synchronized to allow all samples to be innoculated on the agar gel simultaneously. After loading, a constant current was immediately applied to the cell. The results of this experiment are given in Figure 9.

Using antifibrinogen as the antiserum, two products were

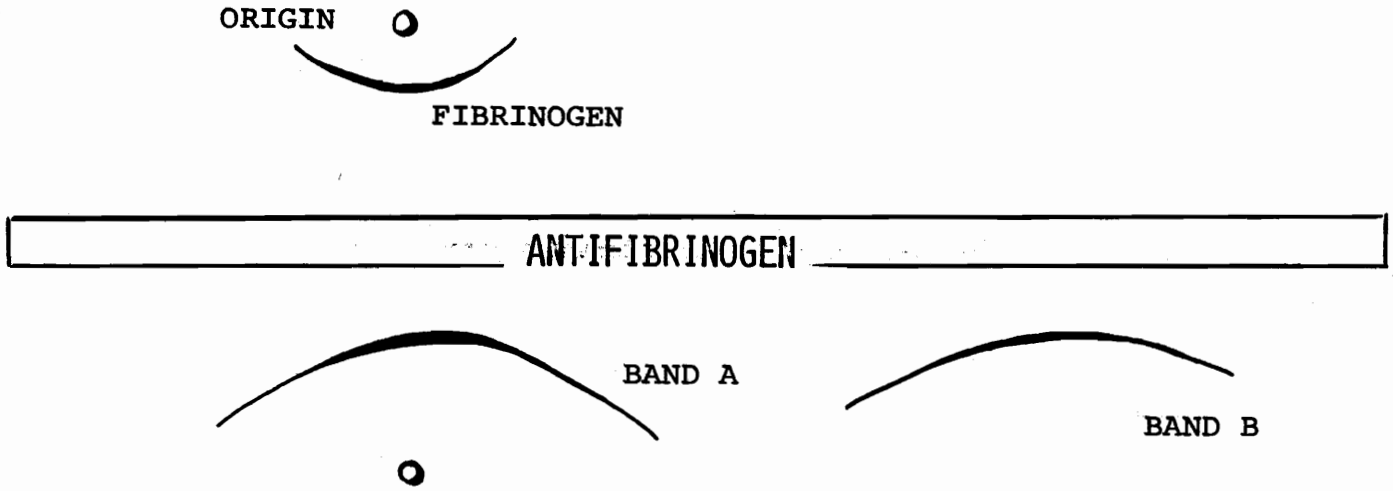


Fig. 8. A diagramatic representation of the immunoelectrophoretic pattern of human fibrinogen before digestion (upper well) and after (lower well) exhaustive plasmin.

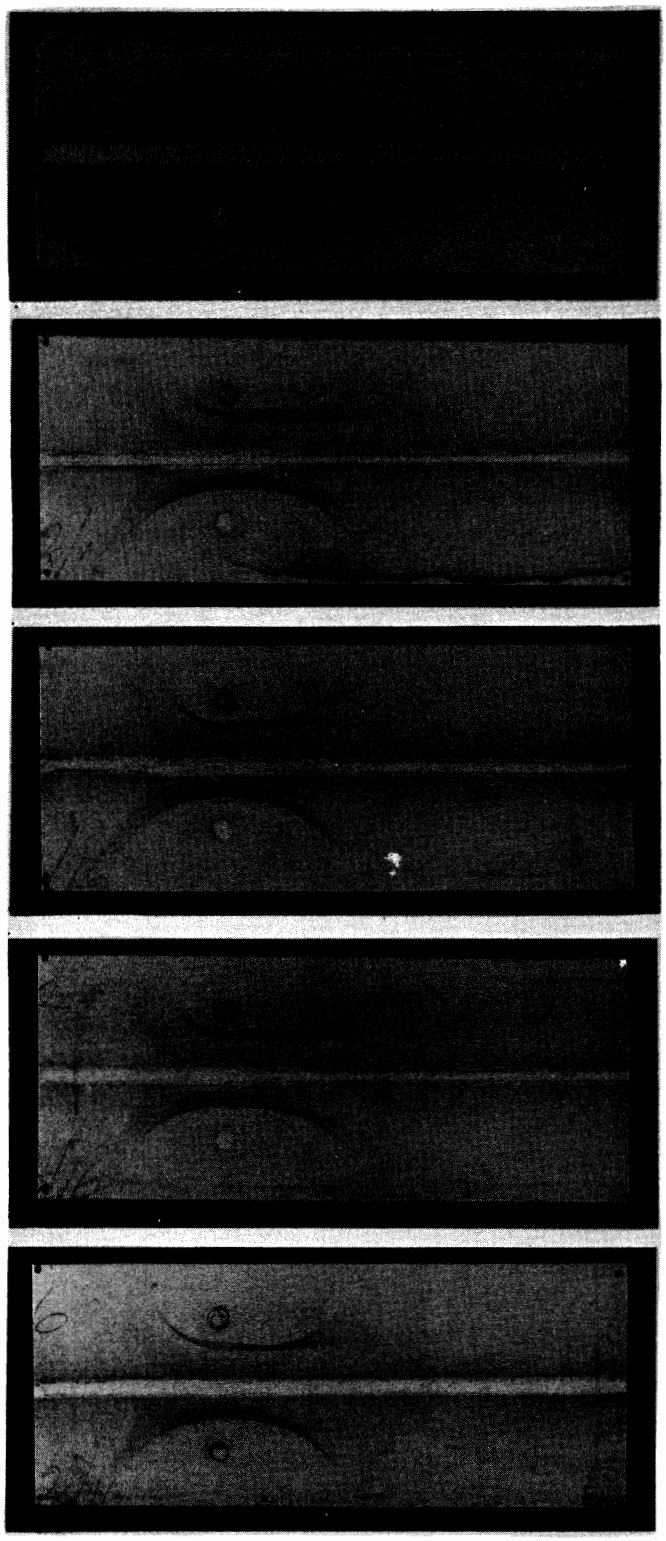


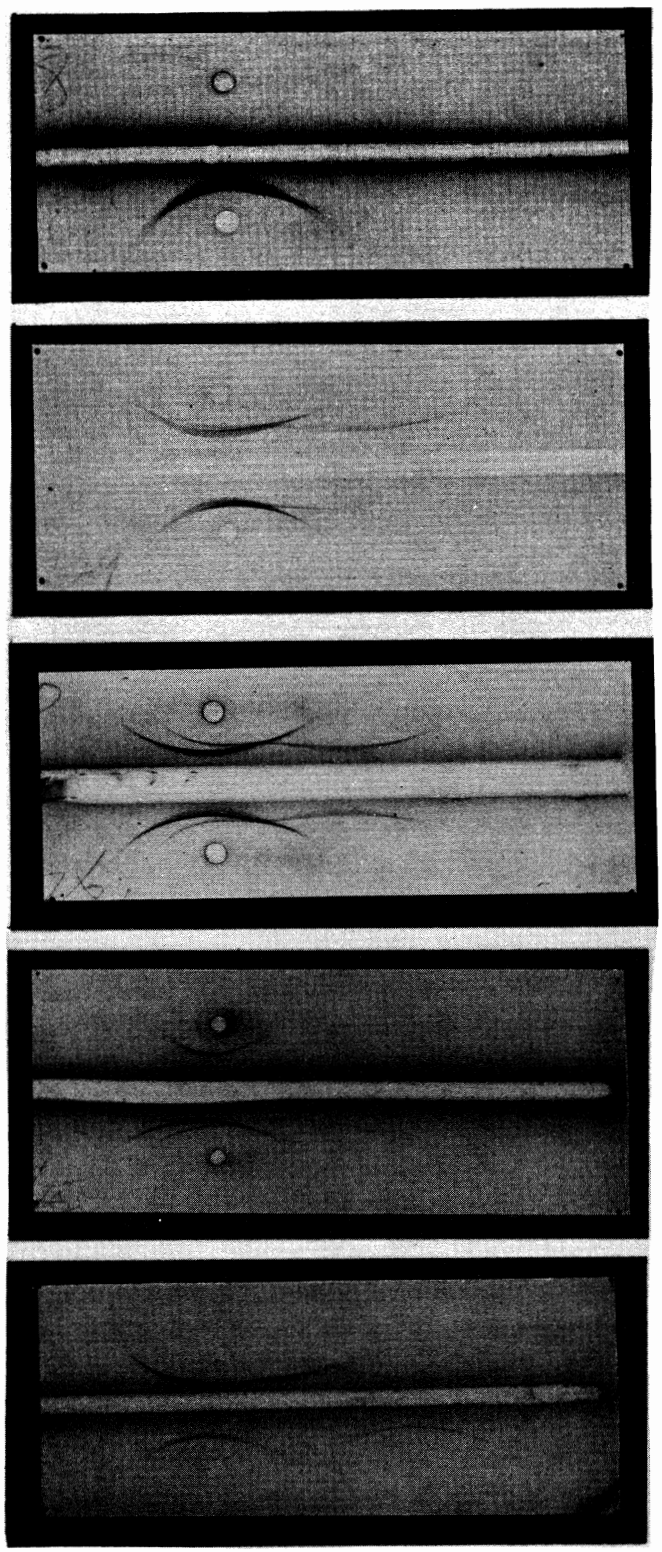
Fig. 9. Streptokinase activation of normal human plasma (NHP) at varying periods of time.

suggested in each case by the presence of two precipitin bands. Band A, nearest the well, remained in a stable position regardless of timing. However, Band B, migrating furthest from the well, varied in mobility depending upon the time allowed for proteolysis to proceed. Streptokinase was added to normal human plasma immediately before loading. Figure 9 illustrates that proteolysis may have occurred instantaneously (note time zero), or that the plasminogen may have been activated sometime during the electrophoretic procedure.

The above treatment of normal human plasma was repeated substituting 100 μ/ml of plasmin in lieu of streptokinase. In Figure 10 it can be seen that the action of plasmin differs from streptokinase. Although the same two products are evident, the mobility of the E components differ. Comparing Figure 9 with Figure 10, it appears that proteolysis of fibrinogen occurs at a slower rate when plasmin is used. Repeated experiments supported this finding and also indicated that plasmin gave more reliable and reproducible results. For these reasons, plasmin was used in subsequent experiments.

IV THERMAL PROPERTIES OF FIBRINOLYTIC PRODUCTS

Using simple radial diffusion technique, Beller and Maki (1967) demonstrated two products resulting from the degradation of fibrinogen by plasmin and identified these products as the D and E fractions originally reported by Nussenweig (1960). The above



NORMAL HUMAN SERUM
ANTIFIBRINOGEN
TIME 0

1 HR
ANTIFIBRINOGEN
15 MIN

30 MIN
ANTIFIBRINOGEN
30 MIN

NHP
ANTIFIBRINOGEN
1 HR

6 HR
ANTIFIBRINOGEN
48 HR

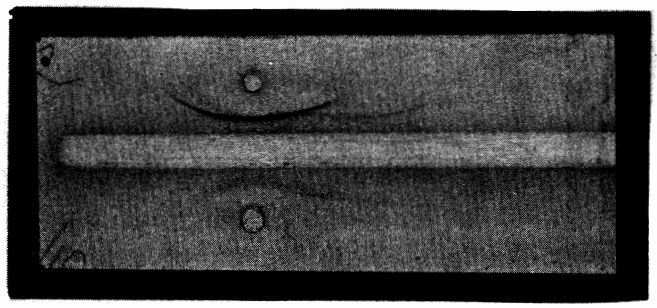
Fig. 10. Serial digestion of fibrinogen in normal human plasma (NHP) by plasmin.

investigators also reported that after heating the fibrinolytic products, the product designated by these workers as the D fraction, could no longer be demonstrated. The thermolabile properties of fraction D and the thermostabile properties of fraction E were later substantiated by Kowalski (1968).

Plasmin (100 µ/ml) was added to two samples of pooled normal human plasma. Both samples were allowed to incubate at room temperature for one hour. Following incubation, one of the samples (sample 2) was heated in a 56 C water bath for 30 minutes. Sample 1 was used as a control. Immunoelectrophoresis was performed on both samples (see Figure 11). The control sample was placed in the upper well, whereas the heated sample was placed in the lower well. Two products were demonstrated in the upper well and only one product was demonstrated in the lower well, suggesting that the heavier band, Band A, migrating nearer the origin, represents the proteolysis product previously described as the D fraction.

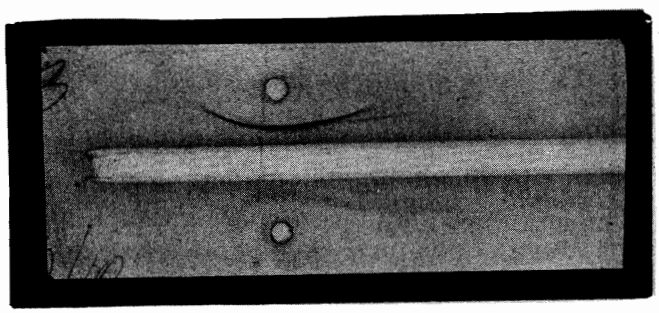
V INHIBITION OF PROTEOLYTIC ACTIVITY

Alkjaersig et al. (1959a) reported that the proteolytic action of plasmin is inhibited by ε-amino caproic acid; however, attempts in this study to inhibit the proteolytic action of plasmin in normal human plasma were unsuccessful. Thus, an experiment was designed to determine the threshold of ε-amino caproic acid plasmin inhibitory activity. Purified human fibrinogen was reconstituted to 55 mg/ml in distilled water giving a final concentration of 10 mg/ml of



PNHP
ANTIFIBRINOGEN
PNHP (56 C)

30 MIN PLASMIN DIGESTION



PNHP
ANTIFIBRINOGEN
PNHP (56 C)

60 MIN PLASMIN DIGESTION

Fig. 11. Plasmin digested normal human plasmin (PNHP) without subjection to 56 C and with 56 C treatment.

clottable protein. ε-amino caproic acid was added to each of seven tubes containing one milliliter of fibrinogen, giving a final concentration of 50 mg/ml of the fibrinogen solution. Varying dilutions of plasmin, as indicated in Table 3, were then respectively added to each tube. Control tubes containing fibrinogen, but not ε-amino caproic acid, were prepared to indicate the proteolytic action of plasmin at each respective dilution. After the tubes were incubated at room temperature for one hour, immunoelectrophoresis was performed on all samples using antifibrinogen as the antisera. When two bands representing fibrinogen breakdown products were observed, the sample was described as not inhibited. It may be noted from Table 3 that proteolysis did occur in each of the control samples. However, when less than 40 units of plasmin was used, the proteolytic action was inhibited by the ε-amino caproic acid.

VI GEL FILTRATION

Normal human plasma treated with plasmin for 48 hours was applied to a column of G-75 Sephadex gel and a column of G-200 Sephadex gel. Fractions were collected and analyzed for protein content. The peaks representing the distribution of protein were ascertained by plotting optical density on a graph (see Figure 12). The tubes containing the absorbance peaks were pooled and concentrated and immunoelectrophoresis was performed on the resultant concentrates. It was observed by inspection of the immunoelectrophoresis slides that partial separation had been accomplished. Samples from the

Table 3. Inhibition of the Proteolyte Activity of Plasmin on Fibrinogen by ε-Amino Caproic Acid (EACA)

TUBE	FIBRIN-OGEN (mg/ml)	EACA (mg/ml)	PLASMIN (in units)	PROTE-OLYSIS*
Control 1	10	--	100	NI
Sample 1	10	50	100	NI
Control 2	10	--	80	NI
Sample 2	10	50	80	NI
Control 3	10	--	60	NI
Sample 3	10	50	60	NI
Control 4	10	--	50	NI
Sample 4	10	50	50	NI
Control 5	10	--	40	NI
Sample 5	10	50	40	NI
Control 6	10	--	30	NI
Sample 6	10	50	30	Inhibited
Control 7	10	--	20	NI
Sample 7	10	50	20	Inhibited

* NI = Not Inhibited

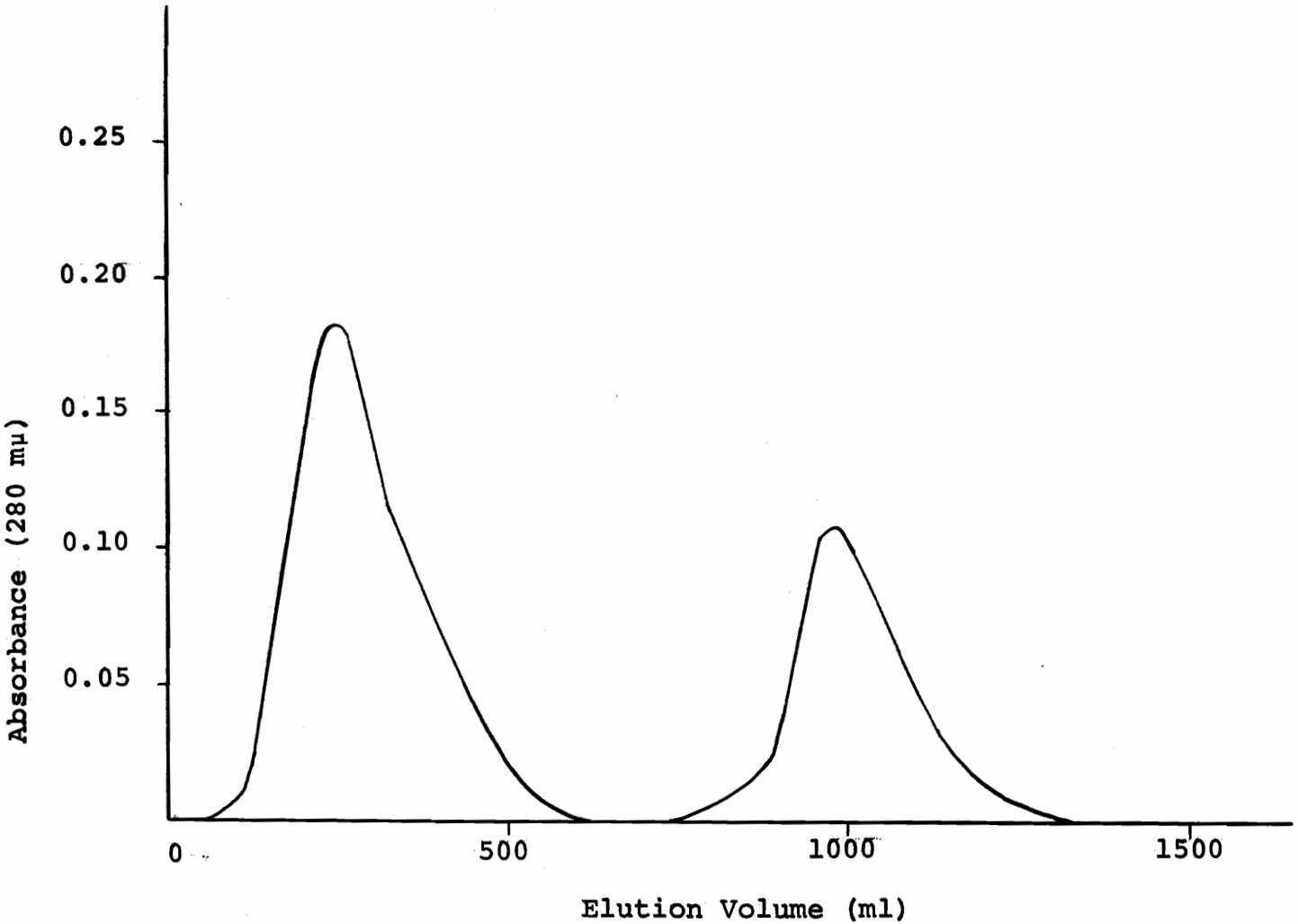


Fig. 12. Pattern obtained after filtration of fibrinogen degradation products through Sephadex G 200.

absorbance peak first eluted from the column contained both the D and the E fractions while samples from the second peak contained only the E fraction.

VII ASSAY FOR FIBRINOGEN AND CRYOFIBRINOGEN

A modified turbidimetric fibrinogen assay (Hunter and Allensworth, 1965) was utilized to determine the fibrinogen levels on plasma samples. Inasmuch as fresh plasma was required for the analysis, two persons with normal plasma fibrinolytic activity and two patients with increased plasma fibrinolysis were selected because of availability at the time of testing. Fibrinogen and/or fibrinogen breakdown products had been demonstrated immunologically from serum samples previously obtained from these patients. The normal range of fibrinogen for this method is 180 to 415 mg/100 ml. Although the patient values were considerably higher than those of the control, only one of the four patients (see Table 4) may be considered as having a slightly increased fibrinogen level. Samples of plasma from the same group of patients were refrigerated at 4 C and observed for cryofibrinogen (Kalbfleisch and Bird, 1960). After 48 hours, no cryoprecipitate was observed in any of the samples.

VIII EUGLOBULIN LYSIS TIME

Increased fibrinolytic activity has been determined by the euglobulin lysis time in patients with a history of abnormal liver function tests and coagulation disorders (Biggs and McFarlane, 1963).

Table 4. Assay for Fibrinogen and Cryofibrinogen

SAMPLE	FIBRINOGEN (MG%)	CRYOPRECIPITATE
CONTROL A	270	NONE
CONTROL B	180	NONE
PATIENT A	450	NONE
PATIENT B	365	NONE

The following fresh plasma samples were studied: five samples with coagulation test values in the normal range, three samples with prolonged prothrombin times, and two samples showing the presence of fibrinogen and/or fibrinogen derivatives by immunodiffusion. The clotting time was recorded as well as the time required for the lysis of the clot. Clot lysis times longer than two hours were considered to be in the normal range. Only one plasma sample (see Table 5) showed increased fibrinolytic activity as demonstrated by the Euglobulin lysis test. A positive test for fibrinogen and/or fibrinogen derivatives by immunodiffusion was previously demonstrated in the serum obtained from this patient.

IX PATIENT DATA

As previously stated, 196 samples of sera were collected from patients hospitalized at the University Hospital and the Veteran's Administration Hospital. Thirty samples of sera were collected from persons previously screened as blood donors by the University Hospital Blood Bank Laboratory and used as normal controls. All samples were screened by immunodiffusion for the presence of fibrinogen and/or fibrinogen breakdown products. These products were not demonstrated immunologically in any of the control sera; however, in ten of the sera obtained from hospitalized patients fibrinogen and/or fibrinogen products were demonstrated. Immunoelectrophoresis was performed on each of these ten samples, using normal human plasma degradated with plasmin (100 units/ml) as a control and antifibrinogen as the

Table 5. Euglobulin Lysis

SERA	SAMPLE	CLOTTING TIME	LYSIS TIME
CONTROLS	CONTROL A	2 MIN.	2 HR., 30 MIN.
	CONTROL B	3 MIN.	2 HR., 50 MIN.
	CONTROL C	2 MIN.	2 HR., 40 MIN.
	CONTROL D	5 MIN.	2 HR., 10 MIN.
	CONTROL E	3 MIN.	2 HR., 10 MIN.
PROLONGED PROTHROMBIN TIME	PATIENT 1	9 MIN.	2 HR., 30 MIN.
	PATIENT 2	7 MIN.	2 HR., 40 MIN.
	PATIENT 3	23 MIN.	2 HR., 5 MIN.
POSITIVE IMMUNODIFFUSION	PATIENT A	3 MIN.	2 HR., 5 MIN.
	PATIENT B	2 MIN.	1 HR., 50 MIN.

antisera. One band, corresponding to the band described as the D fraction, was demonstrated in eight of the ten samples, and in the other two serum samples bands representing the D and E fractions were demonstrated.

Medical histories of these patients were obtained and a diagnostic classification was made. The diagnosis of the patients' illness at the time of this study was performed could be classified into three categories: (1) hepatic disease, (2) kidney disease, and (3) intravascular thrombosis. A subclassification of each of these three categories is presented in Figure 13. Eight of the ten patients were being treated for liver ailments. These included viral hepatitis, cirrhosis of the liver, chronic passive congestion, and biliary disease. The kidney ailments afflicting five patients included kidney infection, myeloma nephrosis, kidney infarction, and nephrosclerosis. Six patients had intravascular thrombosis, which included one patient with an abruptio placentae.

In some instances, evidence of more than one disease process within a single patient was found, i.e., three patients presented with a history of all three disease entities, while one patient had kidney disease accompanied by intravascular thrombosis and one patient had both hepatic and kidney disease. A diagrammatic presentation of the overlap of diseases is given in Figure 14. The arabic numerals indicate the number of patients in each category. It may be noted that there were no cases of kidney problems without the added complication of either liver disease or intravascular thrombosis.

LIVER (8)	VIRAL HEPATITIS (1)
	CIRRHOSIS (2)
	CHRONIC PASSIVE CONJESTION (3)
	BILIARY DISEASE (2)
KIDNEY (5)	MYELOMA NEPHROSIS (1)
	INFECTION (2)
	INFARCTION (1)
	NEPHROSCLEROSIS (1)
INTRAVASCULAR THROMBOSIS (6)	ABRUPTIO PLACENTAE (1)

Fig. 13. A classification of disease states seen in patients with fibrinogen breakdown products in the serum.

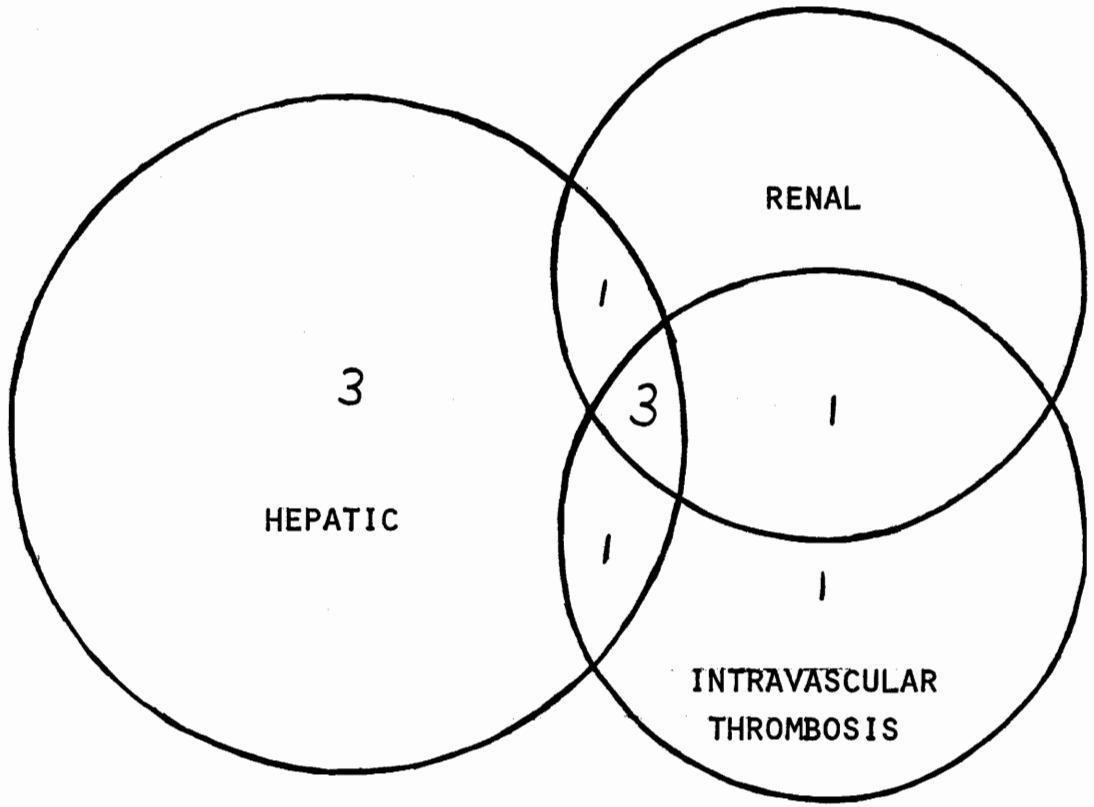


Fig. 14. A diagrammatic representation of the overlap of physiologic sites involved in the disease plates of patients with fibrinogen breakdown products in the serum.

DISCUSSION

Immunological methods have been employed to demonstrate and identify the proteolysis products resulting from the degradation of human fibrinogen by plasmin. The proteolytic breakdown products of fibrinogen studied include products occurring spontaneously and products induced by treatment with streptokinase and plasmin. The fibrinogen degradation products studied were derived from: (1) normal human plasma treated with the fibrinolytic agent plasmin, (2) normal human plasma treated with streptokinase, an activator that converts the proenzyme plasminogen to plasmin, and (3) spontaneous products occurring in the serum of ten hospitalized patients.

Direct observation of the digestion process was facilitated when immunoelectrophoresis was performed on normal human plasma subjected to varying periods of exposure to the proteolytic agent, plasmin. At time zero, the fibrinogen molecule had already begun to split, indicating the occurrence of rapid proteolysis that may have occurred during the electrophoretic procedure. At fifteen minutes Band A representing the D fraction was observed. This band remained stable and did not differ in immunoelectrophoretic position regardless of the time allowed for proteolysis to proceed. The second band, Band B, varied in immunoelectrophoretic position depending on the length of exposure to the proteolytic agent. From these results it appeared that this band may represent an intermediate product containing the E fragment rather than a plasmin resistant product. Furthermore, it

appeared that this intermediate product is still susceptible to the action of plasmin. However, exhaustive proteolysis (48 hours) produces an E fragment resistant to further action by plasmin. This postulate is further substantiated by the increased electrophoretic mobility after prolonged exposure of this intermediate to the proteolytic enzyme. The appearance of a spur on the anodal end of the precipitin band of the E fragment with extended digestion suggests that polypeptides are being continually split from the intermediate product containing the E fragment. With exhaustive proteolysis of 48 hours, the spur disappears and the E fragment, resistant to plasmin, now appears in the prealbumin electrophoretic region rather than the original alpha-beta position.

The concordant opinion as to the exact rate and mechanism involved in the formation of the D and E fractions from the parent fibrinogen molecule has not been established. The recent work of Kowalski (1968) has indicated that during plasmin proteolysis of fibrinogen, fractions D and E are formed at the same rate (see Figure 6). However, the study presented herein indicates that fragment D is formed more rapidly and remains stable, while E is formed at a slower rate. Kowalski formulated his composite hypothesis of this mechanism (see Figure 6) by compiling several discreet experimental studies. In the present study, a sequential degradation of the parent molecule was serially observed. This digestion yielded a rapid formation of the D fragment and a sequential alteration of

the E moiety. This produced a migratory band originating in the locus of the D fragment and progressing across the electrophoretic field to the prealbumin region at the completion of the digestion. A diagrammatic representation of this process is given in Figure 15.

This mechanism is supported by the earlier work of Fisher et al., (1967). Using zone electrophoresis in polyacrylamide gel these investigators indicated that the electrophoretic mobility of the E moiety continues to change until there is a clear separation of both precipitin lines. Complete separation appears to require a digestion period of approximately 24 hours. This work substantiates the postulate that the bands seen in Figure 10 (note 48 hour) represent the final products resulting from the proteolysis of fibrinogen by plasmin, and indicates that the rapid formation of the D fragment may be a more reliable observation of plasmin digestion.

Recent studies performed by Beller and Maki (1967) using radial diffusion confirm an earlier proposal, i.e., when fragment D is subjected to temperatures of 56 C or higher, the fragment precipitates, altering its molecular structure. To substantiate the identification of Band A, previously described in this work as the D fragment, the thermolabile properties were studied by heating plasma and plasmin treated plasma to 56 C for 15 minutes. Immunoelectrophoresis of this sample demonstrated that the product migrating near the well was no longer visible, thus establishing the identification of Band A as fragment D. (Refer to Figure 11)

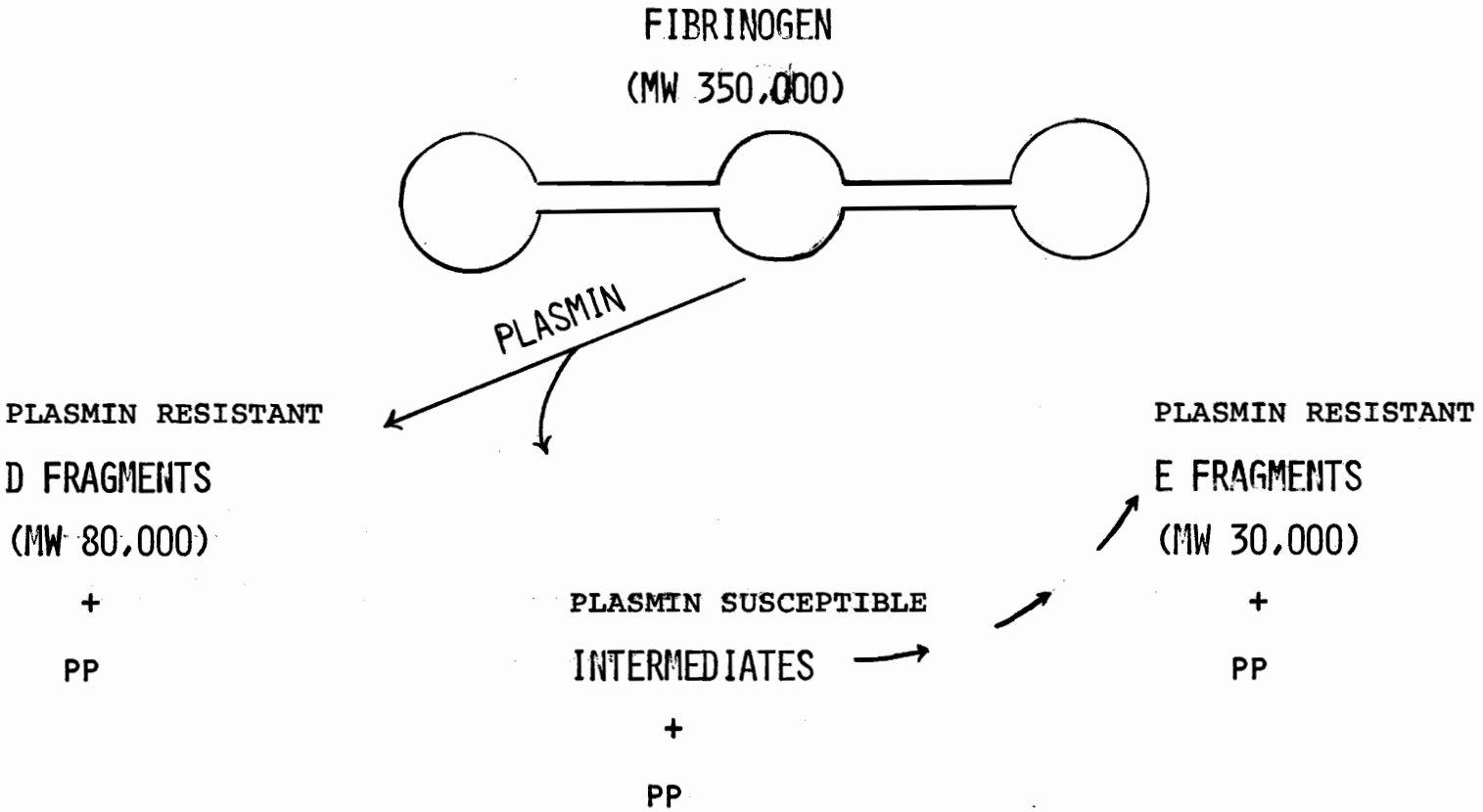


Fig. 15. Model of fibrinogen digestion by plasmin. Plasmin digestion appears to give immediate rise to two D fragments and a sequential degradation to two E fragments. (pp indicates small polypeptides or peptides and amino acid residues.)

Gel filtration chromatography using Sephadex was employed to separate and further identify the larger fragments formed during the proteolysis of fibrinogen, i.e., fragment D (molecular weight 80,000) and fragment E (molecular weight 30,000). Following fractionation on the column, immunoelectrophoresis was performed on the two absorbance peaks. Both Band A and Band B were observed in elution samples contained in the first absorbance peak. The D fragment appeared to be present in a greater concentration than the E fragment. Only Band B was demonstrated in the elution samples contained in the second absorbance peak, indicating that partial separation had been accomplished. The larger molecular weight fragments would be expected to be eluted from the column before the smaller molecular weight fragments. Since partial separation was obtained and Band B appeared as the exclusive component in the second peak, Band B most probably represents the E fragment.

When electrophoresis was performed on normal human plasma following the addition of streptokinase (refer to Figure 9) a different pattern was observed. Two precipitin bands were visible, one appearing in the beta region, just above the origin, and the second emerging initially from the anodal end of the first into the alpha region. The presence of two precipitin bands at time zero, instead of one, would indicate that the action of streptokinase is faster than the action of plasmin. However, at 30 minutes the band representing the E moiety demonstrates a slower electrophoretic

mobility and greater identity with fragment D. This spur could possibly result from reconjugation of the E moiety into an intermediate product, a "reversal" effect. Further degradation of the D fragment would not be a plausible explanation for the spur, in that the plasmin resistant end product of the E fraction is not seen. The reversal effect exhibited by streptokinase has not been explained from the data presented herein. However, the occurrence of this effect has been substantiated by Hunter and Allensworth (1966). These workers demonstrated by serial fibrinogen assays a biphasic effect on fibrinogenolysis resulting from the addition of streptokinase, i.e., after one hour fibrinogen levels were approximately 25 to 33% of the starting material, whereas with prolonged streptokinase treatment fibrinogen levels returned to approximately 75% of the original assay. (see Figures 16 and 17) This effect was not seen when fibrinogenolysis was initiated using commercial plasmin in excess. A possible explanation for the differences observed in the type of reactivity of plasmin and streptokinase may relate to the qualitative and quantitative kinetics of the enzyme systems. Mathematical and experimental studies by Liniger and Ruegsegger (1967) showed that when streptokinase (an activator) enzymatically activates plasminogen generating the active enzyme plasmin, an antiactivator inhibits streptokinase and plasmin is inhibited by reacting stoichiometrically with antiplasmin. It appears, however, the excessive extrinsic plasmin will completely hydrolyze fibrinogen and/or fibrin to stable

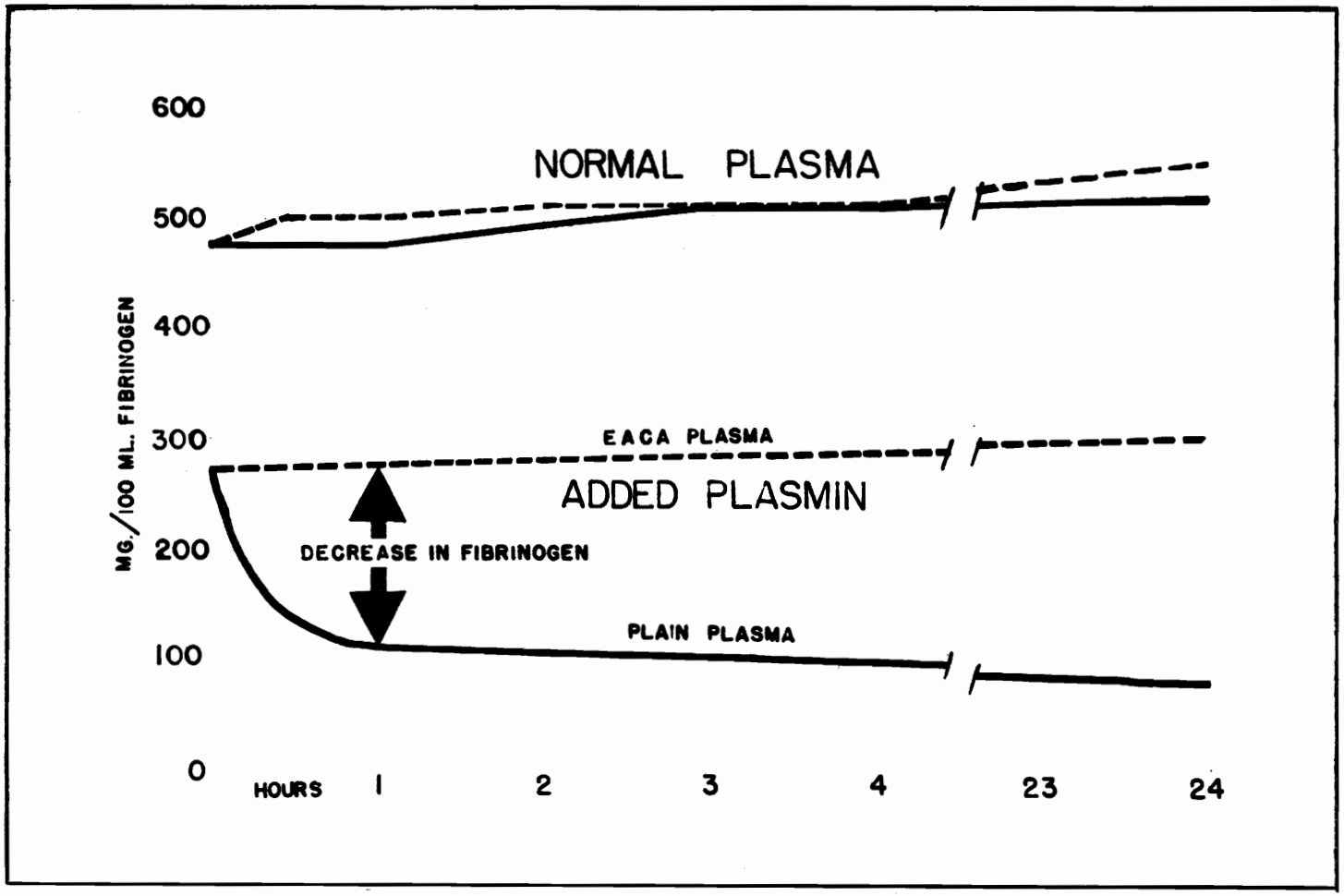


Fig. 16. Fibrinogenolysis is demonstrated by serial fibrinogen assays in a specimen of plasma containing added commercial plasmin.

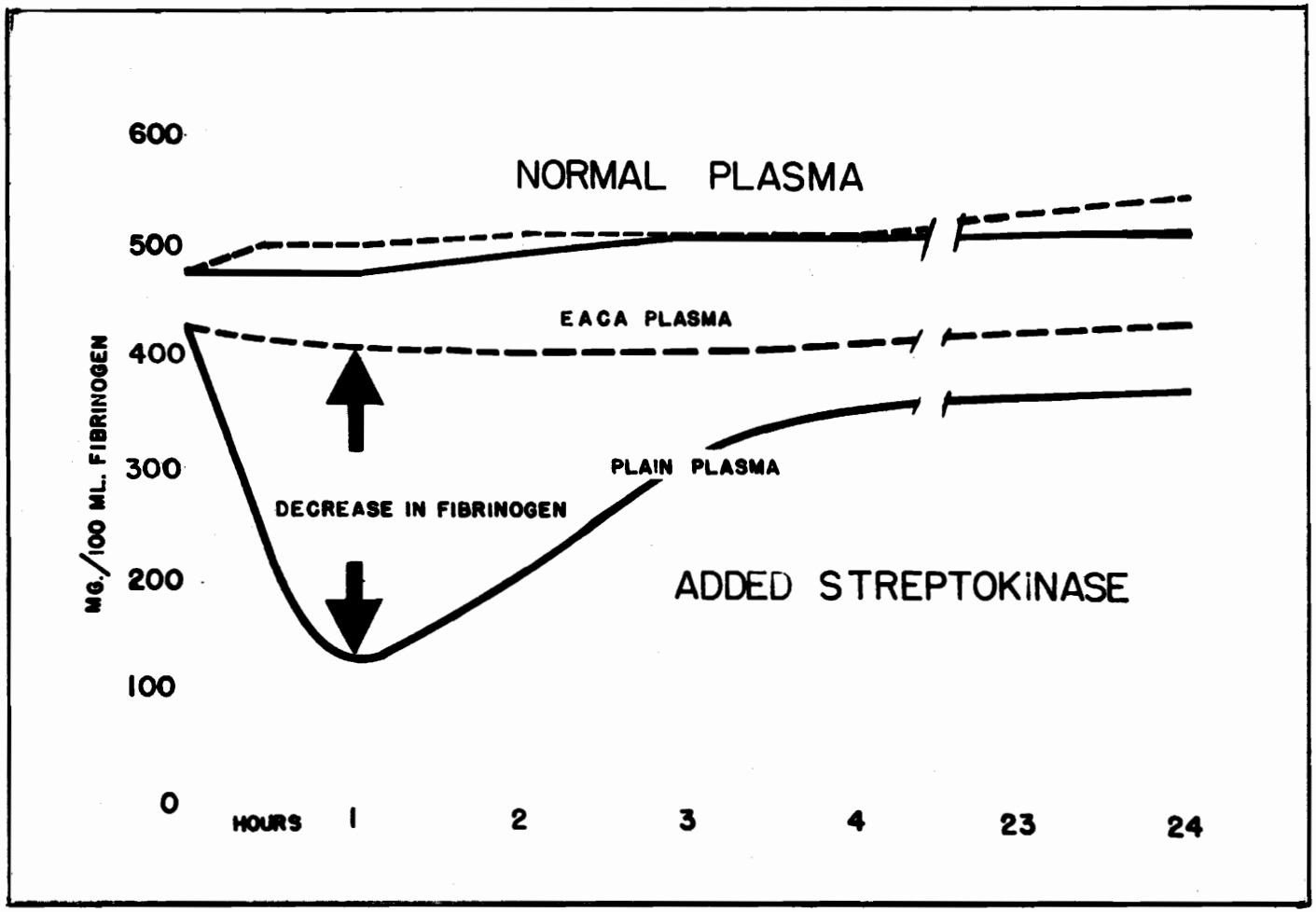


Fig. 17. Serial fibrinogen assays demonstrate a biphasic effect on fibrinogenolysis resulting from the addition of excess streptokinase.

D and E end products.

Serum samples from 196 patients were collected at random without prior knowledge of the admitting diagnosis. Immunodiffusion was employed to screen these sera for the presence of fibrinogen and/or breakdown products of fibrinogen. Since fibrinogen is completely converted during the clotting process, (Rodman, 1968) the presence of fibrinogen and/or products of fibrinogen in the serum in traceable amounts were considered abnormal, indicating a disease state. Fibrinogen and/or breakdown products of fibrinogen were found in the serum of ten patients, or approximately 5% of the 196 serum samples. Immunoelectrophoresis was employed to further characterize the products demonstrated by immunodiffusion. A band appearing in the same immunoelectrophoretic position as the D fraction was demonstrated in eight of the ten sera, while bands occupying the immunoelectrophoretic positions previously observed for both the D and E fractions were demonstrated in the remaining two sera. Since the filtering threshold of the glomerulus has been reported at a molecule weight of approximately 40,000 (Fulton, 1956), the demonstration of only the D fraction in 80% of the sera tested is not surprising. The molecular weight of this fraction is around 80,000, whereas the molecular weight of the E fraction is 30,000. If kidney excretion of the E fragment is consistent with the behavior patterns of other proteins of similar molecular weight, a clearance from the blood stream of the E fraction by the kidney would be predicted and expected. It is interesting to

note that the serum samples containing both the D and E fragments were collected from patients having a history of renal disease.

A modified fibrinogen assay was used to correlate increased levels of plasma fibrinogen with the presence of fibrinogen proteolytic products in the serum, whereas the euglobulin lysis time was employed to correlate increased fibrinolytic activity with the presence of degradation products in the serum. The lack of patient material precluded an adequate evaluation of fibrinolytic activity and fibrinogen levels; however, the presence of fibrinogen breakdown products in the serum does not necessarily accompany increased fibrinogen levels or prolonged euglobulin lysis time. Only the plasma from one of the two available patients exhibited an increased fibrinogen level and prolonged euglobulin lysis time.

Fresh plasma samples from the two patients just described were cooled to 4 C. and observed for the appearance of a cryoprecipitate. Cryoprecipitation was not evident in the plasma of either patient, suggesting that in addition to increased fibrinogen levels and fibrinolytic activity cryofibrinogen is not necessarily correlated with fibrinogen breakdown products demonstrated in the serum.

McKee et al. (1962) studied samples of plasma from 670 hospitalized patients. Twenty-nine patients in this study demonstrated a cryoprecipitate termed by these workers as cryofibrinogen. Later work by Lipinski et al. (1967) suggested that the cryoprecipitate is a soluble complex formed in the presence of increased fibrinogen

breakdown products and intermediate products formed in the enzymatic conversion of fibrinogen to fibrin. The studies that have been reported on the degradation products of fibrinolysis do not confine increased fibrinolytic activity to any single disease entity (Menon, 1969) or physiologic site. Indeed, fibrinolytic activity has been demonstrated to be distributed throughout the tissue components of the body, with the exception of the liver (Todd, 1959). However, the greatest preponderance of digestion products appears in instances where the liver, renal and intravascular systems are involved. Doubtlessly, as further studies are undertaken and procedures for studying fibrinogen breakdown products become more refined, the relationship of these products to specific disease entities will be elucidated and the diagnostic and therapeutic implications will be understood. Although current methodology for detecting fibrinogen breakdown products in the serum cannot readily be employed as a diagnostic tool, those instances of bleeding diathesis secondary to intravascular fibrinolysis could be rationally and specifically treated if fibrinolytic products were identified.

SUMMARY

1. Human fibrinogen was degraded by the addition of commercial fibrinolytic agents to pooled normal human plasma. Agar gel immunoelectrophoresis was employed to identify and characterize the products resulting from the proteolytic action. Two products were observed and identified as the D and E fragments. The D fragment, represented by a band in the beta area of the electrophoretic field, is formed rapidly and remains stable. The E moiety is formed at a slower rate and is represented as a migratory band originating from the locus of the D fragment at the onset of digestion and progressing across the electrophoretic field to the prealbumin region at the completion of digestion.
2. Gel filtration was used for the separation of the D and E fractions. These fractions were identified by immunoelectrophoretic technique.
3. Serum samples from 196 hospitalized patients were studied by immunodiffusion for the presence of spontaneous fibrinolytic products. The spontaneous products were detected in the serum of 10 of the 196 patients tested.
4. Immunoelectrophoresis was employed to further characterize the fibrinolytic products occurring spontaneously in the serum of 10 patients. Evidence of the D fraction was demonstrated in 80% of the sera, whereas in 20% of the sera both the D and E

fractions were observed.

5. Fibrinogen levels, euglobulin lysis time and cryofibrinogen were determined in plasma samples from 2 of the 10 patients. Only one patient exhibited an increased fibrinogen level and euglobulin lysis time.

6. The clinical evaluation of the patient's disease state was correlated in the instances where fibrinogen breakdown products were present in the serum. Physiologic sites predominently associated with fibrinogen breakdown products included the liver, the kidney, and the intravascular system.

BIBLIOGRAPHY

Alimi, S.Y., J.W. Hampton, G.J. Race, and R.J. Speer. 1968. Fibrin stabilizing factor (Factor XIII). Amer. J. Med. 44: 1-7.

Alkjaersig, N., A. P. Fletcher, and S. Sherry. 1958a. The activation of human plasminogen. I. Spontaneous activation in glycerol. J. Biol. Chem. 233: 81-85.

Alkjaersig, N., A.P. Fletcher, and S. Sherry. 1958b. The activation of human plasminogen. II. A kinetic study of activation with trypsin, urokinase, and streptokinase. J. Biol. Chem. 233: 86-90.

Alkjaersig, N., A.P. Fletcher, and S. Sherry. 1959a. ε-Amino caproic acid: an inhibitor of plasminogen activation. J. Biol. Chem. 234: 832-837.

Alkjaersig, N., A.P. Fletcher, and S. Sherry. 1959b. The mechanism of clot dissolution by plasmin. J. Clin. Invest. 38: 1086-1095.

Alkjaersig, N., A.P. Fletcher, and S. Sherry. 1962. Pathogenesis of the coagulation defect developing during pathological plasma proteolytic ("fibrinolytic") states. II. The significance, mechanism and consequences of defective fibrin polymerization. J. Clin. Invest. 41: 917-934.

Alkjaersig, N. and S. Fischer. 1964. Proteolysis of fibrinogen by plasmin. Thrombos. Diathes. Haemorrh. 11: 284-285.

Bang, N.U., A.P. Fletcher, N. Alkjaersig, and S. Sherry. 1962. Pathogenesis of the coagulation defect developing during pathological proteolytic ("fibrinolytic") states. III. Demonstration of abnormal clot structure by electron microscopy. J. Clin. Invest. 41: 935-948.

Beck, E.A. and D.P. Jackson. 1966. Studies on the degradation of human fibrinogen by plasmin and trypsin. Thrombos. Diathes. Haemorrh. 16: 526-540.

Beller, F.K. and M. Maki. 1967. Properties of fibrinogenolysis and fibrinolysis products in immune assays. Thrombos. Diathes. Haemorrh. 18: 114-132.

Bergstrom, K., B. Blomback, and G. Kleen. 1960. Studies on the plasma fibrinolytic activity in a case of liver cirrhosis. Acta. Med. Scand. 168: 291-305.

Biggs, R. and R. G. McFarlane. 1963. Human blood coagulation. 3rd Ed. Blackwell Scientific Publications, Oxford.

Byers, B. 1966. Model R-103 immunoelectrophoresis accessory instruction manual. Beckman Instruments, Inc., Fullerton, California.

Christensen, L.R. 1945. Streptococcal fibrinolysis: a proteolytic reaction due to a serum enzyme activated by streptococcal fibrinolysin. J. Gen. Physiol. 28: 363-383.

Crowle, A.J. 1961. Immunodiffusion. Academic Press, New York.

Dellelbach, H.R. and S.E. Ritzmann. 1967. Lab synopses, Vol. 2. Hoechst Pharmaceutical Co., Kansas City.

DeNicola, P. and F. Soardi. 1958. Fibrinolysis in liver diseases. Study of 109 cases by means of the fibrin plate method. Thrombos. Diathes. Haemorrh. 2: 290-299.

Finkbiner, R.B., J.J. McGovern, R. Goldstein, and J.P. Bunker. 1959. Coagulation defects in liver disease and response to transfusion during surgery. Amer. J. Med. 26: 199-213.

Fischer, S., A.P. Fletcher, N. Alkjaersig, and S. Sherry. 1963. Fibrinogenolysis in vivo: Identification, occurrence, and characterization in man. J. Clin. Invest. 42: 931. (Abstr.)

Fisher, S., A.P. Fletcher, N. Alkjaersig, and S. Sherry. 1967. Immunoelectrophoretic characterization of plasma fibrinogen derivatives in patients with pathological plasma proteolysis. J. Lab. Clin. Med. 70: 903-922.

Fletcher, A.P., N. Alkjaersig, and S. Sherry. 1959a. The maintenance of a sustained thrombolytic state in man. I. Induction and effects. J. Clin. Invest. 38: 1096-1110.

Fletcher, A.P., N. Alkjaersig, and S. Sherry. 1959b. Pathogenesis of hemorrhagic diathesis developing during "fibrinolytic" states: The significance of defective fibrin polymerization. J. Clin. Invest. 38: 1005. (Abstr.)

Fletcher, A.P., N. Alkjaersig, and S. Sherry. 1962a. Fibrinolytic mechanisms and the development of thrombolytic therapy. Amer. J. Med. 33: 738-752.

Fletcher, A.P., N. Alkjaersig, and S. Sherry. 1962b. Pathogenesis of the coagulation defect developing during pathological plasma proteolytic ("fibrinolytic") states. I. The significance of fibrinogen proteolysis and circulating fibrinogen breakdown products. J. Clin. Invest. 41: 896-916.

Fletcher, A.P., O. Biederman, D. Moore, N. Alkjaersig, and S. Sherry. 1964. Abnormal plasminogen-plasmin system activity (fibrinolysis) in patients with hepatic cirrhosis: Its cause and consequences. J. Clin. Invest. 43: 681-695.

Fletcher, A.P. 1965. Fibrinolytic dysfibrinogenemias. Fed. Proc. 24: 822-826.

Fletcher, A.P., N. Alkjaersig, S. Fisher, and S. Sherry. 1966. The proteolysis of fibrinogen by plasmin: The identification of thrombin clottable fibrinogen derivatives which polymerize abnormally. J. Lab. Clin. Med. 68: 780-802.

Forman, W.B., and M.J. Barnhart. 1964. Cellular site for fibrinogen synthesis. J. Amer. Med. Assoc. 187: 128-132.

Fulton, J.F. 1956. Textbook of Physiology. W.B. Saunders Co. Philadelphia p. 923.

Gallimore, M.J. and J.T.B. Shaw. 1967. Some aspects of fibrin clot lysis and its inhibition by human serum. Thrombos. Diathes. Haemorrh. 13: 101-113.

Gladner, A., P.A. Murtaugh, J.E. Folk, and K. Laki. 1963. Nature of peptides released by thrombin. Ann. N.Y. Acad. Sci. 104: 47-52.

Glueck, H.J. and L.G. Herrmann. 1964. Gold precipitable fibrinogen "cryofibrinogen". Arch. Int. Med. 113: 748-757.

Godal, H.C. and I. Helle. 1963. The influence of fibrinogenolytic and fibrinolytic split products on the last stage of coagulation. Scand. J. Clin. Lab. Invest. 15: 327-330.

Goodpasture, E.W. 1914. Fibrinolysis in chronic hepatic insufficiency. Johns Hopk. Hosp. Bull. 25: 330-336.

Gormsen, J. and B. Laursen. 1967. Fibrinogen breakdown products and clotting parameters. Thrombos. Diathes. Haemorrh. 17: 467-481.

Grossi, C.E., A.H. Mareno, and L.M. Rausselot. 1961. Studies on spontaneous fibrinolytic activity in patients with cirrhosis of the liver and its inhibition by epsilon amino caproic acid. Ann. Surg. 153: 383-393.

Grossi, C.E., L.M. Rausselot, and W.F. Panke. 1962. Coagulation defects in patients with cirrhosis of the liver undergoing portasystemic shunts. Amer. J. Surg. 104: 512-526.

Hall, C.E. and H.S. Slayter. 1959. The fibrinogen molecule: Its size, shape, and mode of polymerization. J. Biophys. Biochem. Cytol. 5: 11-15.

Hirschfield, J. 1960. Immunoelectrophoresis: Procedure and application to the study of group-specific variations in sera. Sci. Tools 7: 18-25.

Hirsh, J., A.P. Fletcher, and S. Sherry. 1965. Effect of fibrin and fibrinogen proteolysis products in clot physical properties. Amer. J. Physiol. 209: 415-424.

Hunter, D.T. and J.L. Allensworth. 1965. A modified fibrinogen assay. Amer. J. Clin. Path. 44: 359-363.

Hunter, D.T. and J.L. Allensworth. 1966. Plasmin. A new method for the estimation of physiologically active plasmin. Hemostase 1: 201-206.

Jacobsen, C.D. 1968a. Proteolytic capacity in human plasma. Scand. J. Clin. Lab. Invest. 21: 216-226.

Jacobsen, C.D. 1968b. Proteolytic capacity in human plasma. II. Genetics and clinical study. Scand. J. Clin. Lab. Invest. 21: 227-237.

Jamieson, G.A. and J.H. Pert. 1963. Studies on the digestion of human fibrinogen with plasmin. Vox Sang. 8: 460 (Abstr.)

Jerushalmy, Z. and M.B. Zucker. 1966. Some effects of fibrinogen degradation products (FDP) on blood platelets. Thrombos. Diathes. Haemorrh. 15: 413-419.

Kalbfleisch, J.M. and R.M. Bird. 1960. Cryofibrinogenemia. New England J. Med. 263: 881-886.

Kaplan, M.H. 1944. Nature and role of the lytic factor in hemolytic streptococcal fibrinolysis. Proc. Soc. Exp. Biol. (N.Y.) 57: 40-43.

Kaulla, K.N. von and R.L. Schultz. 1958. Methods for the evaluation of human fibrinolysis. Amer. J. Path. 29: 105-111.

Kopec, M., E. Kowalski, and J. Stachurska. 1960. Studies on paracoagulation. Role of antithrombin III. Thrombos. Diathes. Haemorrh. 5: 285-295.

Kopec, M., A.Z. Budzynski, J. Stachurska, Z. Wegrynowicz, and E. Kowalski. 1966. Studies on the mechanism of interference by fibrinogen degradation products (FDP) with the platelet function. Role of fibrinogen in the platelet atmosphere. Thrombos. Diathes. Haemorrh. 15: 476-490.

Kowalski, E. 1968. Fibrinogen derivatives and their biologic activities. Hematology 5: 45-59.

Kowalski, E., M. Kopec, and Z. Wegrzynowicz. 1964a. Influence of fibrinogen degradation products (FDP) on platelet aggregation, adhesiveness and viscous metamorphosis. Thrombos. Diathes. Haemorrh. 10: 406-423.

Kowalski, E., A.Z. Budzynski, M. Kopec, Z.S. Latallo, B. Lipinski, and Z. Wegrzynowicz. 1964b. Studies on the molecular pathology and pathogenesis of bleeding in severe fibrinolytic states in dogs. Thrombos. Diathes. Haemorrh. 12: 69-86.

Kwaan, H.C., A.J.S. McFadzean, and J. Cook. 1956. Plasma fibrinolytic activity in cirrhosis of the liver. Lancet 1: 132-137.

Kwaan, H.C., A.J.S. McFadzean, and J. Cook. 1957. On plasma fibrinolytic activity in cryptogenic splenomegaly. Scot. Med. J. 2: 137-150.

Laki, K., J.A. Gladner, and J.E. Folk. 1960. Some aspects of the fibrinogen-fibrin transition. Nature 187: 758-761.

Latallo, Z.S., A.P. Fletcher, N. Alkjaersig, and S. Sherry. 1962a. Influence of pH, ionic strength, neutral ions, and thrombin on fibrin polymerization. Amer. J. Physiol. 202: 675-680.

Latallo, Z.S., A.P. Fletcher, N. Alkjaersig, and S. Sherry. 1962b. Inhibition of fibrin polymerization by fibrinogen proteolysis products. Amer. J. Physiol. 202: 681-686.

Latallo, Z.S., A.Z. Budzynski, B. Lipinski, and E. Kowalski. 1964. Inhibition of thrombin and of fibrin polymerization, two activities derived from plasmin-digested fibrinogen. Nature 203: 1184-1185.

Laursen, B. and J. Gormsen. 1967. Spontaneous fibrinolysis demonstrated by immunological technique. Thrombos. Diathes. Haemorrh. 17: 42-50.

Lewis, J.H. and J.H. Wilson. 1964. Fibrinogen breakdown products. Amer. J. Physiol. 207: 1053-1057.

Liniger, W. and P. Ruegsegger. 1967. Fibrinolysis. Thrombos. Diathes. Haemorrh. 17: 412-417.

Lipinski, B., Z. Wegrzynowicz, A. Z. Budzynski, M. Kopec, Z.S. Latallo, E. Kowalski. 1967. Soluble unclottable complexes formed in the presence of fibrinogen degradation products (FDP) during the fibrinogen-fibrin conversion and their potential significance in pathology. Thrombos. Diathes. Haemorrh. 17: 65-77.

Lorand, L. and A. Jacobsen. 1958. Studies on the polymerization of fibrin. The role of the globulin: Fibrin-stabilizing factor. J. Biol. Chem. 230: 421-434.

Lorand, L. 1965. Physiological roles of fibrinogen and fibrin. Fed. Proc. 24: 784-793.

McFarlane, R.G. and R. Biggs. 1948. Fibrinolysis: Its mechanism and significance. Blood 3: 1167-1187.

Menon, J.S. 1969. A study of fibrinolytic activity in subjects with different diseases. Lab. Pract. 18: 427-428.

Merskey, C., G.J. Kluner, and A.J. Johnson. 1966. Quantitative estimation of split products of fibrinogen in human serum related to diagnosis and treatment. Blood 28: 1-18.

Miale, J.B. 1967. Laboratory medicine hematology. 3rd Ed. C.V. Mosby Co., Saint Louis.

Milstone, Haskell. 1957. A factor in normal human blood which participates in streptococci fibrinolysis. Thrombos. Diathes. Haemorrh. 1: 264-286.

Mullertz, S. 1955. Formation and properties of the activator of plasminogen and of human and bovine plasmin. Biochem. J. 61: 424-434.

McKay, D.G., A. Kilman, and B. Alexander. 1959. Experimental production of afibrinogenemia and hemorrhagic phenomena by combined fibrinolysis and disseminated intravascular coagulation. New. England J. Med. 261: 1150-1154.

McKay, D.G. 1965. Disseminated intravascular coagulation. pp. 8-9. Harper and Row, New York.

McKee, P.A., J.M. Kalbfleisch, and R.M. Bird. 1962. The incidence and significance of cryofibrinogemia. Clin. Res. 10: 56. (Abstr.)

Nanninga, L.B. 1966. Preparation of anticoagulant split-product of fibrinogen and its determination in plasma. Fed. Proc. 25: 445. (Abstr.)

Nilehn, J.E. and J.M. Nelsson. 1964. Demonstration of fibrinolytic split products in human serum by an immunological method in spontaneous and induced fibrinolytic states. Scand. J. Haemat. 1: 313-330.

Nilehn, J.E. 1967a. Split products of fibrinogen after prolonged interaction with plasmin. Thrombos. Diathes. Haemorrh. 18: 89-100.

Nilehn, J.E. 1967b. Separation and estimation of split "products" of fibrinogen and fibrin in human serum. Thrombos. Diathes. Haemorrh. 18: 487-498.

Nolf, P. 1905. Des modifications de la coagulation du sang chez le chien apres exterpation du foie. Arch. Int. Physiol. 3: 1 (as reported by Sherry, S. and N. Alkjaersig. 1957. Studies on the fibrinolytic enzyme of human plasma. Thrombos. Diathes. Haemorrh. 1: 264-296.)

Nussbaum, M. and B.S. Morse. 1964. Plasma fibrin stabilizing factor activity in various diseases. Blood 23: 669-678.

Nussenweig, V. and M. Selegman. 1960. Analyse par des methodes immunochemiques de la degradation par la pasmine du fibrinogene humain et de la fibrine, a differentes stages. Rev. Hemat. 15: 451 (as reported by Gormsen, J. and B. Laursen. 1967. Fibrinogen breakdown products and clotting parameters. Thrombos. Diathes. Haemorrh. 17: 467-481.)

Osbahr, A.J., J.A. Gladner, and K. Laki. 1964. Studies on the physiological activity of the peptide released during the fibrinogen-fibrin conversion. Biochem. Biophys. Acta. 86: 535-542.

Pharmacia Fine Chemicals Incorporated. 1966. Sephadex-gel filtration in theory and practice. Pescataway, New Jersey.

Purcell, G. and L.L. Phillips. 1963. Fibrinolytic activity in cirrhosis of the liver. Surg. Gynec. Obstet. 117: 139-144.

Quick, A.J. 1966. Hemorrhagic disease and thrombosis. Lea and Felieger, Philadelphia.

Ratnoff, O.D. 1949. Studies on a proteolytic enzyme in plasma. IV. The rate of lysis of plasma clots in normal and diseased individuals with particular reference to hepatic disease. Johns Hopk. Hosp. Bull. 84: 29-42.

Ratnoff, O.D. 1954. An accelerating property of plasma for the coagulation of fibrinogen by thrombin. J. Clin. Invest. 33: 1175-1182.

Ratnoff, O.D. 1955. The effect of clotting on the spontaneous activation of plasmin. J. Clin. Invest. 34: 958-959.

Rodman, T. 1968. Blood coagulation and clot lysis. pp. 113-153. In J.L. Rabinowitz and R.M. Myerson Topics in Medicinal Chemistry. Interscience Pub., New York.

Sasaki, T., I.H. Page and J.R. Shainoff. 1966. Stable complex of fibrinogen and fibrin. Science 152: 1069-1071.

Sawyer, W.D., N. Alkjaersig, A.P. Fletcher, and S. Sherry. 1960a. Studies on the thrombolytic activity of human plasma. J. Clin. Invest. 39: 426-434.

Sawyer, W.D., N. Alkjaersig, A.P. Fletcher, and S. Sherry. 1960b. A comparison of fibrinolytic and fibrinogenolytic effects of plasminogen activators and proteolytic enzymes in plasma. Thrombos. Diathes. Haemorrh. 5: 149-161.

Scheidegger, J.J. 1955. Une micro-methode de l'immunoelectrophorese. Intern. Arch. Allergy Appl. Immunol. 7: 103-110.

Schultze, H.E. and J.F. Heremans. 1966. Molecular biology of human proteins. Elsevier, Amsterdam, London, New York.

Searcy, R.L. 1969. Diagnostic biochemistry. McGraw-Hill Book Company, New York.

Seegers, W.H. 1962. Prothrombin. Harvard, Cambridge.

Seegers, W.H. 1967. Blood clotting enzymology. Academic Press, New York.

Shainoff, J.R. and T.H. Page. 1960. Cofibrins and fibrin-intermediates as indicators of thrombin activity in vivo. Circulation Research 8: 1013-1022.

Shainoff, J.R. and T.H. Page. 1962. Significance of cryofibrin in fibrinogen-fibrin conversion. J. Exp. Med. 116: 687-707.

Sherry, S. and N. Alkjaersig. 1957. Studies on the fibrinolytic enzyme of human plasma. Thrombos. Diathes. Haemorrh. 1: 264-288.

Sherry, S., A.P. Fletcher, and N. Alkjaersig. 1959a. Fibrinolysis and fibrinolytic activity in man. Physiol. Rev. 39: 343-382.

Sherry, S., R.T. Lindemeyer, A.P. Fletcher, and N. Alkjaersig. 1959b. Studies on enhanced fibrinolytic activity in man. J. Clin. Invest. 38: 810-822.

Solum, N.O. 1966. Platelet aggregation during fibrin polymerization. Scand. J. Clin. Lab. Invest. 18: 577-587.

Tillett, W.S. and R.L. Garner. 1933. The fibrinolytic activity of hemolytic streptococci. J. Exp. Med. 58: 485-502.

Todd, A.S. 1959. The histological localization of fibrinogen activator. J. Path. and Bact. 78: 281-283.

Triantophyllopoulos, E. and D.C. Triantophyllopoulos. 1962. Coagulation studied on the electrophoretic fraction AFIF. Amer. J. Physiol. 203: 595-599.

Troll, W. and S. Sherry. 1955. The activation of human plasminogen by streptokinase. J. Biol. Chem. 213: 881-891.

Weerdt, C.M. van der and J. Vreeken. 1965. Influence of cryofibrin on the antiglobulin consumption test with platelets. Vox. Sang. 10: 536-542.

Wilson, P.A., G.P. McNicol, and A.S. Douglas. 1968. Effect of fibrinogen degradation products on platelet aggregation. J. Clin. Path. 21: 147-153.

Zlotnick, A. and S. Landau. 1966. Immunoelectrophoretic studies in patients with cryofibrinogenemia. J. Lab. Clin. Med. 68: 70-80.

Zucker, M.B., M. Siegel, E.E. Cliffton, J.W. Bellville, W.S. Howand, and C.E. Grossi. 1957. The effect of hepatic lobectomy on some blood clotting factors and on fibrinolysis. Ann. Surg. 146: 772-781.

VITA

Molly Tucker Maxwell was born on November 6, 1936, in Ephraim, Utah, a daughter of Byron E. and Zetta A. Tucker. Her early childhood was spent in Ephraim and her elementary and secondary education was obtained in the public schools in that city. After graduation from Ephraim High School in 1955, she attended Snow College for two years. In June, 1959, after completing a medical technology internship at the Latter Day Saints Hospital in Salt Lake City, Utah, she received a Bachelor of Science Degree, graduating with honors, from Utah State University in Logan, Utah. In July of the same year, she passed the requirement for registry as established by the American Society of Clinical Pathologists.

Following university graduation, Mrs. Maxwell has been employed as a medical technologist in the clinical laboratories at the Latter Day Saints Hospital and the Veterans Administration Hospital in Salt Lake City. Previous to her return to study at the University of Utah, she was employed in the Psychiatric Research Laboratories at the Veterans Administration Hospital.

Mrs. Maxwell is married to Dr. Kameron W. Maxwell, formerly of Mt. Pleasant, Utah. They currently reside at 3241 Millcreek Road, Salt Lake City, and have three children: Anne (9), Nancy (6), and John Byron (1).