SELECTED TOPICS ON BLOOD COAGULATION *

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INTRODUCTION

While the events which start with the release of tissue thromboplastin from damaged tissues and lead to blood clotting are more or less well understood and a general agreement exists about their sequence, the same cannot be said about the activation of factor X by the intrinsic pathway. The cause of this uncertainty is our poor knowledge about the early stages of blood coagulation, i.e., about the activation of the contact system by foreign surfaces – surfaces other than physiological vascular endothelium.

The factors of the intrinsic system which lead to the activation of factor X are factor XII (Hageman factor), the recently described Fletcher factor, factor XI, factor IX, and factor VIII. Since factor IX belongs to the prothrombin complex and together with factor VIII is immediately connected with the activation of factor X, the characteristics and function of these two factors will be discussed in the chapter on the components of the prothrombin complex.

CONTACT SYSTEM

Factor XII (Hageman Factor)

The existence of factor XII was discovered by its very absence from the blood of a patient, Mr. Hageman.[2,1] Congenital deficiency of factor XII (inherited as an autosomal recessive trait) is characterized by a prolongation of the whole blood clotting time, partial thromboplastin time, recalcification time, and defective generation of intrinsic prothrombin activator (plasma thromboplastin).[1,2] What is remarkable about this deficiency is that in spite of impaired in vitro clotting the patient has no hemorrhagic tendency and he is not protected from the occurrence of intravascular thrombosis. The laboratory findings are due to a genuine, immunologically established absence of factor XII and not to the development of an inhibitor or the synthesis of an inactive form of factor XII.[3]

Isolation and Physicochemical Properties

Factor XII has been isolated from human or

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bovine blood by four different groups of investigators.

Ratnoff and Davie[4] isolated factor XII from human plasma by a procedure involving removal of the prothrombin complex and fibrinogen by adsorption on aluminum hydroxide and diatomaceous earth first and then adsorption of factor XII on carboxymethyl cellulose, elution, ammonium sulfate fractionation, chromatography on DEAE-cellulose, and rechromatography on carboxymethyl cellulose. The purified material had a specific activity 3,000 to 5,000 times greater than the activity of the original plasma. It was, however, contaminated with plasminogen and traces of factor IX and was partially activated. The molecular weight of the purified factor depended on the degree of activation. During sucrose density gradient ultracentrifugation the nonactivated protein showed a sedimentation constant of 4.5 to 5.5 Svedberg units,[5] while the activated factor (XIIa) sedimented at the bottom of the centrifuge tubes indicating formation of aggregates. Aggregate formation was also demonstrated with Sephadex G-200 filtration where the activated factor was eluted with the viod volume.[5] It was found on the other hand, that the activated factor XII in its native milieu, the plasma, retains a sedimentation constant of 5 to 7 Svedberg units.[5]

The preparation of Ratnoff and Davie was not inactivated by 10^{-3} M diisopropylfluorophosphate (DFP). Nevertheless, high concentrations of factor XII hydrolyzed tosylarginine methylester.[21] Casting doubt on the last finding, however, is the observation that ellagic acid and kaolin, both powerful activators of factor XII, were unable to increase further the esterolytic activity of the purified preparations, which were only partially activated.[21] The question therefore arises whether the esterolytic activity was due to active factor XII or to contaminating enzymes (plasmin, factor XI, or kallikrein). Against the enzymic nature of factor XII is the observation that soybean trypsin inhibitor was unable to inhibit the procoagulant activity of the purified factor.[6]

Schoenmakers et al.[7] isolated factor XII from deprothrombinized bovine plasma by adsorption on a glass-powder column. The eluate was fractionated with cold 25% ethanol at pH 6.9 and 5.8 and then chromatographed on Sephadex G-25 and rechromatographed on CM- and DEAE-Sephadex. The final product had a specific activity 7,000 times higher than the activity of the original plasma and showed only a single band on disc electrophoresis. The sedimentation constant of the final product was 7.08 and the diffusion coefficient 7.14×10^{-7} cm^2/sec. The molecular weight calculated from these two values and an assumed specific volume of 0.7 ml/gm was 82,000. A similar molecular weight was estimated by Haanen et al.[8] by determining the sensitivity of the purified product to ionizing radiation. Schoenmakers et al. found that their product hydrolyzed tosylarginine methylester (TAMe) and benzoylarginineethylester (BAEE). This esterolytic activity was not sensitive to the inhibitory effect of soybean trypsin inhibitor, Trasylol, ϵ-aminocaproic acid and heparin. It could be inhibited, however, by lima bean trypsin inhibitor and DFP. What effect these two inhibitors exerted on the clotting activity of factor XII is not mentioned.

The same group of investigators[9] claim that activated factor XII can hydrolyze specific arginyl bonds of the β-corticotrophin and of the β chain of oxidized insulin and, in addition, can activate chymotrypsinogen to chymotrypsin. The purified factor was rich in carbohydrates[7] (10.7%) and specifically sialic acid (4.4%). Splitting, however, of the sialic acid with neuramidase did not affect its clotting or esterolytic activities.

Grammens and Mammen[10,512] adsorbed factor XII from bovine plasma on glass wool and eluted it with 10% sodium chloride. They fractionated the eluate with ammonium sulfate (25 to 60% saturation) and cold (40%) ethanol and chromatographed the precipitate on DEAE-cellulose. The purified protein gave only one band on disc electrophoresis which had the mobility of the gamma globulins and an isoelectric point of 7.9. Its molecular weight derived from a sedimentation constant of 7.04S, a diffusion coefficient of 4.45×10^{-7} cm/sec, and an experimentally determined partial specific volume of 0.724 ml/gram was 142,000. After sulfitolysis the sedimentation coefficient dropped to 1.97S.[35] The preparation of Grammens and Mammen was not inhibited by DFP and soybean trypsin inhibitor and lacked esterolytic activity. Its carbohydrate and sialic acid content were 2.89% and 0.35%, respectively. Both these values are considerably lower than the corresponding values of Schoenmakers et al.[7] (carbohydrates 10.7% and sialic acid 4.4%). It is not clear whether this purified protein corrects Hageman factor deficiency as efficiently as prepa-

rations obtained by other methods.[11] It seems as if the purification procedure itself inactivates the protein.[4,12,512] The amino acid composition of the final purification product has been determined.[512]

Speer et al.[12] isolated factor XII from outdated ACD human plasma by adsorption on celite. The eluate in 0.007 *N* ammonium hydroxide was fractionated by isoelectric precipitation and the pH 7.0 to pH 5.2 precipitate was fractionated with ammonium sulfate. The 50 to 67% saturation precipitate was then chromatographed on CM-Sephadex. The final material, although lacking esterolytic activity, was able to correct the clotting abnormalities of Hageman factor deficient plasma and was not inhibited by DFP. Calculation of the molecular weight from ultracentrifugal data gave a value of 20,000, while gel filtration indicated a value larger than 100,000, in agreement with the findings of Donaldson and Ratnoff who detected similar formation of aggregates by gel filtration.[5] The amino acid composition of this preparation was also determined.

It is obvious that the four groups of investigators were not working with exactly the same protein. The comparison of their preparations is hampered by the different origin of the starting material. As mentioned earlier, Schoenmakers et al.[7] and Grammens and co-workers[10,512] isolated factor XII from bovine plasma and both used glass to adsorb it. In spite of these basic similarities, Schoenmakers et al. found that their factor was inhibited by DFP and hydrolyzed TAMe and BAEE, while Grammens and Mammen found the exact opposite for their preparation. Suggestive of proteolytic activity, however, in the protein of Grammens and Mammen is the observation that in the presence of calcium their preparation inactivated bovine prothrombin complex.[13] Further differences have been observed concerning the clotting activity of these preparations. Schoenmakers et al. reported that their material corrected Hageman factor deficient plasma, while doubts have been expressed as to whether the protein isolated by Grammens and Mammen can do so.[11]

Comparison of the human material of Ratnoff and Davie[4] with that of Speer et al.[12] indicates that although the two groups used different methods of purification with significantly different results in purity, their preparations seem similar regarding the absence of sensitivity to DFP. On the other hand, there is obviously no agreement on the molecular size of the factor except that it forms aggregates. The activated preparation of Ratnoff and Davie was excluded from Sephadex G-200 and similarly Speer et al. found that their factor XII had a molecular weight greater than 100,000 by gel filtration. Another difference is that the material of Ratnoff and Davie had the mobility of the beta[513] while the protein of Speer et al. had the mobility of the gamma globulins.[12]

Of these four groups of investigators only Schoenmakers et al. made the unequivocal claim that their factor is inhibited by DFP and lima bean trypsin inhibitor and that it has esterolytic activity (Table 1). The amino acid composition of the material of Speer et al. and that of the preparation of Grammens and Mammen, although they represent proteins originating from different species, are similar regarding the concentration of 11 amino acids.[12,512] The two preparations, however, differ in the *N* terminal amino acids.[35] The bovine factor has glycine and valine[512] factor while the human has arginine only.[12]

It is interesting that the preparations of Ratnoff and Davie and of Speer et al., while both correct Hageman factor deficient plasma, are not inactivated to any considerable extent by DFP and are believed not to have any intrinsic esterolytic activity (the esterolytic activity of the preparation of Ratnoff and Davie is attributed to contaminants). Similar results were obtained with the preparation of Grammens and Mammen. In view of the very high purity of the preparations of Speer et al. and those of Grammens and Mammen, one is inclined to believe that Schoenmakers' material does not contain Hageman factor but probably some other factor involved in other stages of contact activation.

Activation

Factor XII is activated by an impressive number of materials: glass,[5,7,10] celite,[12] carboxymethylcellulose,[4,5] kaolin,[23] fatty acids,[24] ellagic acid,[25] skin,[26] collagen,[14] elastin,[27] crystals of sodium urate,[28] l-homocystine,[15] etc. Even freezing, thawing, and air bubbling have been reported to activate Hageman factor slightly.[21] According to Donaldson and Ratnoff[5] only ellagic acid can activate factor XII fully; glass activates it only partially. The activation by collagen and CM-cellulose is attributed to the presence of free carboxylic groups[21] in these substances. Calcium is not required for the activation of factor XII

TABLE 1

Factor XII: Physicochemical Characteristics

	Bovine		Human	
	Schoenmakers et al.[7]	Grammens et al.[5,12]	Speer et al.[1,2]	Ratnoff and Davie[4]
Sedimentation constant	7.08	7.04 (0.1 M KCl) 5.84 (6 M urea)		4.55–5.5[5]
Partial specific volume		0.724[a] or 0.740[b] ml/g		
Diffusion coefficient ($\times 10^{-7}$, cm^2/sec)	7.14	4.45 (or 3.88)		
Molecular weight	82,000 (sediment diffusion)	142,000 (sediment diffusion)[c]	20,000 (sediment equilibrium) > 100,000 (gel filtration)	
Isoelectric point		7.9		
Sensitivity to diisopropylfluorophosphate	+	–	–	–
Esterase activity	+	–	–	
Sensitivity to soybean trypsin inhibitor	–	–		–
Sensitivity to lima bean trypsin inhibitor	+			

[a] From the amino acid composition
[b] By differential sedimentation equilibrium in 6 M urea-H_2O and 6 M urea-D_2O solution
[c] Similar values were obtained by sedimentation equilibrium and Sephadex-gel filtration

since the latter can occur in the presence of EDTA. The activation of factor XII by sodium urate and homocystine has obvious clinical implications since it may initiate the deposition of fibrin inside joints and blood vessels.

Of great clinical importance is the in vivo activation of factor XII by epinephrine.[16] McKay et al. found that intravenous infusion of epinephrine into rabbits at 5 μg/kg body weight per minute for four hours resulted in a statistically significant decline in the concentration of factor XII and in disseminated intravascular coagulation by stimulating the α-adrenergic receptors. If these findings apply to man they may offer an explanation for the high incidence of myocardial infarction in societies where people are subjected to frequent social and economic stresses. Activation of factor XII has been suggested to occur after addition of endotoxin in vitro[17] or infusion of sodium polyethanol sulfonate (Liquoid) in vivo.[18] The effect of the latter can be prevented by lysozyme, a known inhibitor of the activation of factor XII.

Biological Properties

Iatridis and Ferguson[19] have presented evidence that factors XII and XI are adsorbed on the surface of the platelets where they contribute to the so-called "plasmatic atmosphere" of these cells and where they become activated. In this connection it is interesting to note that Mammen and Grammens[20] showed both in vitro and in vivo that factor XII causes aggregation of platelets and that this aggregation is potentiated by the presence of ADP. Ratnoff,[21] on the other hand, found that purified human factor XII which had been activated with ellagic acid was unable to agglutinate platelets. Intriguing are the experiments of Seegers et al.,[13] who found that preparations of factor XII obtained by the method of Grammens and Mammen increase significantly the amount of thrombin which is generated from bovine prothrombin complex in the presence of calcium

chloride platelet factor 3 (platelet lipoproteins) and factor V. The generation of thrombin in this case is due to the activation of factor X contained in the prothrombin complex.[13] It is obvious that factor XII in this instance behaves like a weak preparation of factor VIII. Shamberge found that factor XIIa, either directly or by the intermediary action of another component of the contact system, can shorten the one stage prothrombin time by activating factor VII.[29] Hemker et al.[30] similarly attributed this procoagulant effect to activation of factor VII. This conclusion is probably connected (in the sense that factor XII affects components of the prothrombin complex) with the observation of Seegers et al.[13] that Hageman factor renders bovine prothrombin complex irresponsive to the activating effect of tissue thromboplastin. It appears that Hageman factor affects one or possibly more of the components of the prothrombin complex which take part in the extrinsic generation of thrombin.

Fletcher Factor

The contact activation of plasma requires not only factors XII and XI but still another recently discovered factor which has been named Fletcher factor from the name of the first patient who was found to be deficient in this coagulation component. The first indication for the existence of this factor was obtained by Schiffman et al.[32] who showed that the clotting abnormality of plasma rendered free of active factors XII and XI by celite adsorption is not completely corrected by purified factors XII and XI, but that an additional factor, present in factor XII or factor XI deficient plasmas, is also needed.

Fletcher factor has been partially purified by Hathaway and Alsever[31] by CM-Sephadex chromatography of intact normal plasma by increasing the concentration of NaCl (0.05 M to 0.3 M) in the eluting buffer. Patients deficient in this factor exhibit a prolonged partial thromboplastin and recalcification time, but like the factor XII deficient patients do not bleed. However, in contradistinction to factor XII or XI deficiencies, the clotting abnormalities of Fletcher factor deficiency are corrected by prolonged contact with activators of Factor XII. In the sequence of contact activation Fletcher factor is probably interposed between factors XII and XI, since contact activation of Factor XI deficient plasma and addition of this plasma to Fletcher deficient plasma corrects the defect of the latter[31] while addition of "activated" factor XII deficient plasma to Fletcher deficient plasma is without effect. Obviously, in the first case activated factor XII from the factor XI deficient plasma activates the Fletcher factor which is also present in this plasma, while in the second set of the experiments the absence of factor XII makes impossible the contact activation of Fletcher factor, which is present in the factor XII deficient plasma. Another peculiarity of Fletcher factor is that it is both adsorbed on and inactivated by celite[31] since neither the celite adsorbed plasma nor the eluate contains Fletcher factor.[31] It has been shown that celite eluates of both normal and Fletcher factor deficient plasmas correct the prolonged partial thromboplastin time of Fletcher factor deficient plasma.[31] The correction apparently is effected by active factor XI. It seems as if factor XI becomes activated on the surface of the celite by active factor XII alone and in the absence of Fletcher factor. This conclusion is also supported by the observation that ellagic acid shortens the recalcification time of factor XI and Fletcher factor deficient plasmas, but not of factor XII deficient plasma.[31]

The relationship of Fletcher factor to Hageman factor co-factor described by Ogston et al.[33] has not been explored yet. Hageman factor cofactor has been shown to be necessary for the activation of the proactivator of plasminogen by factor XII.[33] The Hageman factor co-factor has been partially purified by adsorption on celite 512, elution with 0.5 M NaCl followed by chromatography on CM-cellulose and elution with a sodium chloride gradient from 0 to 0.5 M.[33] Fletcher factor, as mentioned earlier, has been purified directly from the plasma by chromatography on CM-Sephadex and elution with a sodium chloride gradient from 0.05 to 0.3 M.[31]

Factor XI

Isolation and Properties

Factor XI was first discovered by Rosenthal, Dreskin, and Rosenthal.[34] These authors studied members of a family suffering from a mild hemorrhagic condition characterized by impaired prothrombin consumption. The affected individuals were homozygous for the defect. The concentration of Factor XI in normal individuals varies between 0.55 to 1.85 plasma equivalent units per

ml.[21] One plasma equivalent unit is the concentration of 1 ml pooled normal plasma.

The electrophoretic mobility of factor XI determined in unfractionated serum[37] lies between those of the beta and gamma globulins. Factor XI activity in human plasma subjected to Sephadex G-200 chromatography is exhibited by proteins of a molecular weight of 100,000 to 200,000.[36] This observation is in essential agreement with the findings of Donaldson and Ratnoff who determined the sedimentation constant of a preparation of factor XI isolated from Factor XII deficient plasma by sucrose density gradient ultracentrifugation and found it to be 5 to 6 Svedberg units.[5] Diatomaceous earth (Filtercell) selectively adsorbs first factor XI and at higher concentrations, factor XII.[23]

Active factor XI has also been purified by Kingdon et al.[38] Their most pure preparations were obtained from serum by chromatography on CM-cellulose followed by ammonium sulfate fractionation and chromatography on hydroxyappatite. The active factor hydrolyzed TAMe and benzoylarginine ethylester[38] and was inhibited by diisopropylfluorophosphate.[6,38]

Activation

The activation of factor XI by factor XII or by Fletcher factor does not seem to be of proteolytic nature since it is not blocked by potent antiproteases like soybean trypsin inhibitor or by benzoylarginine methylester.[6] Significantly, the sedimentation coefficient of factor XI is not changed after activation with factor XIIa.[5] Ratnoff claims that the clot promoting activity is proportional to the amount of factor XI present, while the rate of its formation is proportional to the concentration of factor XIIa.[6] These findings of Ratnoff are in essential disagreement with those of Haanen et al.[9] The latter investigators have shown that the activation of factor XI does not follow zero order kinetics and that the amount of factor XIa formed is proportional also to the concentration of activated factor XII present.

Strong solutions of sodium chloride (1 M) inhibit the activation of factor XI to factor XIa,[6] presumably by eluting factor XIIa from its attachment to glass or kaolin.

Adsorption on glass is not an exclusive property of factors XII and XI, but is shared by other plasma proteins as well.[9,40] By becoming adsorbed these nonspecific proteins can prevent competitively the adsorption of factors XII and XI and thus prevent their activation. This explains why normal plasma is not activated as well in glass tubes previously coated with factor XII deficient plasma as in new uncoated tubes.[39,515] Haanen et al.[9] believe that for the activation of factor XI the formation of a complex with factor XII on the surface of the activating glass is necessary. In this complex the active site of factor XII is covered. After activation, factor XI deteriorates, probably while still complexed with factor XII on the surface of the glass. In favor of such a complex formation and of the role of Hageman factor in the activation of factor XI are also the experiments of Schoenmakers (quoted from Haanen et al.[9]), who used purified factor XII, and chymotrypsinogen instead of factor XI. As in the case of the activation of factor XI, both the rate of formation and the amount of chymotrypsin formed were affected by the concentration of factor XIIa. Chymotrypsinogen acted as an inhibitor of its own activator.

Inhibitors

After being activated factor XI is released into the plasma. There, on the one hand it activates factor IX in the presence of calcium and on the other, it becomes inactivated by a plasma inhibitor.[514-517] This inhibitor, anti-factor XIa, has been purified 100-fold by a combination of zinc acetate precipitation, anion exchange chromatography on DEAE-cellulose and starch block electrophoresis.[41] The final product gave nonhomogeneous patterns in disc electrophoresis and immunoelectrophoresis. The protein with anti-XIa activity had an α-globulin mobility and a sedimentation constant of 3.69S. The anti-XIa inhibitor is inactivated only after heating above 56°C and is relatively stable over a wide range of pH values retaining full activity between pH 7 to 9. Purified anti-factor XIa did not inactivate factor XIIa and it must be different from antithrombin III as indicated by the observation that a normal concentration of anti-XIa was found in persons with reduced levels of antithrombin III.[41] Anti-XIa is most likely an enzyme since its concentration is not reduced as it inactivates factor XIa.[41]

Factor XIa is also inactivated by an inhibitor of the esterolytic activity of the first component of complement.[42] This inhibitor is of broad specificity and inactivates in addition chymotrypsin, kallikrein, plasmin, plasma permeability factor,

and factor XII. The inhibition of factor XII by an antiprotease is difficult to explain, since this factor is not considered a protease by most investigators.

THROMBIN GENERATION

Prothrombin Complex

Since 1892 when Alexander Schmidt[43] postulated the existence of prothrombin, several efforts were made to isolate this proenzyme from plasma. The methods involved isoelectric precipitation by diluting the plasma with water and adjusting the pH to 5.3,[44] adsorption of prothrombin on an insoluble salt of an alkaline earth metal and elution with CO^2,[45] precipitation with acetone,[46] or precipitation first with acetone and then with ammonium sulfate,[47] or removal of the fibrinogen by heating the plasma to 55°C.[47] Seegers et al.[48] in 1938 modified the isoelectric precipitation method of Mellanby[44] and combined it with the adsorption on $Mg(OH)^2$ and elution by CO^2 technique of Fuchs.[48a] They thus obtained the first preparations of prothrombin which were stable and relatively very active (151 Iowa units/mg dry weight). Seegers and his colleagues improved this method several times in the course of 14 years by introducing modifications and further purification of the carbon dioxide eluate by 50 to 67% saturation with ammonium sulfate and additional isoelectric precipitations first to pH 5.4 to remove impurities and then to pH 4.6 to obtained the prothrombin.[49] The best preparations obtained by this procedure show specific activities up to 2,200 Iowa units/mg of protein[50] and appear homogeneous by several physiochemical criteria: ultracentrifugation,[50,231] free boundary electrophoresis below pH 6.9,[50,123] phase rule solubility,[51] electron microscope shadow casting,[52] and *N* terminal amino acid analysis (only alanine).[53-55] Therefore, these preparations were considered to represent one molecular species, i.e., purified prothrombin. Since they became activated spontaneously and formed thrombin when they were dissolved in 25% sodium citrate or other concentrated salt solutions, the activation was considered autocatalytic in nature.[56]

When the congenital coagulation defects which are due to deficiencies of factors VII, IX, and X were discovered, purified prothrombin was shown to correct all the clotting abnormalities attributed to the lack of these factors.[51,96,245] It also corrected the abnormalities which develop in the plasma of patients receiving dicumarol and other oral anticoagulants.

In an attempt to separate the newly discovered factors or to simplify the purification procedure several investigators introduced modifications or incorporated new techniques in the isolation procedure: adsorption of the plasma or plasma fractions on barium sulfate,[57,58] barium citrate,[59-63,67] tricalcium phosphate,[64] DEAE-cellulose,[65] ECTEOLA,[66] elution with sodium citrate,[57,58,64] disodium EDTA,[60,61] or decomposition of the barium citrate with Amberlite IRF-97 (Na^+),[63] XE-64 (Na),[62] Dowex-50,[58,67,68] or ammonium sulfate.[65,69] Further purification of prothrombin has also been obtained by gel filtration (Sephadex),[62,66,68] filtration through asbestos Seitz filters,[57,85] ion exchange chromatography (amberlite IRC-50,[49,54,67] DEAE- or TEAE-cellulose,[70,71]), DEAE-Sephadex,[68] hydroxyapatite,[72] electrophoresis (Starch block,[73] polyacrylamide gel,[74] etc.), isoelectric focusing[75] or crystallization with barium salts.[68] Heparin[76] and soybean trypsin inhibitor or diisopropylfluorophosphate[77,111] have been added by other investigators to the plasma or during the purification procedure to inhibit the activity of the spontaneously generated factor Xa and thrombin. Factors X[74,77-80] and VII[74,81] have been isolated as separate protein entities[75] and fractions with factor IX[75] activity separate from prothrombin activity have also been obtained by combinations of these techniques, mainly by chromatography on amino-ethyl-cellulose and amino-ethyl-Sephadex, gel filtration, or isoelectric focusing.

In the meantime, additional experimental evidence has been accumulated highlighting the homogeneity of the prothrombin complex, i.e., of preparations exhibiting the activities of all four factors. Shapiro and Waugh[65] showed that their step 5 material (human), which could undergo spontaneous activation in sodium citrate (and should therefore contain all four factors), was homogeneous by chromatography on Sephadex G-100 and G-200, acrylamide gel electrophoresis in 7.2 M urea, and immunoelectrophoresis. No evidence for dissociation into subunits was found by Tishkoff et al.[68] when they studied their bovine prothrombin complex preparations by ultracentrifugation in a solution of 6 M guanidine HCl in the presence of mercaptoethanol.

Similarly, Magnusson[82] showed that reduction and carboxymethylation followed by maleylation of prothrombin prepared essentially by the method of Seegers failed to produce more than one peak when filtered through Sephadex G-200 even in the presence of 7 M urea. The effluent volume of this peak corresponded to a molecular weight of 68,000 which is the same as that found by ultracentrifugation and quantitative *N* terminal amino acid analysis.[82-84] These findings considered together with the fact that the prothrombin complex has only one *N* terminal amino acid (alanine) could be taken as evidence that the four factors are all parts of the same polypeptide chain.

It is difficult, however, to understand how covalent bonds could be split by chromatography on DEAE-cellulose or DEAE-Sephadex or by polyacrylamide gel electrophoresis or starch block electrophoresis, i.e., by procedures which can separate the prothrombin complex into its constituents. In addition, the sum of the molecular weights of the isolated factors in their native form is much greater than the molecular weight of the prothrombin complex. Both DEAE-prothrombin and factor X in the inactive form have been shown to have a molecular weight and a molecular radius more or less in the same range as that of the prothrombin complex;[66,68,76-78,98,99] similarly, a value of 59,000 has been reported for the molecular weight of factor VII.[81] There are also differences in the halflives of the four factors. Factor VII has a halflife of 35 min to 5 hr,[86] factor IX, 18 to 40 hr,[87-89] factor X, 20 hr to 2 days,[87] and prothrombin (factor II), 2 to 5 days.[87]

One should also take into consideration that although only one mole of alanine/mole of prothrombin complex has been detected,[53-55] two C terminal amino acids have been revealed by the ammonium thiocyanate method.[55] The fact that these C terminals are inaccessible to the A and B carboxypeptidases[55,90] is no proof that the prothrombin complex is a single chain molecule.

The apparently controversial findings seem to suggest, as Tishkoff et al.[68] have remarked, that the prothrombin complex represents a family of glycoproteins which have similar physicochemical properties (including probably *N* terminal amino acids) but different clotting activities. This explains their remarkable homogeneity in ultracentrifugation and many forms of gel filtration and chromatography and the difficulty in separating one factor from the other by chemical means; in contrast, their differentiation in terms of biological activity poses no difficulty. Concerning this group of substances nature has been partial: it has favored the biologist and handicapped the biochemist.

Physicochemical Characteristics

Prothrombin complex has been isolated from bovine, human, canine, rat, and buffalo blood. Most of the studies, however, have been performed with bovine and human complexes. Both are glycoproteins and in the plasma migrate with the α_2-globulins.[66,91,92] The isolated human prothrombin complex[59,66,93] and a small fraction of the bovine complex[68] retain the same mobility. On the other hand, the major part of the bovine complex shows the mobility of the α_1-globulins and there is also another minor component which migrates as a β_2-globulin.[68]

It has been shown that in buffers of low ionic strength ($\Gamma/2 = 0.15$)[59,68] and low pH (<9),[59] the prothrombin complex undergoes reversible association and forms aggregates. As a result, the sedimentation constant and other physicochemical characteristics exhibit a negative concentration dependence.[91] On the other hand, in buffers of high ionic strength, the complex undergoes changes in shape and hydration and the sedimentation constant decreases.[68] Thus, depending on the ionic strength and the pH of the solution, the sedimentation constant of the bovine complex has been found to vary from 3.3S[68] to 5.22S,[84] the diffusion coefficient from 5.17[68] to 6.24 ($\times 10^{-7}$, cm^2 sec^{-1}),[231] the Stokes radius from 3.75 to 4.15 mμ[68] and the frictional ratio from 1.32 to 1.63.[68] Similarly the sedimentation constant of the human prothrombin complex in buffers of 0.15 ionic strength can vary from 3.4S at pH 9.0 to 5.9S at pH 6.0.[59] Molecular weights determined in various buffers of high and low ionic strength varied from 54,000 to 86,600.[66,68,95] Values corrected for concentration dependence[83,91,126] or obtained in dissociating solvents (6 M guanidine – HCl containin 0.5% mercaptoethanol) or by sedimentation equilibrium[68] or values calculated from the amino acid and carbohydrate composition[94] were within a very narrow range: 66,000 to 70,000[68,83,91] for the bovine complex and 69,000 to 70,000[94,95,126] for the human complex.

TABLE 2

Prothrombin Complex: Physicochemical Data

	Bovine	Human
Sedimentation constant	3.3–5.22 S[68,84]	3.4–5.9[59,95] ($\Gamma/2 = 0.15$)
Diffusion coefficient ($\times 10^{-7}$, cm^2, sec^{-1})	5.17[68]–6.24[231]	5.14[95] ($\Gamma/2$ 0.15, pH 6.0)
Stokes radius mμ	3.75–4.15[68]	4.18[95]
Partial specific volume ($\bar{V}$) ml/g	0.70[231]–0.719[68]	0.71[94]–0.719[95]
Isoelectric point	4.25[123] ($\Gamma/2 = 0.2$) 4.1[68] ($\Gamma/2 = 0.1$)	3.8[95] (isoelectric focusing)
Isoinic point	7.6[68]	
Intrinsic viscosity	0.041[231] ($\Gamma/2 = 0.15$)	
Frictional ratio	1.17–1.63[68,231]	1.54[95]
Axial ratio	3.4[231] (viscosity increment) 3.7[231] (sediment diffusion)	
Length Å	119[231]	
Width Å	34[231]	
Height Å	98–107[52]	
N content, percent	14.7[533]	10.04–14.9[66,95,94,111]
Carbohydrate content, percent	10.2[533]–11.8[65]	6.4–10[66,95,94]
Extinction coefficient ($E^{1\%}_{1\ cm}$ at 280 mμ)	15.3[68]	12.7–15.2[94,111,65] 8.84[95]
Specific activity (units/mg protein)	2,200–3,000[68,50] (Iowa)	1,000–2,400 (NIH)[59,95,111,65]

Additional physicochemical data are presented in Table 2.

As mentioned earlier, the prothrombin complex appears homogeneous in the ultracentrifuge (sedimentation velocity pattern, sedimentation equilibrium analysis) even in the presence of dissociating agents[68] and also by gel filtration through Sephadex G-100.[65,68,76] Heterogeneity, however, has been detected by immunoelectrophoresis (one major component with the mobility of α_1-globulins and two minor with the mobilities of α_2- and β_2-globulins) and also by disc (two major and 3 to 4 minor components[68]) and starch gel electrophoresis (at least six components[66]).

Prothrombin

Prothrombin essentially free of factors VII and X was prepared by Goldstein et al.[57] in 1959 by Seitz filtration of bovine plasma before adsorption on $BaSO_4$. This prothrombin could not be activated by the regular two-stage reagents (tissue thromboplastin, calcium and factor V) without the addition of serum or serum fractions. It had a specific activity of 1,250 to 1,630 NIH units/mg protein and was free of thrombin, fibrinogen, factors V and VIII but it contained factor IX (PTC).

Prothrombin, chromatographically pure, was first obtained in 1960 by Seegers and Landaburu by chromatography of bovine complex on DEAE-cellulose.[70] Similar preparations were obtained later by Lechner and Deutsch,[96] Marciniak and Seegers,[97] Tishkoff et al.,[68] Ingwall and Sheraga,[98] Marciniak,[76] Mann et al.,[99] and others, using either DEAE-cellulose or DEAE-Sephadex. Such preparations could not be activated in concentrated solutions of sodium citrate or solutions of protamine sulfate; they could, however, become activated by the two-stage reagents which indicates that they contained some factor VII and factor X. Tests with deficient plasmas confirm this conclusion.[68] The tests also indicated that the preparations contained some factor IX. It has been found very difficult to separate the latter factor from prothrombin. Some separation of their activities was obtained recently by Pechet and Smith[75] by isoelectric focusing and also by chromatography on DEAE-cellulose (DE-52). In the latter case two kinds of prothrombin were differentiated: inert and reactive factor II. The reactive contained also all the activity of factor VII. The inert (it could not be activated to thrombin without the addition of serum) may represent either very pure prothrombin, free of

other factors, or the prethrombin of Seegers and Marciniak[100] or the modified zymogen of Tishkoff et al.[68]

The amino acid composition[98,101] and other physicochemical characteristics of chromatographed bovine prothrombin are very similar to those of the prothrombin complex: sedimentation coefficient: 4.80[98] and 5.3S[101] depending on the ionic strength and pH of buffers; molecular weight: 65,500 to 74,000;[68,98,99,101] intrinsic viscosity: 3.4 ml/g;[98] extinction coefficient at 280 nm: 16.5;[98] specific activity: 1,100 to 1,300 NIH units (or 2,500 to 3,000 Iowa units)/mg protein;[68,76] hexoses: 2.3% and a low helix configuration.[98]

Like the prothrombin complex, purified prothrombin has alanine at the *N* terminal.[70,535] The identity of the C terminal residue, however, is uncertain. Landaburu and Seegers[70] found serine, while Malhotra and Carter[535] found glutamate.

Human prothrombin proved more difficult to separate. Neither Seitz filtration of the plasma nor DEAE-cellulose chromatography of the prothrombin complex alone was sufficient since the products could be easily activated in concentrated solutions of sodium citrate. Aronson et al.[72] were able to fractionate human prothrombin complex into two fractions by chromatography on hydroxyapatite. One fraction contained mainly prothrombin with traces of factor X and the other contained factor X essentially free of prothrombin. Derleth and Penner[66] isolated human prothrombin free of factor VII which, however, contained factors IX and X by a combination of ECTEOLA- and DEAE-cellulose chromatography.

Thrombin

Thrombin is generated from prothrombin through the formation of one[56,100,109,110] or more likely of two intermediates,[69,97,99,111,112] by activation with activated factor X (Xa), which may act either alone at relatively high concentrations,[76,97,102,119,220,222,223] or together with factor V, phospholipids and Ca^{++}.[102,103,105-108] Trypsin,[85,220,221] cathepsin,[113] and the venom of Echis Carinatus[114,217] can also activate prothrombin to thrombin.

Isolation

The first relatively pure preparations of thrombin were obtained by Seegers et al.[115] by activation of purified prothrombin complex with tissue thromboplastin and calcium ions, precipitation of the generated thrombin with acetone, extraction of the precipitate with water, acid precipitation and a second precipitation with acetone. These preparations, however, contained activated factor X (autoprothrombin C) and other proteins.[103] Thrombin, like prothrombin and the other coagulation factors, was purified only after column chromatography was introduced. Rasmussen in 1954 chromatographed commercial preparations of thrombin on Amberlite IRC-50 and achieved a 50-fold purification over the starting material.[117] His method was soon adapted by others.[112,118] Seegers and Landaburu introduced chromatography on phosphate cellulose[70] with good results and other investigators used DEAE-cellulose,[120] sulfoethyl-Sephadex C-50[121] or other polycarboxylic resins (Bio-Rex 70)[122] similar to Amberlite IRC-50. Thrombin adsorbs on the cation exchangers (Amberlite or Bio-Rex 70, and phosphate cellulose) at a neutral pH and is eluted last with 0.15 to 0.3 M, pH 8.0 buffers. It does not adsorb on the anion exchangers and is obtained with the void volume.[70] Experiments with carboxymethylcellulose showed that thrombin is adsorbed on this weakly acidic cation exchanger, but the activity is lost during chromatography.[70]

Physicochemical Characteristics. Heterogeneity of Thrombin

Thrombin, chromatographed on Amberlite IRC-50,[118] was found to be homogeneous by several physicochemical criteria: ultracentrifugation,[84,118] free boundary electrophoresis,[123] amino acid analysis,[84] and electron microscopy.[52] Harmison et al.[84] performed a detailed physicochemical study of chromatographed thrombin in 0.1 M KCl (sedimentation constant) and in sodium acetate-potassium chloride, pH 5.6 buffer of 0.2 ionic strength. They found a sedimentation constant (S_{20w}) of 3.76S, a diffusion coefficient (D_{20w}) of 8.76 x 10^{-7} cm^2/sec, an intrinsic viscosity of 0.0376 ml/g and a partial specific volume ($\bar{V}$) of 0.69 ml/g. The sedimentation constant was concentration dependent but unlike the constant of prothrombin and the other members of the prothrombin complex (factors X and Xa) the slope of the dependence was positive. Harmison et al. calculated a molecular weight of 33,700 from the physicochemical data and 33,900 from the amino acid composition. From the

frictional ratio (1.16) and the viscosity increment they estimated an axial ratio of 2.8. They further found that the thrombin molecule has the shape of a proloid ellipsoid 84 Å long and 30 Å wide (prothrombin has been found to be 119 Å long and 34 Å wide[231]). In shadowcast preparations viewed by the electron microscope thrombin has a mean particle height of 91 ± 11 Å.[52]

Values for the molecular weight of bovine thrombin have also been reported by Baughman and Waugh (36,000)[124] from data obtained by filtration through Sephadex G-100, by Magnusson (29,000 to 34,000)[90,112] from *N* and C terminal amino acid analyses, and by Murano (32,000)[125] from thin layer gel filtration data. The molecular weight of human thrombin has been found to be 35,000 by Lanchantin et al.[126] by gel filtration (Sephadex G-100) and 32,000 by Kezdy et al.[127] from kinetic studies. Somewhat lower values were obtained by Magnuson (26,000 to 32,000)[112] from *N* terminal amino acid analyses and by Miller et al. (26,000)[128] from studies with diisopropylfluorophosphate.

In contradistinction to these values, values as low as 8,000 have been reported by other investigators. Shrier et al.[129] found this value when they determined the molecular weight of thrombin by ultracentrifugation in depolymerizing solutions of guanidine hydrochloride. Similarly, Gladner et al.[130] calculated a molecular weight of 9,900 to 17,000 for bovine thrombin (purified from the Parke-Davis preparation) by determining the amount of ^{32}P–DFP which combined with thrombin, assuming that the two substances combined in equimolar concentrations. It seems likely that these investigators were working with degradation products of thrombin.

In spite of the fact that the preparations of chromatographed thrombin appear homogeneous by traditional physicochemical criteria,[49,84,123] additional purification steps or the introduction of new and more discriminating techniques have indicated that there are several forms of thrombin. Seegers et al.[131] subjected thrombin purified by chromatography on Amberlite IRC-50 to a second identical chromatography. They found that the twice chromatographed material was essentially different from the once chromatographed preparations: the sedimentation constant was decreased from 3.76S to 3.2S and the slope of the concentration dependence became negative; the isoelectric point was increased from 5.75 to 6.2 ($\Gamma/2$=0.1) and the specific activity had doubled from 4,200 to 8,230 Iowa units/mg protein. After filtration through Sephadex G-75 in 10% acetic acid the original 3.7S thrombin separated into three protein peaks which had molecular weights of 37,500, 22,000, and 8,100 and three (threonine, isoleucine, lysine), two (lysine, threonine) and one (isoleucine) *N*-terminal amino acids, respectively.[132] The first of these peaks which had the three *N*-terminal amino acids was reduced by mercaptoethanol, alkylated with iodoacetic acid, desalted by Sephadex G-10, and then filtered through Sephadex G-75 under identical conditions as the parent 3.7S thrombin (in 10% acetic acid) and also in 0.1 M pH 8.0, ammonium bicarbonate solution containing 8 M urea. Three peaks corresponding to individual chains and some aggregated material were obtained. The heavier chain (peak 2) which had a molecular weight of 37,000 and isoleucine as the *N* terminal was labeled β(B) chain and corresponded to the B chain of Magnusson;[82] the medium size chain had a molecular weight of 22,500 and lysine at the *N* terminal and was labeled the β chain; the last chain (fourth peak) which had threonine as the *N*-terminal amino acid and a molecular weight of 5,400 (calculated from the amino acid composition) was labeled α chain and corresponded to the A chain of Magnusson.[82] From the molecular weights, the *N*-terminal amino acids and the sequence of 4 to 5 amino acid residues at the *N* terminal of the chains, Seegers et al. concluded that an acidic polypeptide of a molecular weight of approximately 8,100 to 9,500 (composed of approximately 75 amino acid residues), which they called the B fragment, had broken off from some of the β(B) chains by the cleavage of an arginine-lysine bond and the remaining chain was transformed into the β chains. The authors speculated that their 3.7S thrombin preparations contained at least two kinds of molecules, $\alpha\beta$(B) thrombin and αB thrombin mixed together with B fragments. The B fragments remained attached by non-covalent linkages to the thrombins and were separated by filtration in the acid medium and 8 M urea or by the second chromatography on IRC-50. Since the specific activity doubled after the second chromatography on IRC-50, the authors concluded that fragment B was an inhibitor.[131] It is interesting that Baughman and Waugh[124] detected the presence of a nonenzymic inhibitor in preparations of once

chromatographed (carboxylic resin or cellulose phosphate) bovine thrombin.

Recently Seegers and his associates isolated the $\alpha\beta$-thrombin (designated as AB thrombin) free of the larger $\alpha\beta$ (B) thrombin[536] by activation of purified prethrombin, chromatography on Amberlite IRC-50, and gel filtration through Sephadex G-75 at pH 5.0. This thrombin crystallized on standing in 2 M ammonium sulfate solutions of pH 5.8, thus becoming the first thrombin to be obtained in crystals (Figure 1). The crystals strongly diffracted x-rays and were tentatively assigned to the space group $P4_322$. The unit cell dimensions were a=b=87 Å and c=101 Å. The β chain of this thrombin had 208 amino acid residues, i.e., 56 residues less than the B chain of Magnusson.[82] The preparations had a specific activity of 10,000 Iowa units/mg of dry weight, which is the highest ever obtained for bovine thrombin.

Spontaneous heterogeneity of bovine thrombin has been reported also by Mann and Batt,[133] Batt et al.,[134] and Mann et al.[121] These investigators studied the composition of purified preparations of "Thrombin Topical" of Parke-Davis and also of thrombin obtained by activation of the prothrombin complex or by activation of purified preparations of prothrombin in 25% sodium citrate solutions. (To induce activation of purified prothrombin traces of defibrinated plasma were added to the incubation mixture.) In their latest work Mann et al.[121] purified thrombin by chromatography on sulfoethyl Sephadex C-50 and studied its composition by polyacrylamide gel electrophoresis in urea-acetic acid and sodium dodecylsulfate (SDS) and also by sedimentation equilibrium and gel filtration in 6 M guanidine-HCl before and after reduction of the disulfide bonds by mercaptoethanol and alkylation. Their findings support and extend the findings of Seegers et al.[132] They found that at least three active forms of thrombin are formed and that these are related to the degree of activation of the thrombin zymogen. The first form of thrombin which they isolated was a molecule of 39,000 daltons which was composed of two disulfide-linked chains of 33,000 daltons and 6,000 daltons. These authors found that further activation resulted in the formation of two smaller thrombin molecules both having molecular weights of 28,000 daltons. The most active (in terms of clotting activity) of these molecules was composed of two disulfide-linked chains of approximately 18,000 and 10,000 daltons. The least active thrombin was a three chain structure composed of polypeptide chains of 14,000, 4,000, and 10,000 daltons. The clotting specific activity of the larger thrombin (39,000 daltons) was found to be 2,700 NIH units/mg of protein and the activities of the two smaller thrombins were about 0.50 and 0.25 of this value, respectively. At variance to the clotting activity all the thrombins had identical TAMe (tosyl-L-arginine methylester) esterase specific activities.

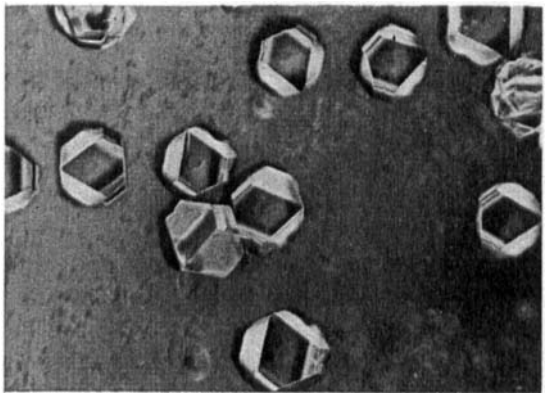


FIGURE 1. Crystals of bovine thrombin magnified 86X. (From Tsernoglou, D., Walz, D. A., McCoy, L. E., and Seegers, W. H., *Thromb. Res.*, 1, 533, 1972. Photograph by Charles A. Baechler. Provided by Dr. W. H. Seegers.)

The two types of thrombin, the $\alpha\beta$(B) and $\alpha\beta$ which were studied by Seegers and his associates,[132] should have molecular weights of 37,000 + 5,400 = 42,400 and 22,500 + 5,400 = 27,900, respectively. They must therefore correspond to the first two forms of thrombin of Mann et al.[121] or possibly to the first 39,000 dalton molecule and to an intermediate with a 23,000 dalton major chain. In comparing molecular weights obtained by various groups of investigators or even by the same investigators in various experiments, one has to take into consideration that: (a) determinations obtained by gel filtration or electrophoresis are not very accurate, (b) sedimentation and diffusion constants of the members of the prothrombin complex and their derivatives determined in nondissociating solvents of low ionic strength are concentration and pH dependent[59,83,84,135] which indicates that these proteins have the tendency to associate and form aggregates. The specific activity of the $\alpha\beta$(B) thrombin has not been determined yet, since this

thrombin has been obtained only in denaturing solvents. It appears, however, that its activity is lower that the activity of the $\alpha\beta$ thrombin since the 3.2S thrombin which is a mixture of $\alpha\beta$(B), and $\alpha\beta$ thrombin has a lower specific activity (8,200 Iowa units/mg[131]) than the $\alpha\beta$ thrombin (10,000 Iowa units/mg dry weight[535]). Taking into consideration that one NIH unit has been found to correspond to 1.13,[65] 1.25,[49] 1.73[68] and 1.8[116] Iowa units by various investigators, the specific activities of the 3.2S and $\alpha\beta$ thrombins should correspond approximately to 5,500 and 6,800 NIH units, respectively. These values are much higher than the specific activity of the thrombin preparations of Mann et al.[121] Another difference between the two laboratories is that, as mentioned earlier, the latter investigators found that the larger thrombin (39,000 molecular weight) was the most active in terms of clotting activity while Seegers and his associates have found the opposite, i.e., that the smaller (28,000 to 30,000 molecular weight) thrombin was the most potent.

Rosenberg and Waugh[136] were able to detect even greater heterogeneity in purified bovine thrombin isolated from the same sources as the thrombins studied by Mann et al.[121] (Parke-Davis Thrombin Topical and from bioactivation of prothrombin complex). Using polyacrylamide gel electrophoresis (pH 8.9, $\Gamma/2$ = 0.16), they detected six types of thrombin (T_1 to T_6 in order of increasing mobility), which they isolated partially by cellulose phosphate chromatography. They found that the slower moving thrombins (T_1, T_2, and T_3) had significantly higher specific activities that the faster moving thrombins. In blood obtained from single pedigreed animals only the first three types of thrombin (having the greater specific activity) were found. The proportions of these thrombins varied with the age of the animal: the older the animal, the higher the thrombin type. Rosenberg and Waugh concluded that the smaller (higher numbered) thrombins were produced from the larger thrombins by peptide cleavage. The specific activities of these thrombins are given as clotting times and cannot be compared with the activities found by the other investigators.

Primary Structure, Active Center

The primary structure of bovine thrombin has been elucidated by Magnusson and his colleagues. Magnusson has reported[82] the amino acid sequence of a molecule of thrombin which is composed of a short chain (A chain) made up of 49 amino acid residues, and of a longer chain (B chain) which has 264 amino acid residues. Taking into consideration the reports just discussed about the heterogeneity of thrombin, Magnusson's group must have studied the $\alpha\beta$(B) thrombin of Seegers et al.[132] or the 39,000 dalton, two-chain structure of Mann et al.[121] Summation of the amino acid residues[121] in the sequence reported by Magnusson gives molecular weights of 5,727 and 30,275 for the A and B chains, respectively. The two chains are linked together by a disulfide bond between two half cystines. The smaller chain has threonine at the *N* terminal and arginine at the C terminal and its amino acid sequence does not bear resemblance to the sequence of the pancreatic proteinases. The larger (B) chain has isoleucine at the *N* terminal and serine at the C terminal. It is approximately 20 to 30 amino acid residues longer than the pancreatic proteinases and its amino acid sequence is by 40 to 50% homologous to the sequence of the latter enzymes. The B chain contains both the active serine, which is inactivated by diisopropylfluorophosphate,[137,191] and the active histidine which is inactivated by TLCK (1-chloro-3-tosylamido-7-amino-2-heptanone).[138] This chain shows a high degree of sequence homology with the pancreatic serine proteinases around histidine-57 (using the chymotrypsinogen convention) and aspartic acid-102 as well as around the active serine-195. This similarity in structure lends support to the hypothesis that their catalytic mechanisms are basically identical.[140,144]

Enzymatic Activity

Thrombin is a hydrolytic enzyme and has both proteolytic and esterolytic activities. Substrates for the proteolytic activity of thrombin are fibrinogen and fibrin, prothrombin, the precursor of the inhibitor originally detected by Mammen et al.,[139] factors V and VIII, and possibly factor IX. These actions of thrombin are discussed individually with the pertinent substrates.

The esterolytic activity of thrombin was first described in 1954 by Sherry and Troll.[141] Thrombin is able to hydrolyze arginine and lysine acyl amides, i.e., α-tosyl-L-arginine methyl ester (TAMe), α-benzoyl-L-arginine methylester (BAMe), α-tosyl-L-lysine methylester (TLMe),

etc.[142] and also nitrophenylesters[143] as carbobenzoxy-L-tyrosine *p*-nitrophenylester, *p*-nitrophenyl acetate, etc. Lorand[140] and Chandrasekhar and Laki[144] have advanced the hypothesis that hydrolysis by thrombin proceeds through an acyl-enzyme intermediate as in the case with trypsin and chymotrypsin.

Although the unit of clotting activity has been well defined as the Iowa[91] or the NIH unit,[91] the unit of esterase activity is still arbitrary and variable.[142] Therefore, esterase activities determined by various methods cannot be compared. Some authors tried to equate the unit of esterase activity to the unit of the clotting activity.[145] Due, however, to the independent variability of the two activities during prothrombin activation,[121,122] which can be attributed to the sequential formation of thrombins with varying clotting specific activities but with almost identical TAMe esterase activities,[121] the two units (clotting and esterase) cannot be identical except for preparations having compositions similar to the composition of the standard.

Clotting and Esterase Thrombin

It has been shown that both the ability of thrombin to clot fibrinogen and to hydrolyze TAMe develop simultaneously during the activation of bovine[121,146] and human[122] prothrombin complex in 25% sodium citrate[121,122,146] or in 0.33% protamine sulfate[146] solutions and also during the activation by tissue thromboplastin, calcium ions and factor V.[146] In the case of bovine prothrombin, both activities, reach a maximum and then decline, first the clotting and then the esterase activity.[121]

Under conditions of slow prothrombin activation, esterase activity has been found to develop before clotting activity appears.[145] Since factor X becomes activated before thrombin can be formed and since factor Xa is also able to hydrolyze TAMe, part of the initial esterase activity in all probability must be due to the formation of factor Xa.

Lanchantin et al.[122] using chromatography on weakly acidic polycarboxylic cation exchangers (Bio-Rex 70 or Rexyn CG-51) found that thrombins obtained during the early stages of prothrombin activation in 25% sodium citrate (at 16 hours) exhibit both clotting and esterolytic activity. Thrombins obtained during advanced stages (500 hr), on the other hand, are of two kinds: thrombins which exhibit almost exclusively clotting activity and thrombins which are almost exclusively esterolytic. These three types of thrombin could not be differentiated by electrophoresis on cellulose acetate at pH 8.6, gel filtration through Sephadex G-100, or ultracentrifugation. All seemed to have molecular weights of approximately 30,000 and the same electrophoretic mobility.[122] Titration experiments, however, with phenylmethylsulfonyl fluoride and diisopropylfluorophosphate revealed a striking difference between the preparations having both clotting and esterase activity and the preparations which had only esterase activity.[122]

Seegers and his associates have succeeded in destroying preferentially either the clotting or the esterolytic activity of thrombin and have thus obtained preparations with only clotting (clotting thrombin) or with only esterolytic activity (esterase thrombin) at will. Esterase thrombin has been obtained by: (a) allowing thrombin preparations to stand at room temperature for a few days,[147,149] (b) acetylation,[148] (c) activating prothrombin in saline containing 0.05 M calcium chloride,[149] and (d) activating acetylated prothrombin.[150] Clotting thrombin has been obtained by acidifying purified thrombin to pH 5.0.[264] This treatment destroys the esterolytic activity permanently but leaves the clotting potential unimpaired. From a functional point of view then, three types of thrombin can be obtained regardless of the stage of prothrombin activation: (1) complete or physiological thrombin, possessing both clotting and esterolytic activities, (2) esterase thrombin with only esterolytic activity, and (3) clotting thrombin with only clotting activity. It has been shown that only preparations which have both clotting and esterase activity can accelerate the activation of prothrombin. Esterase thrombin cannot do this.[122,149]

The acetylation of thrombin or prothrombin is performed in the presence of 25% sodium acetate by addition of acetic anhydride to pH 5.7 to 5.6. The resulting acetylated thrombin has 42 to 51% of its amino groups acetylated and although it is unable to clot fibrinogen, it can lyse fibrin and digest clots twice as fast as the original thrombin.[148] If the acetylation is carried out in 15% potassium carbonate instead of in sodium acetate, more amino groups (up to 96%) and a considerable number of the OH groups become acetylated and O-acyl derivatives are formed. This

thrombin has neither clotting nor esterase activity. It appears then that for the clotting activity both the OH groups as well as all or at least certain of the amino groups, presumably those of serine, are indispensable. (The importance of some amino groups cannot be excluded, however.)

Acetylated thrombin is extremely interesting for its in vivo effects as well. Seegers and his associates[151] injected this modified thrombin into dogs and found that their blood either did not clot at all or it formed clots which lysed quickly. Recently McCoy et al.[104] and Pechet et al.[152] made the remarkable observation that neither the reduction in the fibrinogen concentration nor the lysis of the clots was prevented by administration of ϵ-aminocaproic acid or Trasylol.[104,152] Injections of heparin in quantities sufficient to achieve an in vivo concentration of approximately 50 USP units/ml of plasma were also ineffective.[104] The plasminogen level declined rapidly at the beginning of the infusion but it quickly returned to normal shortly after the infusion was finished.[104] No fibrinolytic activity could be detected on unheated or heated fibrin plates.[152] The euglobulin lysis time, however, became shorter.[104,152] The thrombin clotting time in the experiments of McCoy et al. was greatly prolonged but it was only occasionally affected in the experiments of Pechet et al. Both groups of investigators detected signs of intravascular coagulation: reduction in the number of platelets[104,152] and a drop in the concentration of factors II, VII to X, V, and VIII[152] and of fibrinogen.[104,152] It is significant that thrombocytopenia was not observed when acetylated thrombin was injected into dicumarolized animals.[104] From this observation one is inclined to attribute the thrombocytopenia as well as the other signs of intravascular coagulation not to the small amounts of normal thrombin which contaminated the acetylated enzyme, but to in vivo generation of thrombin. The inability of Trasylol and EACA to prevent the fibrinolytic phenomena excludes plasmin as the enzyme mainly responsible for them. On the other hand, it is well established that both normal and acetylated thrombin are capable of slowly digesting and lysing fibrin in vitro.[148,149,153,154] It is conceivable, therefore, that by partially digesting fibrin they may increase its susceptibility to other proteolytic enzymes and perhaps to plasmin itself so it can digest this altered fibrin even in the presence of EACA and Trasylol.

Factor X

One of the most significant contributions of the sixties concerning blood coagulation was the isolation and characterization of factor X and the realization that it is the substrate of tissue thromboplastin and Russell's viper venom and that the activation product, Factor Xa, is essentially the activator of prothrombin, i.e., the thrombokinase which was postulated by Morawitz in 1905. Thrombokinase, factor Xa, and autoprothrombin C were shown to be synonyms.

Isolation

Factor X and prothrombin (Factor II) are the most extensively studied components of the prothrombin complex. Bovine factors X and Xa have been separated from the other factors of the complex by paper,[155] continuous flow paper,[156] and starch gel electrophoresis[73] or chromatography on Amberlite IRC-50 (factor Xa).[103] The best separations, however, have been obtained by chromatography on DEAE-[77-80,96,97,157] or TEAE-cellulose[71] or DEAE-Sephadex[68] and these types of chromatography, followed by filtration through Sephadex G-100 or by preparative polyacrylamide gel electrophoresis,[74] are now used exclusively. Human factor X proved more difficult to separate. Only a combination of chromatography on hydroxyapatite, polyacrylamide gel electrophoresis, and another chromatography on DEAE-cellulose have given satisfactory results.[72]

Factor X in the proenzyme form was first isolated by Hougie and Bunting in 1960 by DEAE-cellulose chromatography of barium sulfate eluates from pooled serum.[158] A large part of factor X, however, was eluted together with factor VII. Better separations were obtained later by Esnouf and Williams,[78] Duckert and coworkers,[160] Seegers et al.,[80] Papahadjopoulos et al.,[79] Lechner and Deutsch,[96] and more recently by Hogenauer et al.[74]

Esnouf and Williams[78] isolated factor X from bovine plasma and serum as the substrate of Russell's viper venom using, as Hougie did, DEAE-cellulose chromatography of barium sulfate eluates. Elution from the column was performed with 0.02 M phosphate buffers, pH 7.0, containing sodium chloride in concentrations increasing stepwise from 0 to 1 M. Factor X was eluted at 0.4 M

sodium chloride. Approximately 150 mg protein were recovered from 20 l plasma (7.5 mg/l) corresponding to 44 to 68% of the factor X which was present in the plasma or to about 0.011% of the total plasma protein. This preparation exhibited a specific activity of 4 to 5.6 arbitrary units/μg protein (one unit equals 1/100 of the activity of one ml normal serum) representing a 4,000-fold purification. It was homogeneous by electrophoresis at pH 6.8 and 7.3 but it was heterogeneous in the ultracentrifuge (two peaks). A major component with S_{20w} of 4.2 and a minor component with S_{20w} of 10 could be identified. The components were isolated by sucrose gradient centrifugation and were found homogeneous when reexamined in the analytical ultracentrifuge.

Seegers et al.[80] isolated factor X from bovine prothrombin complex after treatment with thrombin, crude cephalin, calcium chloride, and factor V. The combination of these factors permits the generation of small amounts of thrombin but leaves factor X (autoprothrombin III) essentially in its native form.[50,80] The activation procedure was later simplified by using thrombin alone as the activating agent (it activates prothrombin to derivatives which do not respond to tissue thromboplastin). In a further modification of the procedure introduced independently by Lechner and Deutsch[96] and Marciniak and Seegers,[97] digestion with thrombin was omitted and the native prothrombin complex was submitted directly to DEAE-cellulose chromatography. The preparations obtained were homogeneous in the ultracentrifuge and the molecular weight estimated by thin layer gel filtration was found to be 74,000.[159] Depending on whether factor X was isolated from native or thrombin pretreated prothrombin complex, it could or could not be activated in a 25% solution of sodium citrate.[97,100]

Duckert and co-workers[160] and Papahadjopoulos et al.[79] used essentially the same procedure as Esnouf and Williams,[78] except that they eluted the DEAE-cellulose column with dilute citrate buffers of increasing concentration (0.04 to 0.08 M) instead of phosphate buffers containing sodium chloride.

Jackson and Hanahan[77] and Jackson et al.[170] adapted the procedure of Papahadjopoulos et al.[79] to large-scale purification. They used diisopropylfluorophosphate to inhibit the degradation of factor X during the isolation procedure and achieved a 16,000-fold purification relative to the starting plasma. The material which they isolated was also homogeneous in the ultracentrifuge and by disc gel electrophoresis but showed two peaks when it was rechromatographed on DEAE-Sephadex A-50. The molecular weight of both peaks was found to be the same: 53,000 to 56,000 (sedimentation diffusion). A lower molecular weight, 37,800, was found by Tishkoff et al.[68] by sedimentation equilibrium in a solvent of 6 M guanidine hydrochloride and mercaptoethanol. These investigators believe that they were working with a partially activated form of factor X which in its native state could have a molecular weight similar to that of prothrombin.

Factor Xa, as a separate protein entity, was first isolated as thrombokinase from oxalated bovine plasma by Milstone in 1959[61] by a lengthy procedure. This involved filtration of the plasma through diatomateous silica, adsorption of the prothrombin complex on $BaSO_4$, elution first with 0.1 M phosphate to remove most of the prothrombin (some factor X was also removed), and then with 0.4 M phosphate to elute factor X. The proenzyme was then precipitated with ammonium sulfate and activated by alkalinization and storage in the frozen state. The active enzyme was purified by double isoelectric precipitation at pH 5.2 to remove thrombin. The yield was very small and the preparation still contained thrombin and inactive protein. Final purification was achieved a year later[156] by introducing an additional step, continuous flow paper electrophoresis (three times), which was replaced later by chromatography on DEAE-cellulose (once).[157] Factor Xa was eluted with 0.4 M phosphate buffer and was homogeneous in the ultracentrifuge. The yield was 91% of the protein which was applied on the column but only 1 to 2 mg/l of plasma were obtained representing only about 10% of the original factor X content. The preparation was extremely active. Alone it was able to activate prothrombin in microgram quantities and when it was used together with factor V (adsorbed bovine serum), calcium ions, and phospholipids only nanograms were needed.[102] The isolated factor exhibited TAMe esterase activity[156] which was inhibited by soybean trypsin inhibitor.

The activated enzyme was also isolated by Marciniak and Seegers[103] independently as autoprothrombin C from purified preparations of bovine prothrombin complex after activation in 25% sodium citrate. Factor Xa was salted out

together with thrombin by further saturation of the solution to 40% with sodium citrate. It was then reprecipitated first with ammonium sulfate (70% saturation) and then with acetone and chromatographed on Amberlite IRC-50. Factor Xa is not adsorbed on this resin and is eluted first while thrombin is retained. Better purification and better yield were achieved by activating the prothrombin complex with tissue thromboplastin and chromatographing the activated mixture on DEAE-cellulose instead of on Amberlite IRC-50.[163] In contradistinction to the chromatography on the latter resin, factor Xa becomes adsorbed on DEAE-cellulose while thrombin does not and comes out first together with other impurities. The yield by DEAE-cellulose chromatography was 45 to 50% of the activity originally present in the prothrombin activation mixture. The material was homogeneous in the ultracentrifuge. Its sedimentation and diffusion coefficients were found to be concentration dependent and a molecular weight of 21,500 was calculated by determination of the constants at various protein concentrations.[163] This material was also very active: 0.35 μg of it were able to clot bovine plasma in 15 sec (=1 unit) under the conditions of the authors' assay. It was stable at room temperature in 25% imidazole for more than 2 weeks but deteriorated after 8 days at refrigerator temperature. It was quite stable when stored in 50% glycerol at -60°C.

Active factor X of human origin has been prepared by Aronson and Ménaché[69,119] by a modification of the procedure of Milstone. The prothrombin complex was adsorbed on barium citrate which was subsequently disintegrated by the addition of ammonium sulfate to 40% saturation. The prothrombin complex was then precipitated by a further addition of ammonium sulfate to 65% saturation. The precipitate was dissolved in alkalinized water (pH 8.5) and the inactive factor X was spontaneously activated during storage at 5°C for 10 days. This solution was subsequently chromatographed on DEAE-cellulose and Sephadex G-100. Judging from the TAMe esterase and the prothrombin converting ability the authors concluded that their preparation had about 2% of the specific activity of Milstone's bovine material.[69]

Physicochemical Characteristics

Factor X is a glycoprotein having the mobility of α_1-globulins. Upon activation, the factor Xa becomes less electronegative and migrates with the mobility of γ-globulins.[164] As with prothrombin, the sedimentation constants of factors X and Xa in solvents of low ionic strength (Γ/2 0.1) are concentration dependent. Determinations at various protein concentrations gave values of 3.4S[135] and 3.56S[77] for the S_{20w} of factor X and 2.27[163] for the S_{20w} of factor Xa. The respective diffusion coefficients (D_{20w}: x 10^{-7}, cm^2, sec^{-1}) were found to be 5.6[77] and 8.4[163] and the partial specific volumes ($\overline{V}$) 0.690 (from amino acid composition corrected for carbohydrate content),[77] and 0.695 ml/g.[163]

Values for the molecular weight of factor X vary considerably: Seegers et al.[101] reported a value of 52,000 from the amino acid composition; Jackson and Hanahan,[77] 50,000 to 56,000 from sedimentation diffusion data; Murano,[159] 74,000 from thin layer gel filtration and Esnouf and Williams,[78] 84,000 by the approach to equilibrium method and 87,000 from sedimentation diffusion data. The molecular weight of factor Xa (calculated from physicochemical data) has been reported to be 36,000[78] and 21,700.[163]

There is also disagreement regarding the *N*-terminal amino acids of factor X. Esnouf and Williams[78] detected alanine and glycine (DNP procedure of Sanger) while Hogenauer et al.[74] found serine and glycine (dansyl chloride method of Gray and Hartley). The latter investigators have found that the C-terminal residues of factor X are also serine and glycine and that factor VII has similarly serine and glycine at both of its terminals. After activation of factor X an additional *N*-terminal amino acid was detected by Esnouf and Williams. According to these investigators the *N*-terminal amino acids of factor Xa are alanine, glycine, and leucine or isoleucine.[78] None of the data were quantitative. The differences in the *N*-terminal amino acids and also in the molecular weights of factor X may reflect differences in the purity of the various preparations and/or may indicate partial activation of the proenzyme. It appears that factor X is a two chain molecule; it is too early to make conclusions about the structure of factor Xa.

Fingerprints of tryptic digests of factor X show great similarities to fingerprints of factor VII.[74] Similarities in fingerprints have also been detected between factor X and prothrombin. Aronson et al.[72] detected 42 peptides in digests of human

factor X and 47 in digests of human prothrombin. After reduction and alkylation, these authors found that the number of peptides in the digests of factor X increased to 50 and those in the digests of prothrombin increased to approximately 67. Based on visual examination approximately one third of the peptides were common in both factor X and prothrombin.

Concentration in Plasma, Yield, Specific Activity

The concentration of factor X in plasma is very small. It has been calculated that there are approximately 10 to 20 mg of this factor per liter of bovine plasma.[102] The yield in the various preparations varied from 0.06[74] to 7.5 mg/l.[78] The units of specific activity are arbitrary and it is not possible to compare the activity of the various preparations. It is, however, certain that factor Xa is the most potent coagulation substance which has been isolated to date. Alone it can activate prothrombin in microgram quantities; and when factor V, calcium ions, and phospholipids are also present, only nanograms are required. Milstone[162] has found that treatment with chymotrypsin destroys the ability of the factor to activate prothrombin in the presence of factor V, calcium, and phospholipids, but it does not affect its ability to activate the proenzyme in the presence of sodium oxalate. Occasionally, this inactivation may also occur spontaneously.

Bovine factor Xa is inhibited by diisopropylfluorophosphate at concentrations greater than 10^{-2} M[77] and by soybean trypsin inhibitor,[119, 157,165] but not by hirudin.[165] In addition, there is an impressive amount of experimental evidence that antithrombin III inactivates factor Xa by a mechanism analogous to the inactivation of thrombin.[103,166-169] In another similarity to thrombin, activated factor X hydrolyzes tosyl arginine methylester.[78,96,102,103,119,156]

Factor VII

Although concentrates of factor VII[171] can be obtained easily, the isolation of this factor proved to be more difficult than the isolation of factor X or prothrombin. During DEAE-cellulose or DEAE-Sephadex chromatography, factor VII is eluted together with prothrombin or with factor X or between these two factors contaminating one or the other, more commonly contaminating both.[68,73,75,79,158] The fact that purified prothrombin can still be activated by tissue thromboplastin proves that it contains at least traces of factor VII. It is also relevant that Seegers and his associates have shown that factor X (autoprothrombin III) obtained by chromatography on DEAE-cellulose can correct the coagulation defect of factor VII deficient plasma.[97, 135,172]

Isolation of factor VII was first obtained by Hougie and Banting in 1960[158] by DEAE-cellulose chromatography of $BaSO_4$ eluates of human serum. Tishkoff et al.[73] using starch gel electrophoresis and Duckert[160] and his colleagues using DEAE-cellulose chromatography obtained fractions with factor VII activity but they did not isolate any factor VII protein for physicochemical studies.

Prydz[323] isolated factor VII from human serum and plasma by adsorption on $BaSO_4$, elution by chromatography on Sephadex G-25, rechromatography on DEAE-Sephadex A-50, readsorption on $BaSO_4$, elution with citrate, and a final chromatography on Sephadex G-200. The preparations had a specific activity of 30,000 to 60,000 units/mg protein (1 unit = 1/100 of the activity of normal plasma), which represented a 4,500- to 8,000-fold purification, and were free of the activities of factors IX and X and of prothrombin and thrombin. The yield was 0.4 to 1 mg/l serum, approximately 5 to 6% of the amount originally present (calculated original concentration: 8 to 16 μg/ml). More recent preparations[81] have molecular weights of 44,700 when they are isolated from serum and 59,000 when they are isolated from plasma (determined by gel filtration through Sephadex G-200 and sucrose density gradient centrifugation). They show a carbohydrate content of 20 to 25%, which is the highest among the prothrombin complex factors: 16.8 glucose and 6.7% fucose. They have no sialic acid. They contain free SH groups which, however, are not essential for the enzymatic activity. The preparations have a tendency to form aggregates.

Antibodies produced by Prydz[173] against factor VII in rabbits were able to inactivate this factor completely in plasma and in serum, and did not cross-react with factors X and IX or with prothrombin. Further immunological studies showed that the new preparations were free of contaminants. Prydz found that plasmas from patients congenitally deficient in factor VII and from dicumarol-treated normal controls did not

react serologically with anti-factor VII antibodies.[81]

Hogenaeur et al.[74] isolated factors VII and X by chromatography of barium sulfate eluates on DEAE-cellulose followed by preparative polyacrylamide gel electrophoresis. Analytical disc electrophoresis showed that the final product of each factor was a single component. The yield was, however, very small: approximately 3 mg of factor VII and 0.3 mg of factor X were obtained from 5 l of plasma. As mentioned earlier, *N* terminal (dansyl chloride method) and C terminal (carboxypeptidase B) amino acid analysis of these preparations indicated that each factor had at both its ends the same amino acids, namely, glycine and serine. Fingerprints of tryptic digests showed 17 spots for factor VII and 16 for factor X. Of these, 11 seemed to be similar in both.

Partial separation of factor VII was also obtained by Tishkoff et al.[68] by chromatography of bovine prothrombin complex on DEAE-Sephadex A-50. The molecular weight determined by sedimentation equilibrium in a solvent of 6 M guanidine-HCl and mercaptoethanol was found to be 33,900. The authors believed that they had isolated the activated form of the factor.

Factor IX

Factor IX is the most elusive of all the factors of the prothrombin complex. While an extensive literature exists on the clinical manifestations and treatment of the congenital deficiency in this factor (hemophilia B), very little is known regarding its biochemistry. During chromatography on ethyl-celluloses or ethyl-Sephadex, factor IX is eluted together with prothrombin[68] or with factor X.[75] Only Duckert has reported[160] that he and his colleagues obtained factor IX fractions free of the activities of factors VII and X by chromatography of serum eluates on DEAE-cellulose. They did not isolate, however, any protein with factor IX activity for physicochemical studies.

Recently Pechet and Smith[75] using isoelectric focusing obtained partial separation of factor IX from prothrombin. Their factor IX preparations were contaminated with traces of prothrombin and of factors VII and X which did not focus sharply at any pH. Factor IX focused at pH 4.1 to 4.5 and was isolated in the inactive form. The recovery was 20% and the specific activity about 300% of the activity of the plasma. Immunodiffusion experiments showed that prothrombin and factor IX are separate antigenic entities.

Factor VIII

The purification of factor VIII (antihemophilic factor A) has challenged many groups of investigators because of the clinical importance of this factor. Two main difficulties have been encountered: (a) it is very difficult to separate factor VIII from fibrinogen and (b) the purified product is extremely unstable and loses most or all of its activity during purification and especially during lyophilization.[174,175] Most of the preparations which are used clinically are actually mixtures of factor VIII and fibrinogen (Blombäck fraction I-0,[176,177] cryoprecipitate,[178] precipitates obtained by both ethanol fractionation and cryoprecipitation,[179] or by addition of glycine.[179,180] Some products have been further purified by treatment with tannic acid,[181] DEAE-cellulose fractionation,[175] or addition of polyethylene glycol[174,179] to remove most of the fibrinogen. Complete removal of fibrinogen has resulted in a significant reduction of the stability of factor VIII.

Three groups of investigators have recently succeeded in establishing procedures which they claim can isolate active factor VIII completely free of fibrinogen.

Johnson et al.[179,182] fractionated frozen plasma by ethanol (3%), adsorption on aluminum hydroxide (to remove the prothrombin complex), precipitation with polyethylene glycol (twice, to remove fibrinogen), and finally, sucrose density gradient ultracentrifugation or agarose gel chromatography. They obtained a 10,000-fold purification. The product had a molecular weight of over 500,000 (it was excluded by agarose) and showed two components on acrylamide gel electrophoresis. In lyophilized form it was stable at 37.5°C for over six months.[182]

Hynes et al.[175] adsorbed oxalated or resin decalcified (by a cationic type of ion exchanger) plasma on barium sulfate to remove the prothrombin complex and then chromatographed the adsorbed plasma on DEAE-cellulose. Fibrinogen, factors XII and XIII, and other contaminating proteins were eluted first with dilute phosphate buffers. Factor VIII was eluted last by a 0.4 to 0.5 M, pH 5.3 buffer together with factor V, but completely free of fibrinogen. These investigators

found that citrate added to a concentration of 0.4% stabilized the activity during elution and lyophilization. The yield by this procedure was 50 to 92% and the purification 40- to 200-fold.

Hershgold et al.[174] used a procedure very similar to that of Johnson et al.[182] and achieved similarly a 10,000-fold purification. Their starting material was fresh cryoprecipitate rich in factor VIII (6 to 8 units/ml representing a 15- to 25-fold purification from plasma). After adsorption of the prothrombin complex with aluminum hydroxide and most of the fibrinogen with Fuller's earth and bentonite, the solution was fractionated first with ammonium sulfate (35% saturation) and then with polyethelene glycol (3 and 9%). The precipitate was solubilized in citrated saline containing 0.2 M EACA (these investigators confirmed the findings of Casillas et al.[183] that the presence of EACA stabilized factor VIII activity) and finally purified by gel filtration through agarose. The final product was free of fibrinogen α_2-macroglobulin, or β-lipoprotein. The hexose content by the anthrone method was 10% of the amount of the protein and the total lipid 11 to 12% of the dry weight. The preparation was homogeneous by ultracentrifugal and immunological examination. Some impurity (approximately 5%) was detected by refiltration through agarose and by electrophoresis on cellulose acetate. The sedimentation coefficient (S_{25w}) in 6 M guanidine-HCl was 16.3, indicating a very large molecule. The same group of investigators[184] made the interesting observation that phospholipase C, which catalyzes the hydrolysis of the linkage between glycerol and phosphate, inactivates factor VIII by removing the phosphate polar groups together with the base (choline or ethanolamine) and leaving the factor with the diglycerides. On the other hand, phospholipase D, which cleaves only the base moiety leaving the phosphatidic acid behind, increases the activity of factor VIII threefold, because it unmasks the polar groups of the phosphoric acid, which linked the diglycerides to choline or ethanolamine.

Weiss and Kochwa,[270] using gradient ultracentrifugation in 10 to 40% w/v sucrose, separated two fractions with factor VIII activity: a major one in the region of γ M macroglobulin and a minor fraction in the region of fibrinogen.

Concerning the nature of factor VIII the findings of Barrow and Graham are of extreme importance. These investigators were able to isolate a factor VIII-like material from crude leucine amino peptidase preparations from normal dog, hog, and human kidneys[186] and more recently from normal and hemophilic dog kidneys[185] by Sephadex G-200 filtration. The molecular weight estimated from the elution volume was about 25,000. This factor VIII-like material from normal and hemophilic dogs was found to neutralize an anti-factor VIII specific inhibitor present in the plasma of a man suffering from hemophilia A. The authors speculated that plasma factor VIII is a multimer, one subunit of which is synthesized in the kidney and the other in the liver. They concluded that the kidney subunit must carry the antigenic determinants while the liver subunit must be the moiety which is affected by the mutation for hemophilia A. The same investigators acetylated human and bovine albumin with succinic anhydride and obtained an active component with a molecular weight of 25,000 which was similar to the renal factor VIII.[187]

There is considerable evidence[265-268] that factor VIII, like factor V, becomes activated by small concentrations of thrombin (about 0.03 NIH unit/mm), while larger amounts of this enzyme inactivate it.[267,269]

Recent work in the laboratory of one of the authors (D.C.T.) indicates that the breakdown products of fibrinogen potentiate the effect of factor VIII on the generation of thrombin from prothrombin complex. This can explain the prothrombin converting activity of these products when they were added to semipurified preparations of prothrombin.[188] They probably potentiated the effect of small amounts of factor VIII present in the prothrombin preparations.

Factor V

The isolation of factor V[189] or labile factor[190] or accelerator globulin[192,193] has encountered great difficulties because of its instability. As with the prothrombin complex most of the work was performed with bovine material. The reason for this preference is not only the greater availability of bovine plasma, but also the greater instability of human factor V.

Recent attempts to purify and characterize factor V began with the work of Aoki et al.[194] in 1963. These investigators achieved a 1,000-fold purification and a 15% yield starting from bovine plasma. Their method involved adsorption with

barium carbonate to remove the prothrombin complex, isoelectric precipitation (first to remove impurities and then to precipitate the factor), fractionation with ammonium sulfate, repetition of the isoelectric precipitation, Amberlite IRC-50 chromatography, and a second fractionation with ammonium sulfate. The purified material deteriorated spontaneously while attempts were made to obtain physicochemical data. Papahadjopoulos et al.[195] fractionated adsorbed (with barium sulfate) plasma or serum by 33 to 50% ammonium sulfate saturation (this step was sometimes omitted) followed by Sephadex G-200 filtration and obtained a 50-fold purification. Esnouf and Jobin[196] removed factor V from the adsorbed bovine plasma by adsorption on triethylaminoethyl (TEAE)-cellulose and then chromatographed the eluate on a column of phosphorylated cellulose. They reported a purification of 6,000-fold. Barton and Hanahan[197] modified this procedure and obtained a purification of 6,000- to 10,000-fold mainly by introducing a second chromatography on phosphorylated cellulose and calcium chloride precipitation of the final product. They reported a yield of 25 to 40%.

Physicochemical Characteristics

The protein isolated by Esnouf and Jobin[196] gave a single band on immunoelectrophoresis and sedimented as a single component in the ultracentrifuge. Its sedimentation constant (S_{20w}) in 0.1 M phosphate was 8.68S, the partial specific volume ($\bar{V}$) 0.73, and the molecular weight by the approach to equilibrium method 290,000. Gel filtration data have given higher values: 400,000[195] and 350,000.[197] Esnouf and Jobin[196] found that the sedimentation coefficient was concentration dependent which indicates that factor V forms aggregates. Related to this is probably the finding that the factor was eluted from Sephadex G-200 and G-100 columns at a much slower rate than expected.[196] Studies in dissociating solvents have not been reported. Calculations, however, from the amino acid composition have indicated a minimum molecular weight of 98,800.[194] In spite of its high molecular weight, the protein is soluble in water because it contains a large proportion of hydrophilic amino acid residues.[196]

The activity of purified factor V can easily be destroyed by oxidizing and -SH blocking agents but not by reducing agents.[194] All investigators have found that the activity deteriorates on standing especially in dilute solutions.[194,196,197] Stability can be improved by keeping the preparations as concentrated solutions of at least 0.1 mg/ml[197] or by dissolving them in 50% glycerol,[194,196] or by adding either a bivalent metal ion (Mg, Ca, Sr),[198] or crystalline bovine albumin.[197] Esnouf and Jobin reactivated their preparations by rechromatography on the phosphorylated cellulose.[196]

Optical rotatory dispersion data indicated that purified factor V becomes disorganized in concentrated sodium chloride solutions (0.5 M, pH 7.3) and in 50% glycerol.[196] The factor loses its activity in the 0.5 M NaCl solutions. The loss, however, is reversible and it can be restored after dialysis against 0.05 M HCl adjusted to pH 8.0 with tris-hydroxymethane.

The amino acid composition of factor V has been determined by Aoki et al.[194] and by Esnouf and Jobin.[196] The results of the two studies do not agree except for the values of the hydroxylated amino acids (tyrosine, serine, threonine), presumably because of the difference in the degree of purification of the preparations which were used in each study. From specific activity data Aoki et al.[194] have calculated that the concentration of factor V in bovine plasma should be approximately 9 mg/100 ml. Considering the higher degree of purification which has been achieved by more recent studies, the actual concentration of factor V in bovine plasma may be lower.

Activation by Thrombin

Ware et al.[192] and Ware and Seegers[199] found in the late forties that bovine serum has a much higher factor V (Accelerator Globulin) activity than the respective plasma and that addition of small amounts of thrombin to crude preparations of factor V increases the activity of the factor,[192,199] while addition of larger amounts of thrombin inactivates it.[199] Seegers and co-workers postulated that there must be two forms of factor V, a precursor in the plasma and a potent accelerator in the serum, the conversion being mediated by thrombin. These findings were confirmed by subsequent studies and Lanchantin and Ware[200] showed that TCA soluble fragments are liberated during the activation. The two forms of factor V were isolated by Papahadjopoulos et

al.[195] in 1964. These investigators found that the factor V activity of preparations isolated from bovine plasma coincided with the first protein peak, which was eluted from the Sephadex G-200 column. When the starting material was bovine serum, however, only part of the activity continued to coincide with the first protein peak. Another part was eluted with the ascending limb of the second protein peak. The relative amount of the second fraction was larger if the serum was left standing for a long time. The authors confirmed their findings by sucrose density gradient centrifugation and by gel filtration of purified factor V isolated from plasma and subsequently submitted to thrombin digestion. The molecular weight of the plasma factor V was calculated by Papahadjopoulos et al.[195] to be about 400,000 and that of the serum factor between 80,000 and 400,000, most probably near 200,000.

As mentioned earlier, the purification of human factor V has encountered greater difficulties because of the greater instability of the purified factor. Considering also the liability of factor V in human plasma, the finding of one of the authors (D.C.T.) that crude preparations of factor V are resistant to incubation was surprising.[201] Similar results were also obtained by Lewis and Ware[202] who found that partially purified human factor V is more resistant to the inactivating action of thrombin than the factor V activity of plasma. A 30-fold purification of human factor V has recently been achieved by batch chromatography through TEAE-cellulose in the presence of 0.5 M magnesium sulfate and 50% glycerol.[203]

Tissue Thromboplastin

Thromboplastin is found in most tissues in a wide range of concentrations. Its distribution in the tissues of man, rabbit, ox, horse, pig, rat, and calf has been studied by Astrup and his group.[204] It appears that in all these species the lungs have the highest concentration except in man, where the brain is the richest. The liver, heart, kidneys, and muscles have very little thromboplastin. Fibrous capsular tissues and synovial membranes are similarly poor. A high concentration of thromboplastin is found in the intima of both arteries and veins but the adventitia contains very little. A particularly detailed study published recently by Glas and Astrup [205] points out the uneven distribution of thromboplastin within each organ in the rabbit.

The nature of the tissue thromboplastin has been considerably elucidated during the last decade. Deutsch el al.[206] in 1964 separated human brain thromboplastin into a protein and a lipid moiety. The lipid moiety acted as a partial thromboplastin; the protein moiety, however, was inactive. Full activity was restored when the two parts were recombined. More recently Nemerson and Pitlick[207] were able to isolate the protein moiety of lung thromboplastin by solubilizing dehydrated and delipidated bovine lung in 0.25% deoxycholate followed by ammonium sulfate fractionation, DEAE-cellulose adsorption, and gel filtration through agarose (twice). The purified protein still contained 7% phospholipid and still exerted a weak thromboplastic activity. In essential agreement with the observations of Deutsch et al.,[206] Nemerson and Pitlick found that the thromboplastic activity increased 950 times if 1 mg of the protein was recombined with 7.5 mg of a mixture of phosphatidylethanolamine and phosphatidylserine. Both the polar and the nonpolar groups of the phospholipids were necessary for the exertion of procoagulant activity. Analysis of the protein showed the presence of two components, one with a molecular weight of 330,000 and another with a weight of 220,000 and higher specific activity. Both types of protein contained acidic lipid and acid mucopolysaccharides. Recently Pitlick et al.[208] showed that the protein moiety functions as an enzyme and is able to cleave free amino acids and three internal peptides from the oxidized B chain of insulin. In addition, it can hydrolyze synthetic di- and tripeptides. Attempts to separate the coagulant from the peptidase activity by a variety of methods (disc electrophoresis, electrofocusing, sucrose density gradient centrifugation, and gel filtration) proved unsuccessful. The two activities, however, were affected differently by the addition of phospholipids and had different metal requirements. The amount of phospholipids which enhanced the coagulant activity by a factor of 950, increased the V_{max} of the peptidase activity by a factor of 2 to 3. Although calcium was necessary for the coagulant activity, the peptidase activity required cobalt.

Synthesis of the Prothrombin Complex, Factor V and Factor VIII

It was shown experimentally by chloroform[537-539] and phosphorus[537] poisoning as

well as by clinical studies[538] that severe liver damage results in a reduction of the concentration of prothrombin and of factors VII, X, and V indicating that the liver is the site of synthesis of these factors. Immunocytological studies[209] have confirmed this conclusion and have specified the role of the parenchymal liver cell in the production of these factors. In contradistinction, the site of production of factor VIII has been the subject of controversy. While Norman et al.[210] and Dodds[211] recognize the spleen as an important site of production of factor VIII, the liver is considered by others as the main organ of synthesis.[212] Evidence favoring the kidneys[185,213] or the leukocytes[214] has also been presented. Recently Webster et al.[215] published experiments which support the view that the liver is the main site of synthesis of factor VIII, but other sites of limited production must also exist. These investigators transplanted normal livers to hemophilic dogs and found that the operation was followed by an increase in factor VIII to normal levels. Extrahepatic sites of synthesis were, however, revealed by replacing livers of normal dogs with hemophilic livers. The concentration of factor VIII in these dogs never fell below 14%. It is relevant that indirect immunofluorescence microscopy has indicated that the interstitium and the endothelium of the arteries and veins of all human tissues thus far examined contain material immunologically similar to factor VIII.[540] It remains to be seen, however, whether the endothelium synthesizes factor VIII or merely concentrates it from the plasma.

Activation of the Prothrombin Complex

The prothrombin complex can be activated by bioactivators, concentrated salt solutions, trypsin[85,220,221] and some snake venoms.[216,114,217]

Bioactivation

The physiological activators of the prothrombin complex are either normal constituents of the blood initiating the formation of the intrinsic activator or tissue constituents foreign to the blood initiating the formation of the extrinsic activator. The contact system, an integral part of the intrinsic system of activation, and the tissue thromboplastin have been described previously. In both instances the crucial event leading to the activation of prothrombin is the activation of factor X. This step, as well as the activation of the prothrombin itself, has been the subject of intensive studies and of considerable controversy over the past ten years.

Activation by the Intrinsic Pathway

All authors agreed that for the intrinsic activation of factor X four factors are required: activated factor IX (IXa), factor VIII, phospholipids, and calcium ions; and for the activation of prothrombin four factors again: activated factor X (Xa), factor V, and once more calcium ions, and phospholipids. The dispute was over the sequence and the nature of the reactions between these factors. The cascade[218] or waterfall[219] theory postulated that factor IXa activated factor VIII in the presence of phospholipids and calcium ions and that the activated factor VIII (VIIIa) together with calcium ions activated factor X. The factor Xa then in turn activated factor V in the presence of phospholipids and the activated factor V (Va) was the enzyme which activated prothrombin.

Work in several laboratories indicated that the postulated sequence could not be supported by experimental findings. Milstone already in 1952[220] and again in 1963[221] had demonstrated that thrombokinase (factor Xa) alone can activate prothrombin in the absence of ionic calcium, factor V, and phospholipids if it is used in relatively large concentrations (μg/ml). The latter substances act only as accessory factors potentiating the activity of factor Xa so that extremely small concentrations of this factor (thousands of a μg/ml, equivalent to the concentrations generated during blood clotting) become able to achieve the activation of prothrombin. This conclusion is also supported by the findings of Barton et al.,[222] Jobin and Esnouf,[223] and Marciniak.[76] It is also significant that Colman[224] found that factor V in the presence of phospholipids and calcium tripled the TAMe esterase activity of factor Xa. Papahadjopoulos and Hanahan,[105] Esnouf and Jobin,[106] and Cole et al.[107,108] independently in 1964 and 1965 and later other investigators[222,225] showed that factor V, factor Xa, calcium ions, and phospholipids form a complex and it is this complex as a whole that activates prothrombin rapidly. Hougie et al.,[226] Hemker and Kahn,[225] and Barton[228] showed that factor IXa, factor VIII, and ionized calcium become similarly adsorbed on the

phospholipids and form another complex. This complex then as a whole activates factor X.

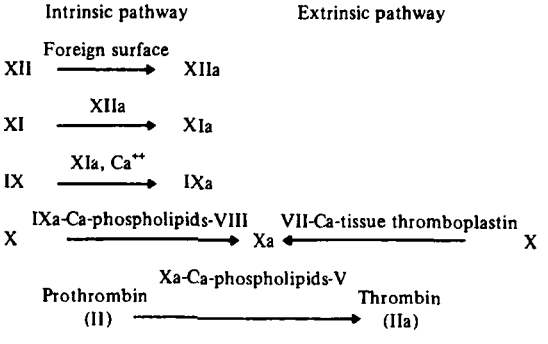
The formation of the complexes was demonstrated by gel filtration through Sephadex G-200,[105,222,226,228] by ultracentrifugation,[106,108,107,223,225] and by sucrose density centrifugation.[227] The pertinent factors, factor V and Xa on the one hand and factors VIII and IXa on the other, were incubated together with calcium ions and phospholipids and then were either filtered through the Sephadex column or centrifuged at 65,000 to 100,000 x G. In the filtration experiments when calcium ions were included in the elution buffer all four factors of each incubation mixture were eluted together in the void volume where, normally, only the phospholipids or factor VIII was expected to emerge. When factors V, IXa, and Xa, and the calcium ions were filtered individually, they were eluted either shortly after the void volume (factor V) or much later, since they are smaller molecules. Similarly, in the experiments with the ultracentrifuge the incubated factors in the presence of calcium precipitated together and formed a pellet at the bottom of the tube regardless of their individual sedimentation rates and the supernatant became free of their respective activities.

For the adsorption of factors IX and X on the phospholipids calcium ions were required[105-107, 222,223,225,226] and the factors had to be activated before they could be adsorbed.[105, 164,225] In contradistinction calcium was not required for the adsorption of factors V and VIII.[105,108,164,222,223,225] In fact the presence of calcium inhibited their adsorption.[108,223,225] Although the rate of prothrombin activation was much greater when thrombin activated factor V was used,[105] this factor could be adsorbed on the phospholipids even in its native form.[105,164] In contrast, factor VIII had to be activated before it could participate in the formation of factor X activator. When factor VIII was added in its native form and hirudin was also present in the activation mixture, only traces of factor X activator were formed.[268] At concentrations of calcium higher than 0.04 an almost quantitative release of factor V from its complex was achieved by Cole et al.[108] leaving factor Xa and the Ca^{++} still attached to the phospholipids. This property was used by these authors to obtain factor V (Ac-globulin) in a high state of purity. Factors IXa or Xa, on the other hand, could be eluted from the respective complexes by omitting calcium from the eluting buffer[105,222,226,228] during the gel filtration experiments or by adding a decalcifying agent (EDTA) to the incubated solution or to the eluting buffer.[222] This teatment left factor V or factor VIII still attached to the phospholipids. Factor Xa has also been eluted from its complex by washing the pellet after ultracentrifugation with 0.01 M $MgCl_2$.[106,223] It has been possible, therefore, to separate each of the factors involved in the formation of these complexes after their interaction with the others and test their ability to activate factor X or prothrombin. There was no evidence that factor V or factor VIII became activated during their interaction with factors Xa or IXa and neither of them could activate its supposed substrate alone with calcium ions, as the cascade theory postulated.[222,228] Nor could factors IXa or Xa alone do the activations at the concentrations at which they were used in these experiments. Activator activity was restored only when all the constituents of a complex were recombined.[106,222,223,228] Activator activity was also lost when the complex factor VIII-phospholipids-calcium^{++}-factor IXa was incubated with antibodies against factor IX or factor VIII.[229]

Activation of Factor X by the Extrinsic Pathway

The activation of factor X by the extrinsic pathway has been shown to proceed with the formation of a similar complex between tissue thromboplastin, calcium ions, and factor VII.[518,519]

The coagulation reactions until the activation of prothrombin can then be summarized as follows:



Activation in Concentrated Salt Solutions. Autoprothrombins

It was shown by Seegers and his associates[56, 230] and has been confirmed by other investigators[65,85,112,122,126,231] that the purified prothrombin complex (originally throught to represent only prothrombin) becomes activated when it is dissolved in 25% sodium citrate or other concentrated salt solutions[56,85] (ammonium sulfate, magnesium sulfate, sodium sulfate, protamine sulfate, potassium citrate, etc.) and forms thrombin. As it was shown later, a second enzyme, activated factor X or autoprothrombin C, is also formed during the same process.[103,542]

The activation of the prothrombin complex is effected through a stage of intermediates which are refractory to the two-stage reagents, i.e., they cannot form thrombin upon addition of tissue thromboplastin, factor V (Ac-globulin), and calcium ions.[56,230] The activation can stop at an intermediate stage if soybean trypsin inhibitor,[232] 3,4,4′-triaminodiphenyl,[230,233] or 2-hydroxy-4,4′ diaminodiphenyl sulfone[230] is added to the solution. Refractory solutions can also be obtained if the activation of the prothrombin complex is attempted in water or saline solutions by adding thrombin[139,234,235] or a combination of platelet factor 3, calcium, and factor V[139,236,237] or small quantities of factor Xa[238] or simply by storage.[239,240] Small amounts of thrombin are formed by the autocatalytic activation of the prothrombin complex and this thrombin inactivates prothrombin even at -10°C.[240] The only way to make the activation go to completion in these cases is to add sodium citrate to a concentration of 25%[236,238,239,242] or larger quantities of factor Xa. Seegers and co-workers found that the refractory solutions, although they themselves could not form thrombin without the addition of salts, could accelerate the formation of thrombin from preparations of intact prothrombin complex, i.e., they could act as procoagulants.[139, 236-241] Seegers and his co-workers called these procoagulants autoprothrombins and distinguished over the years two kinds: autoprothrombin I or plain autoprothrombin, which accelerated the activation of prothrombin by the extrinsic pathway using diluted tissue thromboplastin, factor V, and calcium,[139,236,238,241] and autoprothrombin II which activated the prothrombin complex together with platelet factor 3, factor V, and calcium[139,242] (the last three reagents alone could activate the prothrombin complex only partially forming autoprothrombin I). Three types of autoprothrombin I have been described: autoprothrombin I or plain autoprothrombin of earlier publications which is formed spontaneously during storage[239,240] or when chromatographed (Amberlite IRC-50) prothrombin is activated by thrombin;[239] autoprothrombin I_c obtained by activating the prothrombin complex with small amounts of factor Xa (Autoprothrombin C);[238,241] and autoprothrombin I_p obtained by activating the prothrombin complex with a combination of platelet factor 3, calcium, and factor V.[236,237]

Mammen et al. studied the autoprothrombins in detail.[139] They found that autoprothrombin I formation was more rapid in alkaline solutions being progressively better from pH 5.7 to pH 9.7. The optimum pH for autoprothrombin II formation was 6.7 to 7.9. They further found that thrombin could induce the generation of either autoprothrombin I or autoprothrombin II depending on whether or not the prothrombin (complex) had been chromatographed on IRC-50 before activation. Autoprothrombin I activity formed from the chromatographed prothrombin and autoprothrombin II activity from the non-chromatographed preparations. The autoprothrombins were purified by ammonium sulfate fractionation followed by chromatography on Amberlite IRC-50.[139] Autoprothrombin II[139] and I_c[238] activities precipitated at 50% ammonium sulfate saturation while those of autoprothrombin I and I_p required a 70% saturation to be salted out.[139,238] Autoprothrombin II was eluted from the Amberlite in the first two tubes and autoprothrombin I in the first five. There were also other protein fractions unrelated to the procoagulant effect which were eluted later.[139] Similarly, chromatography of autoprothrombin I_c on DEAE-cellulose showed the presence of several components (three); the procoagulant fraction, however, was eluted from this resin last with the 0.4 M phosphate buffer.[241] It appears from these results that in both types of chromatography the procoagulant fractions of the autoprothrombins had the same elution characteristics as factors X and Xa.[97,100,103,163]

Autoprothrombin I was found to correct factor X and factor VII deficiencies[172,236,241,245] and autoprothrombin II, factor IX deficiency.[244,245] In addition, autoprothrombin II could substitute

for serum in the thromboplastin generation test.[245] One must conclude, therefore, that the procoagulants in autoprothrombin I preparations were factor X and factor VII and those in autoprothrombin II, factors X and IX, which were isolated together as one fraction in each case.

Factor X in autoprothrombin I_c preparations must have been in an intermediate form of activation since by contrast to autoprothrombin C which is the fully activated enzyme (factor Xa), it could not activate prothrombin by itself, not even partially to the refractory stage.[241] Its physicochemical characteristics similarly were intermediate to those of factor X and factor Xa: its sedimentation constant was 3.34S, diffusion coefficient 7.5 ($x10^{-7}$ cm^2/sec), and molecular weight 35,000.[246] The corresponding data for factor X are S_{20w} = 3.4 to 3.6,[77,135] the molecular weight = 53,000 to 56,000,[77] and the data for factor Xa: S_{20w} = 2.27S, D_{20w} = 8.4 x 10^{-7} cm^2/sec, molecular weight = 21,500 to 27,000.[163]

Factor X isolated from prothrombin complex activated by thrombin at pH 7.0 was found to be in the zymogen form (autoprothrombin III)[97,100,172] and showed the same filtration characteristics through Sephadex G-100 as before the addition of thrombin.[76] It was, however, found ineffective in correcting hemophilia B deficiency in contradistinction to factor X preparations isolated from intact prothrombin complex not activated by thrombin.[97,172] Taking into consideration that autoprothrombin II, which also corrects the clotting abnormalities of hemophilia B, is formed by the addition of thrombin to the prothrombin complex or by the clotting of blood,[51,139,245] one wonders whether thrombin does not first activate factor IX and then under certain conditions inactivate it, just as it does factors V and VIII.[195,199,265-269] Alternatively, in the second instance the activity of factor IX could be counteracted by the inhibitor which is formed at the same time. (references 97, 100, see p. 79).

Since the refractory solutions of partially activated prothrombin complex generate thrombin in 25% sodium citrate solutions,[236,238,239,242] they must contain a precursor or precursors of thrombin beside the "autoprothrombins." Such a precursor was first observed by Seegers et al. in 1950 by free boundary electrophoresis[56] and was isolated by Asada et al.[109] and Magnusson[110] in 1961 by DEAE- and TEAE-cellulose chromatography, respectively. These semipurified precursors were insensitive to the two-stage reagents and could give thrombin only in 25% sodium citrate solutions[109] or when serum was also included in the reaction mixture.[110] Seegers and Marciniak[100] and Marciniak and Seegers[97] isolated similarly a thrombin precursor (prethrombin) by DEAE-cellulose chromatography of solutions of prothrombin complex which had been activated partially by the addition of thrombin[97,100] or spontaneously.[97] In one of these publications Marciniak and Seegers[97] isolated two peaks with prethrombin activity. The molecular weight of one of the prethrombins was estimated to be 40,000 (sedimentation diffusion[131]), its isoelectric point (I.P.) 5.5,[135] and the Stokes radius 3.35.[76] The I.P.'s of prothrombin and thrombin have been reported to be 4.1 to 4.25[56,68,123] and 5.3 to 5.8;[123] and the Stokes radii 4.62 and 3.17, respectively.[76] The amino acid composition of prethrombin was found to be very similar to that of thrombin.[245] Tishkoff et al.[68] isolated a thrombin precursor from bovine prothrombin complex, which they called modified zymogen. This had a molecular weight of 52,400 (sedimentation equilibrium in a solvent of 6 M guanidine hydrochloride and mercaptoethanol) and appeared to be similar, if not identical, to the prethrombin of Seegers and Marciniak.[100,243] Prethrombin can be activated to thrombin by activated factor X (autoprothrombin C) alone.[76,97,100,135,243] Phospholipids, calcium ions, and factor V all together accelerate the activation by factor Xa. Marciniak recently drew attention to the fact that prethrombin requires a higher concentration of factor V than prothrombin to be activated to thrombin.[76] Tissue extracts or Russell's viper venom neither alone nor together with factor V and/or calcium ions can activate prethrombin.[100,135,243] Trypsin can do the activation and, in this respect, is more effective than factor Xa.[100] Purified prethrombin cannot be activated "spontaneously" in 25% sodium citrate.[135]

Aronson and Ménaché[69] activated human prothrombin complex with activated factor X (thrombokinase) in the absence of calcium, phospholipids, or factor V. Using chromatography on DEAE-cellulose and gradient elution (0.15 M→0.50 M NaCl), they isolated from the in-

cubation mixture two thrombinogenic intermediates and two others without thrombin potential. The smallest of the thrombin precursors was eluted together with thrombin and had a similar molecular weight (31,800, sedimentation-diffusion data). The non-thrombinogenic fragments had molecular weights of 26,000 and 17,000. The same intermediates were formed when the prothrombin complex was activated spontaneously[111] or after addition of calcium chloride together with inosithin, which had been saturated with plasma.[69] Similar results were obtained by Magnusson[112] after activation of bovine prothrombin complex (in 25% sodium citrate solutions) and by Lanchantin et al.[93] after activation of human prothrombin complex.

Recently Mann et al.[99] activated purified prothrombin in 25% sodium citrate solutions after addition of trace quantities of defibrinated plasma and subjected samples of the incubation mixture to SDS polyacrylamide gel electrophoresis before and after reduction of the disulfide bonds with mercaptoethanol. They found that prothrombin is first cleaved to a single chain intermediate of 65,500 daltons. The intermediate is subsequently broken into two single chain molecules, one of 39,000 and the other of 24,000 daltons. The 39,000 dalton chain is thrombinogenic giving rise to an active thrombin composed of two disulfide-linked chains (33,000 and 6,000 daltons) but the 24,000 dalton intermediate is not thrombinogenic. This last intermediate most likely corresponds to the "pro-piece" of Shapiro.[258] The 39,000 dalton chain must correspond to the prethrombin of Seegers and Marciniak[97,100,243] or to the peak III of Aronson and Ménaché[69] and Aronson[111] or to the second intermediate of Magnusson,[112] peak II; and the 65,000 dalton chain must be the same derivative as the peaks II and IIa of the latter investigators, respectively.[69,111,112]

Shapiro and Connell[247] added ^{125}I labeled prothrombin into plasma. By measuring the radioactivity, they found that very little thrombin was formed during clotting. Most of the prothrombin was split into the "pro-piece" (approximately 35,000 molecular weight) which could not form thrombin and to another derivative "nearly the size of prothrombin" which had a faster electrophoretic mobility and was thrombinogenic. It is interesting to mention here that McClaughry already in 1956[248] had isolated a prothrombin derivative from serum which could generate thrombin in 25% sodium citrate solutions. His findings have been confirmed by Mammen et al.[139]

Anticoagulant from the Prothrombin Complex (Autoprothrombin II-A)

During the fractionation of autoprothrombin II solutions by ammonium sulfate Mammen et al.[139] found that occasionally an anticoagulant was obtained instead of the expected procoagulant. The anticoagulant (autoprothrombin II- Anticoagulant) could be removed by chromatography on Amberlite IRC-50 and the original procoagulant activity recovered. Further work in the same laboratory indicated that freeze-drying of autoprothrombin II concentrates increased the chances of obtaining the anticoagulant, but even so, its generation remained unpredictable.[249] In 1965 Seegers and Marciniak[100] and Marciniak and Seegers[97] succeeded in obtaining this anticoagulant regularly by DEAE-cellulose chromatography of solutions of prothrombin complex activated by thrombin at pH 7.0. The anticoagulant was eluted by 0.175 M, pH 7.4 phosphate buffer (the same buffer which eluted prothrombin from nonactivated preparations) as a middle fraction between prethrombin and factor X (autoprothrombin III). The inhibitor was also obtained directly from plasma[249] and serum.[139,249,250] Its precursor was found to be different from prothrombin.[250] Gel filtration through Sephadex G-200 indicated that the inhibitor is eluted together with factor VII and may consist of particles with a molecular weight of approximately 80,000.[250] This inhibitor interferes with the activation of factor X by the intrinsic pathway[250,252] but it does not affect the activation of this factor by the extrinsic (tissue thromboplastin and factor VII) pathway or by Russell's viper venom.[250-252] It also inhibits the action of factor Xa in a competitive way[251] and thus, impairs the conversion of prothrombin or prethrombin to thrombin. Its action is species specific[250,252] and is rapidly inactivated by plasma or serum.[250,251] The formation of this inhibitor is most likely responsible for the refractoriness of prothrombin during the initial stage of activation. Only when sufficient amounts of factor Xa are formed can the inhibition be counteracted and the prothrombin transformed into thrombin. The finding that the inhibitor does not inhibit the extrinsic activation of factor X,

while it can inhibit the intrinsic activation of this factor, may explain why the prothrombin time of plasma is much shorter than the activated partial thromboplastin time. It may also explain why the generation of thrombin in solutions of 25% sodium citrate never reaches the prothrombin potential. It usually stays at 55 to 67%[99,230] and the most it has been reported to have reached without addition of activators is 75%.[122]

IMMUNOLOGICAL STUDIES ON CONGENITAL AND ACQUIRED COAGULATION "DEFICIENCIES"

The spontaneous production of inhibitors against factor VIII and factor IX and the relatively recent introduction of immunological methods for the detection of coagulation factors have shed new light on the nature of the congenital coagulation defects and on the coagulopathies which result from vitamin K deficiency or from the administration of its antagonists.

Congenital Coagulation Defects

Hemophilia B_M or B^+ and Hemophilia B^-; Evidence for Further Heterogeneity

Fantl et al. in 1956[520] studied a patient with a naturally occurring inhibitor against factor IX. They found that a barium sulfate eluate prepared from one patient with factor IX deficiency neutralized the inhibitor to the same extent as eluates from normal plasmas, while similar eluates from two other patients with factor IX deficiency did not. They concluded that patients with factor IX deficiency could be divided into two groups: those with no factor IX-like protein and those with a circulating protein which was antigenically similar to factor IX but had no procoagulant activity.

Kidd et al.[521] found in 1963 that the thrombotest and the one-stage prothrombin time using ox brain thromboplastin was prolonged in one of their seven cases of hemophilia B. However, the prothrombin time was normal when human brain substituted as the source of thromboplastin. Hougie and Twomey[257] and Denson et al.[522] found that two (brothers) out of their six and three out of their 27 patients with hemophilia B, respectively, had the same abnormality. The prolongation of the prothrombin time with ox brain thromboplastin was shown to be due to the presence of a competitive inhibitor[251,522] which was identified as an abnormal, physiologically inactive factor IX molecule similar to the one detected by Fantl el al.,[520] by immunodiffusion and an inhibitor neutralization test.[522] Hougie and Twomey proposed the term hemophilia B_M (M was the family initial of their patients with the abnormality) and Denson et al., hemophilia B^+, to distinguish this type of hemophilia with the abnormal factor IX molecule from the cases where the patient's plasma did not contain material cross reacting with factor IX inhibitors or antibodies and which they called hemophilia B^-.

Further work indicated that hemophilia B is more heterogeneous. In a subsequent publication Denson et al.[253] mentioned the case of two brothers whose plasma contained an abnormal factor IX protein but only one of the brothers had a prolonged one-stage prothrombin time with ox brain thromboplastin. Meyer et al.[255] described a more complicated picture. They found that in a total of 14 cases classified as hemophilia B, three of the patients had an inhibitor neutralizing antibody in their plasmas but only one of them exhibited a prolonged prothrombin time with ox brain extracts. On the other hand, four of the patients who did not have any inhibitor neutralizing protein, i.e., who were B^-, had this test abnormal. It appears, therefore, that the presence of an abnormal factor IX protein and the prolongation of the one-stage prothrombin time with ox brain thromboplastin represent two different conditions which may or may not appear in the same individual.

Hemophilia A

Reactivity with Spontaneous Inhibitors and Antibodies Induced by Factor VIII Concentrates: Hemophilia A^+ and Hemophilia A^-

Denson et al.[253] and Hoyer and Breckeridge[523] in 1968 studied 48 and 34 hemophilic patients, respectively. They found that the plasma of 4 of the 48 and of 6 of the 34 patients could neutralize inhibitors of factor VIII which had developed spontaneously or after multiple transfusions in other patients and also it could neutralize antibodies produced in the rabbit by the injection of factor VIII concentrates.[253] Similar results were subsequently reported by others.[255,524,525] In an analogy to hemophilia B, Denson et al. proposed the terms hemophilia A^+ for the cases where the patient's plasma contained cross-reacting material against factor VIII antibodies or

inhibitors and hemophilia A^- for the cases where such material was lacking. Using immunoelectrophoresis on agar, Gralnick et al.[525] found that the plasma of patients with hemophilia A^+ produced a precipitin line in the β-globulin area which was similar but not identical to the line which was produced by plasmas with normal factor VIII, thereby demonstrating that the abnormal, factor VIII-like protein was immunologically somewhat different from the normal factor.

The cross-reacting material was found mainly in the plasma. Normal serum had little neutralizing ability.[253] Storage of normal plasma destroyed little (about 14%) of its inhibitor neutralizing ability, while most of the biological activity (88%) disappeared, suggesting that the inhibitor reacted also with an early degradation product(s) of factor VIII. The incidence of hemophilia A^+ is about 15%, 24 cases out of a total of 229 reported in the literature.[253,523-525]

Reactivity with Antibodies Induced by Purified Factor VIII

The production of antisera against purified preparations of factor VIII indicated that material antigenically similar to factor VIII is produced even in the cases of hemophilia A^-. Shanberg and Gore[254] found in 1957 that the plasma of severe hemophiliacs could neutralize antibodies produced in rabbits by the injection of relatively pure preparations of factor VIII. More recently Zimmerman et al.[526] and Stites et al.[527] injected rabbits with factor VIII purified 6,000- to 16,000-fold. Using quantitative immunoelectrophoresis and an antiserum blocking assay[526] or a hemagglutination inhibition test[527] these investigators found that both the plasma and the serum of all hemophilia A patients they tested (22 and 14, respectively) contained material antigenically similar to factor VIII and in amounts comparable to those of normal plasma and normal serum. It appears therefore that the purified factor induces the formation of antibodies which are different from the antibodies which develop spontaneously in patients (inhibitors) or are induced by the injection of factor VIII concentrates into animals. Relevant of the different nature of these antibodies is that sera from three patients with spontaneously occurring inhibitors against factor VIII behaved like normal serum, i. e., they a) inhibited the hemagglutination at titers comparable to those of normal serum and b) did not cause agglutination of red cells coated with purified factor VIII.[527] It seems that the antibodies against the highly purified factor have a broader specificity and are able to combine with early precursors or probably with abnormal forms of factor VIII and also with degraded forms of it (they react with serum) as well as with the biologically active factor. On the other hand, the naturally occurring antibodies have a narrow specificity and react with the biologically active form(s) or its immediate precursor(s) or successor(s). Another difference is that the natural inhibitors inactivate also factor VIII of unrelated species (ox, cat, sheep, goat, etc.) while the rabbit antiserum against the highly purified human factor cross reacts only with the factor of closely related species, the chimpanzee and the gorilla, and weakly with the bovine factor.[526,527]

Von Willebrand's Disease

Only a small number of patients (about 25) with this disease have been studied so far. Inhibitor neutralization studies[253,523] and also immunoelectrophoresis with antibodies induced by highly purified factor VIII preparations[526] indicated that the antigenic reactivity of the plasma of these patients was remarkably low or equivalent to their factor VIII coagulant activity. The hemagglutination inhibition tests showed little or no factor VIII antigenicity.[527] It appears, therefore, that patients with Von Willebrand's disease do not produce biologically inactive factor VIII. The abnormality, as far as this factor is concerned, seems to be reduced production, i.e., a true deficiency.

Dysprothrombinemias

Only two of the reported cases of hypoprothrombinemia have been investigated for the existence of an abnormal prothrombin molecule. In both cases positive results were obtained.

Prothrombin Cardeza

Shapiro et al.[528] studied a family with 32 members, 11 of whom exhibited one half of the normal prothrombin activity by clotting assays but had normal amounts of this proenzyme immunologically (agarose electrophoresis). Prothrombin isolated from one of the affected individuals generated one half or fewer thrombin units than were generated from normal prothrombin, regardless of the mode of activation, intrinsic or

extrinsic, in concentrated sodium citrate solutions or by trypsin. Agarose immunofixation electrophoresis of serum showed the presence of normal and abnormal activation products indicating that the affected family members were heterozygotes synthesizing normal and abnormal prothrombin molecules. Studies with ^{125}I labeled prothrombin suggested that the abnoraml proenzyme, which was called "prothrombin Cardeza" after the name of the foundation where it was studied, was able to undergo the initial steps of the activation and formed an abnormal "pro-piece" and a major intermediate product which could not generate thrombin.

Only two of the members had a hemorrhagic diathesis. Both, however, suffered also from other abnormalities which could cause bleeding: One was afflicted with Ehlers-Danlos syndrome and the other had a reduced level of factor VIII (30%). The inheritance of prothrombin Cardeza was found to be autosomal.

Prothrombin Barcelona

Josso et al.[529] described a family from Barcelona, Spain with eight children, four of whom, two boys and two girls, suffered from a hemorrhagic diathesis clinically resembling mild hemophilia which was, however, transmitted as an autosomal defect. The prothrombin levels of the affected children were 4% and 12% by the one- and two-stage methods, respectively, but 80 to 100% by the staphylocoagulase assay and by immunoelectrophoresis. The parents and a brother, who were clinically normal (heterozygotes), had a prothrombin level of about 60% by the clotting assays and 80 to 117% by the other tests. Experiments with euglobulin precipitates indicated that the total yield in thrombin was normal but that the rate of conversion of the proenzyme to thrombin was about 20 times slower than normal. As a result of the slow activation rate, the newly formed thrombin was most likely neutralized by the antithrombin of plasma as soon as it was formed. The abnormal prothrombin could be adsorbed on tricalcium phosphate and gave a line of identity with normal prothrombin on immunodiffusion (Ouchterlony). It had, however, a greater anodal mobility on immunoelectrophoresis.

The Factor-X Defect

Denson et al.[530] studied the reactivity of factor X of seven patients with factor X deficiency including Mr. Prower and Mr. Stuart, the first patients who were found to suffer from this coagulation abnormality. They distinguished five types of the defect depending on whether the partial thromboplastin, prothrombin, and Stypven times were slightly or grossly abnormal or even normal (Stypven) and on whether or not immunodiffusion and antibody neutralization assays indicated the presence of factor X-like material in the patient's plasma. They found that the abnormalities of Stuart and Prower plasma were similar in that they both displayed prolonged prothrombin, partial thromboplastin, and Stypven times, but they were different immunologically: While the Prower plasma neutralized antibodies against normal factor X and gave a precipitin line of identity with it, the Stuart plasma did not contain material cross-reacting with factor X antibodies.

Factor X Friuli

Girolami et al.[531,532] studied two families from a remote valley, called Friuli in Northeastern Italy, with several severely (homozygotes) and mildly affected or asymptomatic (heterozygotes) members of three generations. The homozygotes suffered from mild spontaneous but severe post-traumatic bleeding. Their coagulation abnormality was characterized by a prolongation of the partial thromboplastin and the prothrombin times, while the Stypven time was normal. Factor X levels were 4 to 9% of normal in assays where tissue thromboplastin was used, but 70 to 92% in assays with Stypven. A factor X antibody neutralization test performed with a sample of one of the patients yielded a value of 117% of normal and his serum extract gave a line of identity with normal factor X. Judging from these tests, factor X Friuli seems identical to one of the cases (D.E.C. patient) studied by Denson et al.[530]

Factor VII Heterogeneity

Congenital factor VII deficiency has been shown similarly to be heterogeneous. Goodnight et al.[256] found that the plasma of two of their four patients neutralized more antibody (induced into rabbits by factor VII concentrates) than was expected from their factor VII clotting activities. In contrast, the other two patients had antibody neutralizing material equivalent to the factor VII activity of their plasmas.

Acquired Coagulation Defects

Although all methods show a significant decrease in the concentration of prothrombin during vitamin K deficiency or during the administration of its antagonists, there is a great discrepancy in the values obtained by the classical clotting assays on the one hand and the immunological and other methods on the other. Müller-Berghaus and Seegers in 1966[261] determined the thrombin potential of the plasma of rabbits before and after the administration of large doses of Warfarin®, by the two-stage method and by the addition of factor Xa (autoprothrombin C) and factor V to the plasma. They found that during the administration of Warfarin the amount of the extra thrombin which was detected by the addition of factor Xa was greater than the amount which was detected by the two-stage reagents (10 vs 5 units) while before the administration of the drug it was the other way around (43 and 199 units, respectively). These investigators concluded that a greater amount of prethrombin, which they viewed both as a pre- and as a meta-prothrombin, than of prothrombin was synthesized during the administration of the drug.

Related to these findings are the results of Soulier and his collaborators.[260,524] The French investigators found that the concentration of prothrombin in the plasma of patients receiving Dicumarol® appears to be three times greater when it is determined by the addition of staphylocoagulase or by immunological methods (immunodiffusion) than when it is determined by the two-stage method or by the thrombotest. They concluded that the relative lack of vitamin K induces the synthesis of an abnormal prothrombin molecule which cannot be activated by the physiological clotting mechanism, which, however, has the same antigenic determinants and the same reactivity with staphylocoagulase as the normal prothrombin. This conclusion seems to be supported by the findings of Malhotra and Carter,[262] who isolated an altered prothrombin from the plasma of dicumarolized steers. Recently Malhotra[541] found that this abnormal prothrombin can be differentiated into three types depending on their adsorbability: "Ba-citrate," "Ba-oxalate," and "alumina" atypical prothrombin. None could be adsorbed on $BaSO_4$. These atypical prothrombins could be further distinguished by their isoelectric points, their R_f values in disc gel electrophoresis and their precipitability by ammonium sulfate. All three had a lower specific activity than normal prothrombin and could be further distinguished from it by agar gel electrophoresis at pH 8.6 in the presence of calcium. The atypical prothrombins migrated between the α_1 and the α_2 globulins while normal prothrombin migrated between the β and α_2 globulins.

Goodnight et al.[256] found that during dicumarol-induced or nutritional vitamin K deficiency, the plasma can neutralize more factor VII antibodies than is expected from its factor VII coagulant activity. In contrast, in liver cirrhosis the immunological reactivity of the plasma is equivalent to its coagulant activity, i.e., there is only a reduction in the production of normal factor VII; there is no synthesis of an abnormal factor VII-like molecule.

Hemker et al.[525] and Hemker and Müller[259] have presented evidence based on enzyme kinetics that vitamin K deficiency induces the production of an abnormal protein analogous to factor X, which acts as a competitive inhibitor during coagulation tests where the concentration of the plasma to be tested is relatively high (10% and over). The presence of this inhibitor may explain, at least in part, the lower levels of prothrombin and factor VII which are found by the classical clotting assays. It is relevant that Hemker et al.[526] in their preliminary report assumed that this inhibitor was a preprothrombin. It would be interesting to see whether there is any connection between this inhibitor and the inhibitor described by Seegers and his collaborators.[97,100,139,250-252]

In view of the above it is evident that the derivatives of Dicumarol affect late stages in the synthesis of the prothrombin complex. Regarding the mechanism of this inhibition, it should be mentioned that Georgatsos and Karemfyllis[263] found that these compounds inhibit the incorporation of orthophosphate ^{32}P and Thymidine ^{3}H into the nuclear and the mitochondrial DNA of baby syrian hamster kidney cells grown in culture.

FIBRINOGEN

With the exception of thrombin, fibrinogen is the best known of the coagulation factors.

Physicochemical Characteristics

The commonly used value for the molecular

weight of human and bovine fibrinogen is 340,000[271-273] calculated from light scattering[274] and sedimentation diffusion data.[275,270] The molecule has a partial specific volume of 0.71 to 0.72,[271,275,277] a sedimentation constant (S_{20w}) of 7.6 to 8.0S,[271,275,276,278] a diffusion coefficient (D_{20w}) of 1.98 to 2.02 x 10^{-7}, cm^2, sec^{-1},[276,278] and an intrinsic viscosity of 0.25.[275,276,278] Its length in solutions was found to vary from 500 to 700 Å[271,272,274] and its axial ratio from 5 to 20[271,272] indicating that fibrinogen is highly asymmetrical, and has the shape of a prolate ellipsoid of revolution. Depending on the ionic strength and the nature of the buffers used for its determination, the isoelectric point has been found to vary from 5.5 to 5.95.[279-281] All these values are compatible with the amino acid composition of human fibrinogen.[277] The native molecule displays a 30 to 33% helical structure calculated from optical rotatory dispersion,[282] ultraviolet spectroscopy,[283] and low angle x-ray diffraction studies.[343] Fibrinogen is stable in the range of pH 5.5 to 10. Denaturation by acids, alkali, and 5 M urea reduces the helical content to about 20% while denaturation by 5 M guanidine-HCl or 10 M urea destroys it completely.[282] During denaturation tyrosine residues become exposed to the solvent.[283]

Morphology

Under the electron microscope[284,285] fibrinogen appears to be made of triads of closely spaced nodules linked together by filaments of about 15 Å in diameter. Each of the outer nodules has a diameter of 65 Å, while the middle nodule is slightly smaller with a diameter of 50 Interestingly, the molecular length is affected by the pH, the shortest length (230 Å) being observed at pH 5.6, the isoelectric point of fibrinogen. As the pH departs from this value, the length of the molecule increases.

Chemical Composition

The Polypeptide Chains and the N Terminal Disulfide Knot

It is now well established that the fibrinogen molecule is a dimeric unit composed of two identical subunits. Each subunit is made of three polypeptide chains,[286-289] the α(A), β(B), and γ chain.* In the human species their respective molecular weights (sedimentation equilibrium) and *N*-terminal amino acids are 63,500, alanine (90%) and aspartic acid (10%); 56,000, pyroglutamic acid; and 47,000, tyrosine.[290,291] The three chains at their *N* terminal ends are linked together by disulfide bridges and this area of the molecule has been appropriately called the *N* terminal disulfide knot.[292,293]

Although the amino acid sequence of the fibrinopeptides A and B components of the knot of various species had been known for several years,[294-299] the elucidation of the structure of the remaining parts of the knot has been completed only recently and only for the human species.[300,301] The knot has been studied extensively by Blombäck and co-workers. It has been isolated by the cleavage of fibrinogen at the sites of the methionine residues with cyanogen bromide (CNBr) and filtration of the resulting fragments through Sephadex G-100.[292,293] Digestion of the eluted fractions with thrombin indicated that the two fibrinopeptides were always released from material rich in disulfide bonds, which was emerging from the column close to the void volume. The structure of the knot was elucidated by reduction of the disulfide bonds with mercaptoethanol and by alkylation with iodoacetic acid followed by stepwise Edman degradation of the intact knot as well as of thrombic, tryptic, and chymotryptic fragments of it.

Each monomeric knot of human fibrinogen is made of 244 amino acid residues of which 11 are half cystines. Fifty-one amino acids are contributed by the α(A), 115 by the β(B), and 78 by the γ chain.[300,301] According to Blombäck,[300] the experimental evidence indicates that the three chains are linked together by four disulfide bridges. Two of the bridges link the α(A) chain to the β(B) chain, the third links the β(B) chain to the γ chain and the fourth bridge links the γ chain to the α(A) chain. The two knots of each fibrinogen molecule are linked together by three separate disulfide bridges: two of these stretch between the two γ chains (at positions 8 and 9,[302]) and the third between the two α(A) chains (at position 28[302]). The two knots together represent 16% of the molecular weight of

*Nomenclature of the International Committee on Thrombosis and Haemostasis, *Thromb. Diath. Haemorrh.*, 35, 161, 1969.

fibrinogen, i.e., the monomeric form of the knot has a molecular weight of 25,000 to 30,000.[300,302]

There are indications that the *N*-terminal disulfide knot is more or less hidden in the intact fibrinogen. Antibodies produced against the knot give only a faint precipitation line with fibrinogen and antifibrinogen antibodies barely react with the knot.[303]

The Carbohydrate Content

The total amount of the carbohydrates of fibrinogen has been reported to be 3 to 5%.[304-307] Mester and co-workers[306-308] degraded bovine fibrinogen by pronase. They filtered the digest through Sephadex G-25 and separated a glycopeptide fraction which contained 80% of the original carbohydrate of fibrinogen. Using paper electrophoresis in pyridine-acetic acid-water at pH 6.4 they fractionated the crude material into three glycopeptides which they labeled A, B, and C in the order of their electrophoretic mobility. Each of the glycopeptides contained three molecules of D-glucosamine, four molecules of D-mannose, three molecules of D-galactose and one (glycopeptide A) or two (glycopeptides B and C) molecules of sialic acid (as *N*-acetyl- and *N*-glycolylneuraminic acid in a ratio of 1:1). Cleavage with neuramindase, methylation, and oxidation with periodic acid before and after the removal of the sialic acid indicated that the sialic acid occupied the terminal positions, each molecule being attached to a D-galactose molecule which in turn was attached to a D-mannose molecule. The third D-galactose molecule was terminal and was bound to a D-glucosamine molecule. The binding to the protein was done by the first D-glucosamine residue. The linking component was isolated and identified as *N*-acetyl-D-glucosamyl-β-aspartyl amide.[307] The molecular weight of the carbohydrate moiety of the glycopeptides B and C was found to be about 2,400 and that of glycopeptide A, 2,100. Sulfitolysis of fibrinogen and electrophoresis of the sulfoderivatives on paper or cellulose[289] or chromatography on carboxymethyl cellulose[309] revealed that all the chains of bovine and human fibrinogen contain carbohydrate. Identification of the glycopeptides indicated that glycopeptide A was linked to the γ chain, glycopeptide B to the α(A) chain and glycopeptide C to the β(B) chain.[307] Amino acid sequence analysis has shown that the glycopeptide of the γ chain (glycopeptide A) is linked to the asparagine residue in the 52nd position.[310]

The Clotting of Fibrinogen by Thrombin

A Two-Step Procedure

Laki and Mommaerts[311] and Mommaerts[312] reported in 1945 that the addition of thrombin to fibrinogen at acid pH values (5.1) does not cause clotting. The thrombin treated fibrinogen, however, clots after neutralization. These investigators suggested that the formation of fibrin must take place in two steps. The primary step involves the action of thrombin on fibrinogen and can take place at pH values which are both on the alkaline and on the acid side of the isoelectric point of fibrinogen. The second step, however, the polymerization, can occur only when the fibrinogen is negatively charged. On the basis of these and later findings that thrombin shifts the isoelectric point of fibrinogen to a more alkaline pH value[313] and that iodinated fibrinogen does not polymerize (it was proven later that it clots very slowly) although its isoelectric point is shifted by the action of thrombin[314] Laki postulated[315] that thrombin takes part only in the first step and that its role is to modify the fibrinogen, probably by splitting a peptide bond and removing an acidic group. The altered fibrinogen then polymerizes during the second step independently of thrombin. The correctness of this hypothesis was proven very shortly. In 1950 Lorand[316] demonstrated that fibrin dissolved in urea can be reclotted if the urea is removed by dialysis. Solution, dialysis, and reclotting of fibrin can be repeated many times. Using large volumes of urea solution for the dissolution of fibrin, thrombin can be eliminated and yet the fibrin reclots each time the urea is replaced by a suitable solvent.

Limited Proteolysis

In 1951 Bailey and Bettelheim and Lorand and Middlebrook[317] discovered independently that the action of purified thrombin on purified bovine fibrinogen results invariably in the disappearance of *N*-terminal glutamic acid and the appearance of *N*-terminal glycine (fluorodinitrobenzene method of Sanger). These results clearly indicated the proteolytic nature of the action of thrombin on fibrinogen and were soon supported by other findings: Lorand[318] and Laki[319] during the same year isolated peptide material from clot super-

natants by paper chromatography and paper electrophoresis, respectively. The liberated material was approximately 3% of the weight of fibrinogen[318,320] and was separated into two peptides by paper electrophoresis at pH 4.15 by Bettelheim and Bailey.[321] The peptide with the higher mobility, peptide A, had glutamic acid at the *N* terminal position but the slower moving peptide, peptide B, did not react with fluorodinitrobenzene. The authors determined the amino acid composition of each peptide and calculated that two moles of peptide A and two moles of peptide B were released per mole of fibrinogen.

In 1959 Folk et al.[322] demonstrated that carboxypeptidase B liberates arginine from the C terminal of each fibrinopeptide. It thus became apparent that the action of thrombin on fibrinogen involved the hydrolysis of four arginine-glycine bonds in the vicinity of the *N*-terminal portion of the molecule with the arginine residues acting as the carboxyl donors and the glycine residues as the amine donors.

In 1968 Blombäck et al.[292,293] found that thrombin can also split – to a limited extent – a third pair of arginine-glycine bonds in the *a* chains of human fibrinogen removing a tripeptide after polymerization has occurred.

Nature of the Enzyme-Substrate Binding

By analogy to the pancreatic proteinases it has been suggested that the cleavage of the fibrinopeptides by thrombin is accomplished by an intermediary acylation reaction between each susceptible arginine of the substrate and a reactive serine residue of the enzyme. According to Lorand,[140] the acylation reaction can be depicted in the following way:

$$\mathrm{RCOY + HOE = (RCOY \cdots HOE)} \xrightarrow{\text{acylation}} \mathrm{RCOOE + HY} \xrightarrow{\mathrm{H_2O,\ deacylation}} \mathrm{RCOOH + HOE}$$

where RCOY = fibrinogen, HOE = free thrombin, RCOOE = acylthrombin intermediate, i.e., combination of the active serine of thrombin with the susceptible arginine of fibrinogen to form arginyl-O-serine; HY =fibrin monomer, RCOOH = fibrinopeptide.

The acylenzyme is stable in acid but is easily hydrolyzed in alkaline pH values.[140]

The scheme describes the reaction of one thrombin molecule with fibrinogen at one of the four fibrinopeptide loci. At sufficiently high enzyme concentrations, two thrombin molecules per mole of fibrinogen would attack at these loci simultaneously. Since peptide B is released at a slower rate, this peptide would probably be removed by subsequent attacks.[140]

Release of the Fibrinopeptides

The hydrolytic cleavage of fibinopeptide A is a first order reaction as far as both thrombin and fibrinogen are concerned.[272,324,351] The Michaelis constant for this reaction has been found to be 1.2×10^{-5}, the optimum ionic strength about 0.1, the optimum temperature 37°C and the optimum pH 8.5 to 9.0.[272]

For the appearance of a clot the splitting of the two types of fibrinopeptides is not equivalent. While thrombin, as already stated, splits both fibrinopeptide A and B, reptilase and other snake venoms split only fibrinopeptide A[324] and yet polymerization still takes place.[324,325] The polymerization pattern of reptilase-fibrin, however, is different from that when thrombin is used to hydrolyze the fibrinogen. Light-scattering studies of the two types of fibrin in urea solution (1 M) have shown that the molecular weight to length ratio is almost double for thrombin-fibrin as compared with reptilase-fibrin (1.8 and 1.1, respectively). Furthermore, the viscosity of the two fibrins in urea solution is different.[303,326] From these studies it can be concluded that the splitting of fibrinopeptide A is important for the initiation of polymerization and that the release of peptide B is required only for later stages of the process. This conclusion is further supported by the findings of Bettelheim[327] and Blombäck and Vestermark[328] that thrombin splits earlier and faster fibrinopeptide A from bovine fibrinogen than fibrinopeptide B. The release of the A peptide runs parallel with the formation of fibrin, whereas peptide B is released at a maximum rate when the formation of fibrin is almost completed. Faster release of fibrinopeptide A has also been observed from rabbit,[329] human,[330,272] and horse fibrinogen.[272] In contrast lamprey thrombin removes both peptides from lamprey fibrinogen at about the same rate.[331]

An interesting observation made by Abildgaard[330] is that the extent to which fibrinopeptide A has to be split from fibrinogen in order

to initiate the formation of a clot depends on the milieu of the fibrinogen. While for the formation of a clot from purified fibrinogen 25 to 40% of the fibrinopeptide A must be split, only 3 to 10% of it is required when the fibrinogen is in the plasma. It appears that the plasma proteins act as nonspecific colloids.[330]

Heparin in the presence of its co-factor can inhibit the splitting of fibrinopeptide A completely. In the absence of the co-factor the inhibition is only partial.[324,332] TAMe as a substrate for thrombin[141] is a competitive inhibitor. Interestingly, the K_m for the esterolytic activity of thrombin on TAMe, calculated by Seegers et al.,[131] is 2.97 x 10^{-4} M, i.e., it is greater than the K_m for fibrinogen. The affinity, therefore, of thrombin for fibrinogen must be greater than its affinity for TAMe. The inhibition of the clotting of fibrinogen by TAMe can then be explained as the result of the great discrepancy in the molar concentrations of the two substrates. Since TAMe is a much smaller molecule, it is usually used at more than 1,000 times higher concentrations than fibrinogen.

The Polymerization

Flow birefringence,[333,334] light-scattering,[335,336] and sedimentation diffusion[337] studies of fibrin obtained from purified fibrinogen have shown that the first intermediate polymers which are formed have a length of approximately 2,500 to 6,000 Å and an average width equal to about twice the width of the fibrinogen molecule. Studies with the electron microscope have given similar results: a length of about 1,000 to 5,000 Å and a width of 50 to 100 Å (or a height twice the height of the fibrinogen molecule[338-340]). 338–340).

Viewed under the electron microscope, fibrin appears like a tangle of fibrils with characteristic cross striation.[339-342] When the preparations are stained with phosphotungstic acid they show regular light and dark cross bands perpendicular to the fibrin axis with a periodicity of 230 to 250 Å.[285,339,342] The same periodicity was revealed by low angle x-ray diffraction studies which showed four orders of a 226 ± 3 Å repeat similar to those of fibrinogen.[343]

Based on physicochemical data Ferry et al.[335,344] suggested that the intermediate polymers are formed by staggered overlapping of fibrin monomers so that the mass per unit length is doubled as compared to the mass of the fibrinogen molecule. The individual polymer chains then aggregate side by side and form the fibrin fibers. A staggered arrangement of the fibrin monomer units has also been suggested by Stryer et al.[343] on the basis of their x-ray diffraction studies. The same type of arrangement is also favored by Bang.[340] Based on his electron microscope observations of the early phases of polymerization, Bang suggested that the intermediate polymers are formed through staggered overlapping of monomeric fibrin molecules in a stepladder fashion. Ferry and co-workers favored the flat overlapping model.[344]

In contradistinction to the staggered overlapping theory, Hall and Slayter[339] and Hall[285] on the basis of their electron microscope studies postulated that the polymerization occurs first by an end to end and then by a side to side association of the monomers in an orderly fashion. During polymerization a shortening of the molecule occurs and its length approaches the length of fibrinogen at its isoelectric point. Because of this shrinking, the mass per unit length seems to double. These authors suggested that the dark striations which are observed in the stained preparations are made by the juxtaposition of two terminal nodules (diameter 65 Å) of two consecutive fibrin molecules, while the thin striations are made by the single central nodules which have a smaller diameter (50 Å).

The main advantage of the theory of staggered overlapping is that there is no need to postulate a shortening of the fibrinogen molecule, a change which is not supported by the physicochemical data. The viscosity, sedimentation coefficient, flow birefringence, light-scattering properties, optical rotation, optical rotatory dispersion, and the x-ray diffraction patterns of fibrinogen and fibrin have been found to be almost identical[336,337,343-349] indicating that there is no fundamental change in the molecular plan of fibrinogen during fibrin formation.[348] The only physicochemical difference which has been detected is that the isoelectric point of fibrin is 0.1 pH unit higher than that of fibrinogen (5.6 vs 5.5).[313]

The nature of the bonds which hold the fibrin monomers together initially has not been clarified yet. It is certain that the linking is of a noncovalent nature. It can be reversed by concentrated urea[313-316] or dilute acid or alkali[350] solutions. It has been suggested that it is electrostatic.[344]

The covalent linking of fibrin monomers, which is necessary for effective hemostasis and wound healing, is effected by factor XIII,[350,352] a proenzyme, after it is activated by thrombin.[353,507] The covalent bond produced is the isopeptide γ-glutaminyl-ε-lysyl,[354-356] which links the γ chains of fibrin in pairs and the α chains in polymers.[357]

Dissociation of Proteolysis and Polymerization

Fibrinogen can be modified to the extent that it loses its ability to polymerize without losing its function as a substrate for the proteolytic activity of thrombin. Acetylation,[358] iodination,[314,359] sulfitolysis,[287,289] or cleavage of fibrinogen with cyanogen bromide[292,293] do not prevent the splitting of the fibrinopeptides. However, the coagulability of the resulting fibrin monomers is reduced or completely inhibited. Additional evidence in favor of the relative independence of polymerization from proteolysis is the divergence of their respective optimal pH values. Polymerization is favored by an acidic pH (6 to 7[630]) while the optimum pH for the cleavage of the fibrinopeptides is 8.5 to 9.0.[272]

Role of the Carbohydrate Moiety

Although the glycopeptides are not directly involved in the proteolysis of fibrinogen by thrombin or in the transformation of soluble into insoluble fibrin by factor XIII,[307] they nevertheless seem to affect clotting. Laki and Mester[361] oxidized selectively the carbohydrates of fibrinogen with periodate and found that the molecule became refractory to the proteolytic action of thrombin. Interestingly, the removal of sialic acid with neuramidase increased the clottability of fibrinogen by thrombin, most likely by accelerating the polymerization process.[362,363] These observations were amplified by Mester and Szabados,[364] who observed that in congenital and acquired dysfibrinogenemias the ratio of sialic acid to hexosamine is increased. They found that the thrombin clotting time of the fibrinogen becomes longer as the relative concentration of sialic acid goes up (see, however, p. 40, acquired dysfibrinogenémies).

The Fibrinopeptides

The first fibrinopeptides which were isolated and characterized were of bovine origin. The complete amino acid sequence of the A fibrinopeptide was elucidated in 1959[294,295] and the sequence of peptide B in 1960[296,297] independently in the U.S. and Sweden. Bovine peptide A has 19 amino aicd residues and a molecular weight of 2,000 (sedimentation diffusion and amino acid analysis). The homologous peptide B has 21 residues and a weight of 2,400.[365] The sequences which were worked out by the two laboratories (phenylthiohydantoin method of Edman) were in perfect agreement except for an amino group at position 12 (counting from the C terminal arginine) and the sequence of the *N* terminal (final) tetra-peptide of peptide B. Blombäck and Doolittle[298] concluded that the *N* terminal of bovine fibrinopeptide B was pyroglutamic acid, while Folk and Gladner[296] maintained that this was acetyl threonine.

The amino acid sequence of fibrinopeptide A from 41 animal species and the sequence of peptide B from 30 species have been elucidated.[299,300,366] Blombäck and his group have found that in a given species only one main type of fibrinopeptide A or B is present with one exception.[300] In a hybrid such as the mule exactly equal amounts of horse and donkey chains were found. The number of residues of fibrinopeptide A in the mammals varies from 15 (marsupials) to 19 (artiodactyls) and the number of residues of fibrinopeptide B from 9 (macaques) to 21 (artiodactyls). All fibrinopeptides have arginine at the C terminal position but all show a preponderance of negative charges, especially the B peptides. The acidic residues are located almost exclusively at the *N* terminal half of the chain, while the positive residues are located at the C terminal half.[299,300,366] Comparison of the sequences of the fibrinopeptides of the various species indicated that the residues which are located close to the C terminal position of the A peptides remained considerably more stable during mammalian evolution than the residues close to the *N* terminal portion of the chain.[272,300,371] The changes in the sequence of fibrinopeptide B were greater. Generally, similarity in sequence seems to be proportional to phylogenetic affinity.[300] Based on sequence data Dayhoff and McLaughlin[367] have constructed a phylogenetic tree of the species studied.

Human fibrinopeptide A has been found to be heterogeneous.[291,293,302] Seventy percent of it is made of 16 amino acid residues, which have alanine at the *N* terminal position (fibrinopeptide A), 10% is made of 15 residues with aspartic acid

(the next in line after alanine) at the *N*-terminal position (fibrinopeptide AY), and 20% have 16 residues but differ from fibrinopeptide A in that the serine at location number 3 counting from the *N*-terminal alanine is phosphorylated (fibrinopeptide AP). Human fibrinopeptide B has 14 residues.

Each tripeptide contains glycine, proline, and arginine, which occupy positions 17, 18, and 19 in the α (A) chain, respectively. The cleavage of the tripeptide takes place at a slower rate than the removal of fibrinopeptide B.[292,293,302]

The fibrinopeptides are able to inhibit the proteolytic action of thrombin on fibrinogen in a competitive manner;[368,369] fibrinopeptide B has been shown to increase the sensitivity of vascular smooth muscles to the action of bradykinin and to electric stimulation.[370]

Binding Sites

As mentioned earlier, thrombin can remove the fibrinopeptides not only from intact fibrinogen but also from fibrinogen denatured and degraded by cyanogen bromide (*N* terminal disulfide knot)[292,293] and from the isolated α (A) and β (B) chains as well.[287,289] This suggests that specific structures in the sequence of these chains are primarily responsible for the binding of the enzyme.[300] Carboxymethylation of the cysteine residues at positions 28, 36, 45, and 49 together with condensation of the tryptophan residues at positions 33 and 41 in the α (A) chains of fibrinogen with Koshland's reagent did not change to any appreciable extent the specificity of thrombin for fibrinogen.[300] These results suggested that a specific binding site resides in the structure preceding the no. 28 residue. Blombäck pointed out that since fibrinopeptide A inhibits the action of thrombin on fibrinogen in a competitive way[368,369] some of the residues involved in the binding must be located in this peptide. The attention of the Swedish investigators had long been attracted to the C-terminal portion of the peptide and particularly to the phenylalanine residue at position 9 because this portion of the fibrinopeptide has remained remarkably stable through the phylogenetic evolution.[371, 272,300] Blombäck et al.[300,372] synthesized a series of peptides in which phenylalanine was incorporated at increasing distances from an esterified arginine residue. These peptides inhibited the clotting activity of thrombin. The most pronounced inhibition was found with the nonapeptide.

Further support for Blombäck's hypothesis about the significance of phenylalanine at position 9 came from studies with secretin. One of the arginyl bonds of this gastrointestinal hormone is susceptible to the action of thrombin and a phenylalanine residue is located 9 residues away from this bond. In further similarity with the structure of fibrinopeptide A, secretin has a glutamic acid residue at a distance of six residues from the thrombin susceptible arginyl bond.[373,374]

Comparison of fingerprints of tryptic digests of the α (A) chains from human, ox, rabbit, sheep, and horse fibrinogen indicated that three ninhydrin positive spots were identical in all the chains.[300] These corresponded to residues 17 to 19 (gly-pro-arg), 41 to 50, (trp-asn-tyr-lys-cys-pro-ser-gly-cys), and residue 51 (met) of the human α (A) chain. As mentioned earlier, the residues 17 to 19 form the tripeptide which is cleaved slowly from human fibrinogen after the removal of the A and B peptides.[292,293] The persistence of these structures in the various fibrinogens may indicate that these sequences are somehow important for the function of fibrinogen. Characteristically, substitution of the arginine in position 19 by serine in fibrinogen Detroit resulted in the inability of this fibrinogen to polymerize, although the release of fibrinopeptide A from this chain is normal.[375] The release of fibrinopeptide B, however, is inhibited.[300,376]

Heterogeneity of Fibrinogen

Fractions I-4 and I-8

On the basis of solubility studies, fibrinogen has been divided into several fractions ranging at one extreme from fibrinogen, which precipitates spontaneously upon standing in the cold, to that which is soluble even in the presence of saturated glycine solutions.[377] By analogy to the nomenclature of Cohn, the higher the number assigned to a given fibrinogen fraction, the higher the solubility of that fraction. Thus, Cohn Fraction I can be subdivided into fractions I-1 to I-4; fraction I-4 constitutes the bulk of the fibrinogen of plasma. Fibrinogen of higher solubility than that of fraction I-4 can be isolated from plasma by glycine precipitation and be further fractionated to yield fractions I-5 to I-9. The two main fractions I-4 and I-8 have been found to have

molecular weights of 325,000 and 269,000, respectively.[377,378] Each of these fractions can be further fractionated by DEAE-cellulose chromatography into two additional subfractions. Fractions I-4 and I-8[378] have been found to have the same *N*-terminal amino acids and UV spectral curves (254 to 330 nm), and thrombin cleaves trichloracetic acid soluble peptides at the same rate per micromole from both fractions. The speed, however, at which each of the fractions forms a clot is considerably different; fraction I-4 clots faster than fraction I-8. Since fibrin monomer obtained from fraction I-4 polymerizes faster than the monomer from fraction I-8, all indications point to different speeds of polymerization as the cause of the different thrombin times.

It has been suggested that fractions I-8 and I-9 are early clottable metabolites of native fibrinogen (fraction I-4), which are produced by the action of plasmin in vivo,[379,380] although they have been found to have structural differences from plasmin digestion products.[380] Mosesson and Finlayson[381] subjected fractions I-8 and I-9 to SDS polyacrylamide gel electrophoresis before and after reduction of the disulfide bonds with mercaptoethanol. They found that none of the α (A) chains of fraction I-9 and very few of the α (A) chains of fractions I-8 were intact. These investigators isolated eight remnants of the α (A) chains with molecular weights ranging from 27,000 to 72,000 or 75,000 (the last figures represent the molecular weight of the native α (A) chains by SDS polyacrylamide gel electrophoresis). They identified the fragments by terminal amino acid analysis and fingerprint patterns and concluded that fractions I-8 and I-9 are formed from fraction I-4 by hydrolysis at several sites along the C-terminal portion of the α (A) chain.

Mills and Karpatkin,[382] also using SDS polyacrylamide gel electrophoresis, found that the patterns of clots obtained from fibrinogen of high solubility, both before and after reduction with mercaptoethanol, closely resembled the patterns of clots of the initial degradation products of fibrinogen I-4 by thrombin. They found no resemblance with plasmin degradation products and concluded that the fibrinogens of high solubility cannot be products of degradation of fibrinogen I-4 by plasmin but most likely are the products of its degradation by an enzyme identical or similar to thrombin. Thrombin, however, first removes the fibrinopeptides, and fractions I-8 and I-9 still have their fibrinopeptides intact.[378]

Cryofibrinogen

Shainoff and Page[383] and later Copley and Luchini[384] showed that the clotting of fibrinogen digested by reptilase or digested partially by thrombin can be prevented by intact fibrinogen or by fibrinogen from which only a fraction of the A fibrinopeptides has been cleaved. Such mixtures form intermediate polymers, which are soluble at room temperature and at 37°C and constitute what is usually called cryofibrinogen. This means that the polymers can further aggregate in the cold to form precipitates which depolymerize and disperse when they are heated to 37°C.

Abnormal Fibrinogens (Dysfibrinogenemias)

Congenital Dysfibrinogenemias

During the last 14 years several cases of congenital abnormalities concerning the function of fibrinogen have been reported.[385-399] In an analogy to the hemoglobinopathies, the abnormal fibrinogens have been named after the city where they were first described.[387] The following abnormal fibrinogens have been reported: fibrinogen Parma,[385] Paris I,[386] Baltimore,[387] Zurich,[388] Cleveland,[389] Detroit,[390] Paris II,[391] St. Louis,[392] Oklahoma,[393] Los Angeles,[394] Louvain,[395] Bethesda I,[396] Bethesda II,[397] Giessen,[398] Montreal,[399] and possibly fibrinogen Vancouver.[390] In all cases but two,[385,392] an autosomal dominant mode of inheritance was established. In the first of the two exceptions no other individual of the same family was found to be affected. In the second[392] the inheritance had not been investigated at the time of publication.

The abnormality is characterized by the synthesis of fibrinogen of poor clottability which, with the exception of fibrinogen Parma and Baltimore, is capable of inhibiting the clotting of normal plasma or normal fibrinogen by thrombin or reptilase. In one half of the cases,[386,388,389,391,394,397,399] the patients had no bleeding tendency and the abnormality was discovered accidentally by the laboratory. Five of the other patients exhibited a hemorrhagic diathesis which varied from mild[387,396] to severe[385,390,398] and which could be attributed to the abnormal fibrinogen unequivocally. The remaining two patients (with fibrinogen Louvain[395] and St.

Louis[392]) had bleeding episodes due to unrelated causes (endometriosis and hemophilia A, respectively) and it is difficult to assess whether or not the fibrinogen abnormality had contributed to the bleeding to any extent. It appears paradoxical but the patient with fibrinogen Baltimore suffered from recurrent thromboses and pulmonary embolism in spite of his bleeding diathesis.[381,400] The patient with fibrinogen Detroit and her half sister were found to be homozygotes by amino acid sequence analysis.[376] In most of the other cases heterozygocity was indicated by immunoelectrophoresis,[389,396] DEAE-cellulose chromatography,[391,403] susceptibility to the action of reptilase,[402] and rate of release of fibrinopeptides.[396,405]

Coagulation Abnormalities

Findings characteristic of the abnormality in the order of frequency were prolongation of the thrombin, reptilase, and arvin times, prolongation of the one-stage prothrombin and partial thromboplastin times, abnormalities of the thromboelastogram (prolongation of r and k, decreased maximum amplitude;[385,386,389,390,405] and less frequently prolongation of the recalcification[385,386,390] and clotting times.[385,386,389-391] Excess thrombin normalized the prolonged thrombin time except in the cases of fibrinogen Los Angeles and fibrinogen Bethesda II.[394,397] All types of abnormal fibrinogen showed defective polymerization of the fibrin monomers with the exception of fibrinogen Bethesda I.[396] The defect in polymerization was characterized by a delay in the onset[388,397,398] and/or a slow rate of polymerization[388,391,397] or even complete inability of the monomers to polymerize and form a visible clot.[385,389,405] The clots were less opaque than normal[401] and showed abnormalities in the thickness and length of the fibrin network.[389,394,398,401] Ultrathin sections of clots from Paris I plasma showed complete disorganization without any fiber structure whatsoever. These clots had a jelly consistency.[401] In most of the cases, however, the abnormality of polymerization was less severe and in the case of fibrinogen Paris II the fibrin appeared normal under the electron microscope.[391]

Although the clotting of normal fibrinogen and normal plasma by thrombin was inhibited by the plasma of patients with fibrinogen Paris I[386] and Paris II,[391] the polymerization of purified fibrin monomers from normal plasma was not inhibited by these abnormal plasmas. Similarly, purified fibrin monomers of fibrinogen Cleveland did not inhibit the polymerization of normal monomers.[389] Monomers from fibrinogen Zurich, however, were inhibitory.[402]

Physicochemical Properties

The fibrinogen concentration in the dysfibrinogenemias was found normal when precipitation or immunological methods were used; it appeared low, however, whenever it was determined as thrombin clottable protein. Most of the abnormal fibrinogens were undistinguishable from normal fibrinogen by ultracentrifugation,[389,390,392,396,397] electrophoresis on paper,[385,386,388,400] cellulose acetate,[390,392,396,397] polyacrylamide gel,[396,397] and agar;[389] by chromatography on DEAE-cellulose,[391,392,396-398] and by immunodiffusion.[386,388,389,400] Minor but reproducible differences from normal fibrinogen were, however, shown by immunoelectrophoresis for most of the abnormal fibrinogens[389,390,394,400] and also between fibrinogens Baltimore, Paris I, Detroit, and Cleveland.[390,400] There were, however, deviations from this general behavior which contributed to the differentiation of each disorder: DEAE-cellulose chromatography of fibrinogen Baltimore revealed that the first peak was eluted later and was broader but more symmetrical than the respective peak of normal fibrinogen.[403] In sharp contrast, the first peak of fibrinogen Paris I was narrower than normal. Both peaks of the latter fibrinogen were eluted from the column later than their normal counterparts.[404] Fibrinogen St. Louis[392] showed the same immunoelectrophoretic behavior as normal fibrinogen but could be distinguished from it by immunodiffusion (spur formation). Fibrinogen Bethesda II exhibited an increased anodal mobility in agar.[397] A sedimentation constant of 8.13S (vs 7.85 for normal fibrinogen) was found for fibrinogen Baltimore. Molecular sieving experiments in acrylamide gels, however, indicated a molecular weight of 325,000 which was the same as that found for normal fibrinogen and suggested that the high sedimentation coefficient most likely was the result of aggregate formation.[403] The rate of the release of fibrinopeptides A and AP from fibrinogen Baltimore was slower than normal and the proportion of phosphorylated relative to the nonphosphorylated fibrinopeptide was high.[405]

The rate of the release of fibrinopeptides from fibrinogen Bethesda I was also slower than normal, especially for peptide A; the aggregation of fibrin monomers, however, was normal.[396] The lack of a polymerization abnormality distinguishes this fibrinogen from all the others. The patient had a mild bleeding diathesis. The rate of the release of fibrinopeptides from fibrinogen Bethesda II was only slightly delayed but the aggregation of the monomers was abnormal. This patient had no bleeding tendency.[397]

The survival time of normal fibrinogen infused into patients with fibrinogen Parma,[385] Baltimore[387] and Bethesda I[396] was found normal; it was, however, shortened in the patient with fibrinogen Bethesda II.[397] The survival of the abnormal fibrinogens Bethesda I and Bethesda II in the respective patients did not differ from the survival of normal fibrinogen.[396,397]

Identification of the Defect at the Molecular Level

Only in the case of fibrinogen Detroit has a defect been elucidated at the molecular level. Blombäck et al.[375] showed that the arginine in position 19 of the α(A) chains is substituted by serine. The rate of release of fibrinopeptides A and AP by thrombin from this fibrinogen was found normal, but fibrinopeptide B was not released.[376] Since the release of only fibrinopeptide A is sufficient to enable normal fibrinogen to clot, Blombäck and Blombäck have suggested that the substitution of the arginine by serine may have resulted in considerable conformational changes in the *N*-terminal disulfide knot, which in turn affected directly or indirectly the polymerization.[376] Mammen et al.[390] have shown that the concentration of lysine, arginine, valine, hexose (orcinol method), hexosamine, galactosamine, and sialic acid was lower in fibrinogen Detroit than in normal fibrinogen, while the concentration of leucine was higher. These findings suggest that more abnormalities in the sequence of this fibrinogen should be anticipated.

Fingerprint analysis of the α(A) chain fragments of the disulfide knot of fibrinogens Paris I and II, Cleveland, Zurich, Baltimore, and Louvain has shown no abnormalities.[376]

The molecular defect of fibrinogen Oklahoma has been uncovered only partially. Polyacrylamide gel electrophoresis of sulfitolyzed samples showed that in addition to the α(A), β(B), and γ chains this fibrinogen has a fourth chain with a faster mobility. Two-dimensional paper electrophoresis-chromatography of tryptic digests of the isolated chains revealed the lack of two basic peptides and the presence of an extra acidic peptide. Cyanomethylation of whole plasma showed that fibrin from this fibrinogen contained a greater ratio of ϵ (γ-glutamyl) lysine than the fibrin from normal fibrinogen.[393]

Acquired Dysfibrinogenemias

In addition to the congenital types of dysfibrinogenemia, cases of acquired fibrinogen abnormalities have been described.[406-409] These cases were associated with advanced cirrhosis[409] and other severe liver diseases (hepatoma,[407] severe viral hepatitis[406]). The laboratory findings due to the fibrinogen abnormality were similar to those of the congenital type. All the reported cases were characterized by abnormal polymerization of the fibrin monomers. Unlike the congenital types, however, none of the patients showed a bleeding tendency which could be attributed to the acquired fibrinogen abnormality itself.

Recently an intermittent type of a nonhereditary dysfibrinogenemia was described in an apparently normal white male with a long history of rebleeding of wounds after initial hemostasis.[410] Most of the fibrinogen of this patient could not form fibrin monomer by the addition of thrombin, indicating that the release of fibrinopeptide A was inhibited. Inheritance of the abnormal fibrinogen could not be established.

Mester and Szabados[364] determined the carbohydrate content in two cases of acquired dysfibrinogenemia and found that the concentration of sialic acid was increased (0.96% and 1.21% vs 0.54% for normal fibrinogen), while the concentration of hexosamine remained normal. They postulated that the increased sialic acid/hexosamine ratio was characteristic of dysfibrinogenemias including the congenital forms, fibrinogen Paris I, Baltimore, and Detroit. However, as it was mentioned earlier, the sialic acid content of fibrinogen Detroit was found to be lower, not higher, than normal.[390] Since the concentration of hexosamine was also decreased in this fibrinogen, the ratio of the acid to the amine was very much the same as in normal fibrinogen according to the results reported by Mammen et al.[390] Mosesson and Beck[403] and Gralnick et al.,[396,397] on the other hand, have shown that fibrinogen Baltimore and fibrinogens Bethesda I and II have normal

sialic acid contents. It appears, therefore, that there are no general rules concerning the sialic acid content of abnormal fibrinogens; it can be higher, lower, or in the normal range.

THE BREAKDOWN PRODUCTS OF FIBRINOGEN AND FIBRIN

Tillett and Garner reported in 1933[411] that when filtrates of human strains of hemolytic streptococci were added to human clots, the clots lysed quickly. The following year these investigators[412,413] proceeded to isolate the active principle, which they called "streptococcal fibrinolysin" (what we now call streptokinase) and showed that it could also attack fibrinogen, rendering it uncoagulable by thrombin.

Seegers, Nieft, and Vandenbelt[279] observed in 1945 that bovine fibrinogen and fibrin left at room temperature under sterile conditions for several days undergo the same changes, due presumably to the activation of the fibrinolytic system. From such solutions Seegers et al. isolated two of the final products.

Systematic study of the breakdown products of fibrinogen and fibrin, however, was undertaken only after it was discovered that these products have anticoagulant properties. Triantaphyllopoulos et al.[414] reported in 1955 that incubation prolongs the thrombin time of plasma and that addition of the incubated plasma to fresh plasma appreciably prolongs the thrombin time of the latter. In 1957 and 1958 Triantaphyllopoulos[415,416] and independently Stormorken[417] demonstrated that the anticoagulant effect is due to degradation of the fibrinogen of the plasma during the incubation. At about the same time Niewiarowski and Kowalski[418,419] found that an antithrombin-like anticoagulant develops when plasmin or streptokinase is injected into cats or when added to plasma or to purified fibrinogen or fibrin in vitro. Since the occurrence of fibrinolytic activity in the blood under physiological (stress, exercise, fear) or pathological (abruptio placentae, amniotic fluid embolism, liver disease, prostatic carcinoma, shock, injections of adrenalin, etc.) conditions[420] had by then been recognized and since the treatment of thrombosis with injections of streptokinase had already been initiated, the importance of these findings was quickly appreciated and intensive research on the breakdown of fibrinogen and fibrin was undertaken in many laboratories.

Fragmentation of the Fibrinogen Molecule

Although trypsin, chymotrypsin, papain, and thrombin[153,154,382,421-427] have been shown to attack fibrinogen and fibrin, most of the studies on the breakdown products of fibrinogen have been concerned with the derivatives which are produced by the action of plasmin. This is obviously due to the fact that the plasminolytic digestion of fibrinogen occurs much more frequently in human pathology than its digestion by the other enzymes.

Fragmentation by Plasmin

Plasmin has been added to fibrinogen either as the active enzyme or has been formed *in situ* from the proenzymic state by the addition of an activator (streptokinase or urokinase) or spontaneously by incubation.

It is known that plasmin, like trypsin and thrombin, splits arginyl and lysyl bonds.[428,429] However, not all of these bonds in the fibrinogen molecule are attacked by plasmin. Garner and Tillett in 1943[413] found that in contrast to the digestion by trypsin and streptococcal streptase, the fibrinolytic degradation of fibrinogen was limited and that the end products still had the characteristics of proteins. Unlike fibrinogen, however, the fragments were no longer precipitable at 50% saturation with sodium chloride or at 25% saturation with ammonium sulfate. They required a 35 to 50% saturation with the latter salt to be precipitated and their thermosensitivity was much lower than that of fibrinogen.

Holmberg in 1944[430] subjected completely lysed fibrin to ultracentrifugation and observed two peaks with sedimentation constants of 5.54 and 4.95, respectively. He could not separate the components, however. He determined the diffusion coefficient of the mixture and calculated an average molecular weight of 100,000.

A year later Seegers, Nieft, and Vandenbelt,[279] using free boundary electrophoresis, detected similarly two final products in solutions of completely lysed fibrinogen and fibrin which they called the α- and β-fibrin or fibrinogen derivatives. These investigators found that the α-derivative had the same mobility as intact fibrinogen (I.P., 5.5), was heat labile (55°C for 30 min) but needed a 35 to 50% ammonium sulfate saturation to be pre-

cipitated from solutions. The β-derivative was heat stable, more electronegative (I.P., 4.2), and could be partially purified by isoelectric precipitation after removal of the α-derivative by heat. Seegers et al. found no difference between the respective fibinogen and fibrin derivatives by these criteria.

Two additional macromolecular fragments were detected as final products by ultracentrifugation in 1955,[431] and in 1957 and 1958 three different laboratories reported that non-protein nitrogen (NPN) and TCA soluble tyrosine were also released during the degradation.[419,429,432]

Kowalski et al.[433] in 1960, using fibrinogen labeled with ^{131}I and paper electrophoresis, found that the degradation proceeded through a stage of intermediates and that at the end fragments with the mobilities of α-, β-, and γ-globulins were produced. Nussenzweig et al.[434,435] also noted the presence of intermediates; they concentrated their studies, however, on the final products. Using chromatography on DEAE-cellulose, they detected five peaks which they separated into four fractions: A, B-C, D, and E. Components D and E corresponded to the α- and β-derivatives of Seegers et al.,[279] respectively. Nussenzweig et al. found as Seegers and co-workers had found before them, that component D was thermosensitive. All other components were thermoresistant. They determined the sedimentation constants and diffusion coefficients (double diffusion in gel) of the components and calculated their molecular weights: B-C (average): 13,400, D: 83,000, and E: 35,000. Then they characterized these fragments immunologically.[435] They found that D and E reacted with antifibrinogen sera, while A and B-C did not react. Using immune antisera which had been adsorbed with either component D or E or fibrinogen, they concluded that: (a) D and E each had a different antigenic determinant which reacted with antibodies of different specificities and did not cross-react, (b) intermediate products possessed both D and E antigenic determinants and (c) fibrinogen should have at least four antigenic determinants and possibly five, two or three being lost during the degradation. The essential findings of Nussenzweig et al. have been confirmed by subsequent studies. Differences have been found by some investigators regarding the molecular weights of components D and E (see Final Products).

Interested mainly in the anticoagulant activity which one of the authors had first described in 1957,[415] the authors followed the degradation of fibrinogen thoughout the incubation using a combination of ammonium sulfate, ether, and heat (60°C for 15 min) fractionation and paper, continuous flow paper, column, cellulose acetate, and polyacrylamide gel electrophoresis.[436-443] They were the first to isolate intermediate products sequentially from the beginning until very advanced stages of fibrinogenolysis and studied their clottability and anticoagulant activity. These studies were supplemented by amino acid[277] and carbohydrate[309] analysis of fibrinogen and some of the larger derivatives, determination of TCA soluble peptides which are liberated by thrombin from them[441,444,445] and also by isolation of the acid soluble or dialyzable fragments which are formed during fibrinogenolysis.[346-348] On the basis of their results the authors concluded[436-438,440,441,446-448] that the degradation of fibrinogen by plasmin is a complicated process involving the formation of changeable clottable and unclottable intermediates and of a large number of micromolecular fragments; and that the D, E, and A, B, C fragments change with incubation.

Clottable Intermediate Derivatives

In 1965 the authors showed that the fraction which precipitates at 25% ammonium sulfate saturation from fibrinogen digests does not represent remaining intact fibrinogen as it was assumed.[437,439] Even at the very beginning when its clotting time and electrophoretic mobility were identical to that of fibrinogen, differences from the parent protein were demonstrated by the difficulty with which this fraction can be dissolved in physiological saline or 0.3 M sodium or potassium chloride.[437,441] The thermostability of this fraction increases steadily with incubation to almost 100% (human specimens) in contradistinction to that of intact fibrinogen which is only 2 to 4%.[439,441] The coagulability deteriorates progressively and finally disappears and at the same time the fraction becomes able to prolong the thrombin time of intact fibrinogen.[439,441] Cellulose acetate electrophoresis could not differentiate early samples of this fraction from fibrinogen but samples isolated at the end of the clottable period formed diffuse bands on the electrophorogram, showing increased mobility towards the anode[441] (Figure 2, upper half, second to fifth patterns). Column electro-

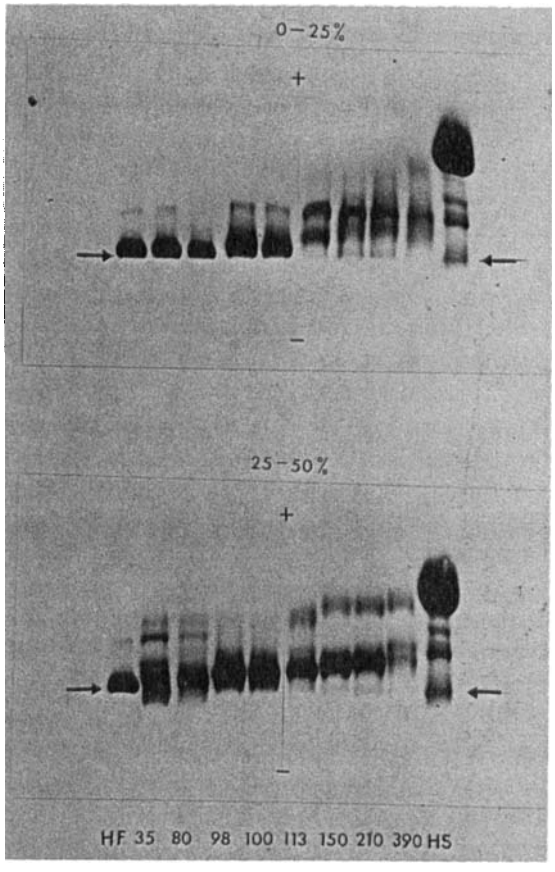


FIGURE 2. Gelatinized cellulose acetate electrophoretic patterns of the two ammonium sulfate saturation fractions obtained from a human fibrinogen solution at different stages of incubation. A sample of human serum is included as reference (0.25 M-tris buffer, pH 8.9, 170 V for 45 minutes). Arrows indicate the points where the samples were applied, the plus and minus signs the direction of the current and the numbers at the bottom the percentage of the clottable period (CP). HF, human fibrinogen; HS, human serum. The concentration of the fractions was adjusted to 24 mg/ml and 0.002 ml aliquots were applied. Human serum was used undiluted. (From reference 441.)

phoresis on cellulose of the same fractions separated the protein into two main, closely associated components and two others in trace amounts.[441] Taking into consideration that by the end of the clottable period, 20% of the original protein becomes nonprecipitable by 50% ammonium sulfate saturation[437] and that this protein contains a large number of acid soluble fragments,[446,447] it must be concluded that at the beginning plasmin removes from fibrinogen small fragments with a preponderance of free positive charges. As a result, successively smaller clottable derivatives are formed which differ from each other in charge and weight only slightly so that conventional methods of isolation and molecular weight determination cannot differentiate each one of the derivatives from the others with the exception of one or two which must have resulted from the removal of relatively large fragment(s). This conclusion is further supported by the results of other investigators. Fletcher et al.,[449] using ultracentrifugation and acrylamide gel electrophoresis, distinguished a "first fibrinogen derivative" with a molecular weight of 265,000 and a mixture of intermediates with sedimentation constants of approximately 5.7S. Recently Mills and Karpatkin[450] and Mills[451] subjected fibrinogen digests to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and separated the clottable products I and II. Both of these products were heterogeneous and had average molecular weights of 317,000 to 325,000 and 271,000 to 275,000, respectively (molecular weight of fibrinogen by this method 362,000 to 365,000). Figure 3 shows results obtained in our laboratory by the same method. The first pattern is the pattern of native fibrinogen. The dark bands at the top of the second pattern represent the clottable remnants of the fibrinogen molecule or clottable residues (CR). The diffuse bands in the middle and lower third correspond to proteins of molecular weight of 15,000 to 45,000 and must represent fragments which were removed from fibrinogen when the clottable residues (CR) were formed. The yield in clottable derivatives (0 to 25% fraction) during this period is 87% of the original protein.[437] Consequently, the fragments together with the acid soluble material which was formed until then (about 2%) must account for the remaining 13% of the mass of fibrinogen, i.e., for about 44,000. These calculations suggest that the diffuse bands most likely represent successive forms of degradation of one fragment only which was removed at different rates from various fibrinogen molecules. Mills[451] had detected a 44,000 single chain fragment, which was successively degraded to 24,000 and to less than 15,000 molecular weight fragments. Alternatively, each band may represent a completely different fragment but for some unknown stereochemical reason, the mobilities of these fragments in SDS polyacrylamide gels are much slower than their molecular weights would

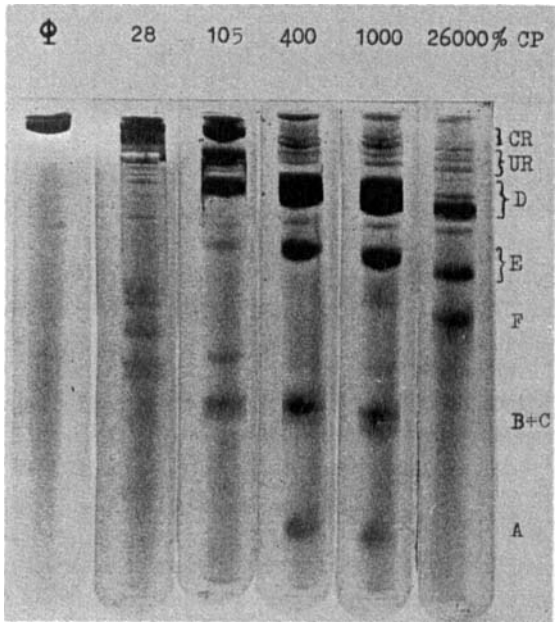


FIGURE 3. Polyacrylamide disc gel electrophoresis of samples of human fibrinogen digested by purified human fibrinolysin, at different stages of incubation. The samples at 50 μg of protein were applied on the top of 9% gels containing 0.1% sodium dodecyl sulfate and a current of 7.8 mA/tube was run for 5½ hours with the top of the gels connected to the negative pole. The first pattern was obtained with a sample of fibrinogen before the addition of fibrinolysin. The numbers on the top indicate the stage of incubation as a percentage of the clottable period. CR, clottable residues; UR, unclottable residues. (From Triantaphyllopoulos and Chandra, 1972, unpublished results.)

justify. It has been found that albumin, a single chain protein, shows a slower mobility after the reduction of S-S bonds than before this reduction. The same may occur after the cleavage of peptide bonds.[451]

A clottable fibrinogen derivative, fragment X, of molecular weight of 240,000 has been identified by Larrieu et al.[452] and by Marder et al.[453] by agar gel electrophoresis. This fragment has been isolated by gel filtration through Sephadex G-200.[453,454]

Nonclottable Intermediate Derivatives

These were first isolated in 1957 as the fibrinogen fraction or as it was called later, the Anticoagulant Fraction of Incubated Fibrinogen, or AFIF, by 25 to 50% ammonium sulfate saturation of solutions of spontaneously lysed (incubated) fibrinogen.[415,416] Later the authors isolated such fractions sequentially from the beginning until very advanced stages of digestion and fractionated them further by paper, continuous flow paper, column, and cellulose acetate electrophoresis.[437,440,441,444] The electrophoresis on cellulose acetate indicated (Figure 2, lower half, second pattern) that the first nonclottable macromolecular derivatives which are formed are several minor components.[441] These are obviously the same components which were mentioned earlier (Figure 3, diffuse bands in the middle and lower third of the second pattern) and must represent fragments which were removed early during the digestion when the clottable derivatives were formed (see previous section). As the incubation progresses a component having an electrophoretic mobility slightly greater than that of fibrinogen becomes gradually predominant among the nonclottable fibrinogen derivatives and forms diffuse bands on the electropherogram while its general mobility increases (Figure 2, lower half, fourth and fifth patterns). This component, in all probability, represents the remnants of the fibrinogen molecule after they become uncoagulable by thrombin (unclottable residues of fibrinogen = UR) and can no longer be precipitated at 25% ammonium sulfate saturation. The diffuse appearance of the bands indicates that the unclottable residues, like their predecessors (the clottable residues), are heterogeneous. On the SDS electropherograms (Figure 3, third pattern) these residues (UR) form three bands. With further digestion the unclottable residues are split into two unequal parts (Figure 2, lower half, sixth pattern). The larger of the fragments has the same electrophoretic mobility as the parent derivatives (unclottable residues) and is similarly heterogeneous: It forms a dark and two diffuse bands on this type of electrophoresis. The smaller fragment is more electronegative. As the digestion progresses both of these fragments become more electronegative and they finally acquire the mobility of the β-globulins and that of albumin and become the final D and E components of Nussenzweig et al.,[434] respectively. The electrophoresis on the SDS polyacrylamide gels (Figure 3) indicated, in addition, that (a) the transition from the clottable to the unclottable residues and the division of the latter to the D and E fragments do not occur simultaneously in all the fibrinogen molecules. There is overlapping of stages and during the second half of the clottable period all four types of derivatives may be seen (third pattern), (b)

some unclottable residues remain until the final stages, (c) the changes of the D and E fragments as the incubation progresses are significant enough to result in demonstrable lowering of the molecular weights (increased mobility) and a decrease in yield at the final stage, while a heterogeneous new fragment designated as fragment F in Figure 3 is formed, (d) the fragments which were removed from fibrinogen at the beginning (diffuse bands on the middle and lower third of the second gel) are further degraded to fragments smaller than 15,000 and are not visible at the end.

Unclottable residues and early forms of fragment D were first isolated in 1962 as the Large Peak of AFIF and early forms of fragment E as the Small Peak of AFIF by ammonium sulfate fractionation and continuous flow paper electrophoresis.[455] These fragments were studied more systematically in 1965[437] and 1966[440] when samples were isolated sequentially from the beginning until very advanced stages of digestion and their antithrombic activity (see section on "Inhibition of the Clotting of Fibrinogen by Thrombin" p. 52) and thermosensitivity were determined. The thermosensitivity of human fragments D was found to decrease as the digestion progressed from 65% to 25%. The thermosensitivity of fragments D of bovine origin on the other hand did not change much with digestion (from 83% to 77%). Fragment E in both instances was completely thermoresistant from the beginning. A nonclottable derivative, fragment Y, with molecular weight of 155,000 was isolated in 1969 by Marder et al.[453] by gel filtration through Sephadex G-200. Mills,[451] using filtration through 5% SDS polyacrylamide gels, detected a similar fragment (product III) with molecular weight of 210,000. He found, as the authors did, that fragments D and E become smaller as the digestion progresses and calculated an apparent molecular weight from 130,000 → 43,000 for fragment D (product IV) and from 67,000 → 58,000 for fragment E. The variability of fragment D has been observed also by Furlan and Beck.[454] These authors isolated fragment D from 15 min and 45 min digests. They found that the former had a molecular weight of 80,000 to 90,000, while the latter had a weight of only 46,000 to 50,000.

Final Fibrinogen Derivatives

The final fibrinogen derivatives vary in size from free amino acids to fragments of about 83,000 molecular weight and can be most conveniently divided into macromolecular and micromolecular fragments.

Macromolecular Derivatives

These are the final forms of fragments D, E, F and A, B, C. Fragments D and E are the largest of the group and have been studied more extensively than the others. They have been isolated by column chromatography on DEAE-cellulose,[434] by continuous flow paper[437,438,455] and column electrophoresis,[440] by gel filtration through Sephadex G-50,[456] G-75,[456] and by Pevikon block electrophoresis.[457] Most authors have reported values of 68,000 to 83,000 for the molecular weight of fragment D and values of 28,000 to 58,000 for the molecular weight of fragment E.[434,458-461,451] There are three reports, however, that the molecular weight of fragment D after exhaustive digestion, especially in the presence of urea, is reduced to 43,000 to 50,000.[343,451,454] The differences in the reported values are most likely due to the isolation and characterization of the fragments at varying degrees of digestion. The more complete the digestion, the smaller the molecular weight.

As mentioned earlier, fragment D is heterogeneous. Jamieson and co-workers distinguished six variants of fragment D by starch and polyacrylamide gel electrophoresis and by the isoelectric focusing technique.[461-463] Each of the variants displayed a molecular weight of approximately 80,000 (gel filtration) and all had the same antigenic specificity. Treatment with neuraminidase did not alter the patterns indicating that the differences in mobility were not due to the partial removal of neuraminic acid from the glycoprotein structure. Similar findings were reported by Nilehn, who separated fragment D into nine fractions by chromatography on DEAE-cellulose and prolonged electrophoresis in agarose gel.[459]

Fragment F was identified only recently by SDS polyacrylamide gel electrophoresis (Figure 3, last two patterns). It may be a degradation product of fragment D. It appears to be smaller than fragment E with a molecular weight of about 45,000 (judging from its mobility on the SDS gel).

The A, B, and C fragments were first identified by column chromatography on DEAE-cellulose.[434] They must be the remnants of the large fragments which were removed from the C

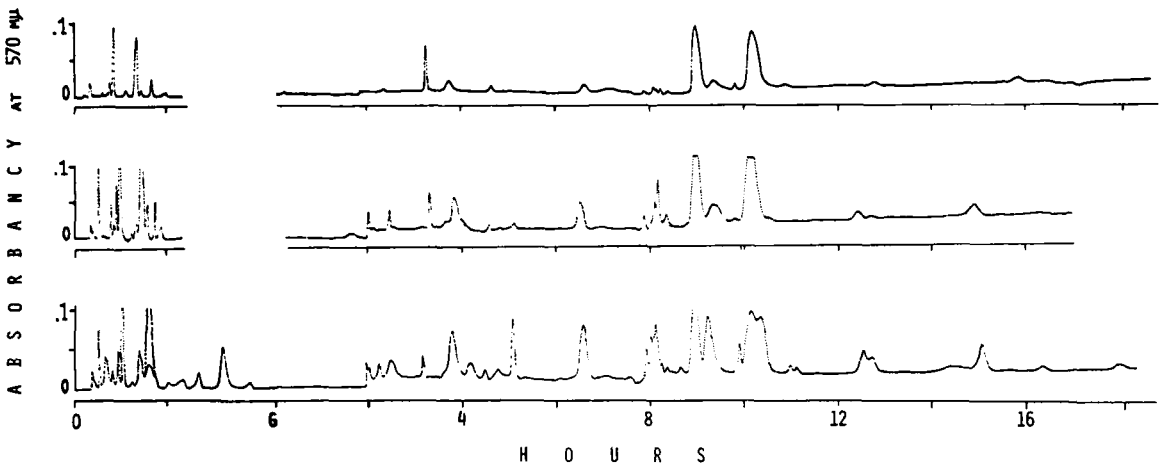


FIGURE 4. Cation exchange chromatography of dialyzable fragments isolated from a human fibrinogen solution digested by human fibrinolysin. Resin, aminex Spinco PA-35; buffers, pyridine acetate from 0.01 M, pH 4.2 to 2 M, pH 8.5. The first chromatogram was obtained with a sample isolated at 25% clottable period (CP), the second with a sample isolated at 100% CP, and the last chromatogram with a sample isolated at about 10,000% CP. The amounts applied on the column were obtained from the digestion of 0.043, 0.043, and 0.023 g of fibrinogen, respectively. (Triantaphyllopoulos, E., 1972, unpublished results.)

terminal portions of the α(A) chains[450,451] at the beginning of the digestion when the clottable derivatives were formed (Figure 3, diffuse bands at the middle and lower third of the second to fifth patterns. They finally became too small and are not detectable in the last pattern). Nussenzweig et al.[434] found an average molecular weight of 13,400 with a sedimentation constant of 1.5S for the B and C fragments together and a sedimentation constant of 1.2S for fragment A. Gardlund et al.[464] recently isolated two single chain fragments by gel filtration on Sephadex G-200 (eluted with 10% acetic acid) and SDS polyacrylamide gel electrophoresis with molecular weights of 20,500 and 18,000, respectively. Which one of the A, B, or C components these fragments represent is not known.

Like fragment D, the A, B, and C fragments were found to be heterogeneous. Nilehn separated fragment A into 7, fragment B into 6 and fragment C into 6 components by agarose gel electrophoresis.[459]

Micromolecular Derivatives

By the end of the digestion, 17 to 20% of the original protein of fibrinogen becomes soluble in trichloroacetic acid.[465,466] Column chromatography of picric acid supernatants of fibrinogen digests on the aminex resins Q-15S, 15A, 150A, and AG 50W-X2[446,447] indicated that these supernatants contain at least 27 peptides and also free lysine and free arginine in small amounts. Recently, using a more discriminating resin (Spinco PA-35 = Dowex 50W-X8) and a recorder of higher sensitivity (expanded to the 4 to 5 mV range) one of the authors (E.T.) found that approximately 47 dialyzable fragments can be differentiated in the original chromatograms of early digests (isolated at about 25% of the clottable period; Figure 4, upper graph), 56 in those isolated by the end of the clottable period, and at least 80 such fragments in digests from advanced stages of incubation (Figure 4, middle and lower graphs, respectively).[448] Filtration of digests of human fibrinogen through Amicon membranes UMO5 indicated that about 21 of the fragments from the final digests have molecular weights of less than 500.[467] These figures confirm the earlier conclusion that the number of the intermediates of large molecular weight, which are actually formed during fibrinogenolysis, is greater than the patterns of Figures 2 and 3 indicate or has been reported by other investigators,[449-451,454,468] since electrophoresis or ultracentrifugation of large molecules cannot differentiate between fragments having such small differences in molecular weight, especially in the range of 100,000 to 300,000.

Susceptibility of the Main Degradation Products to the Action of Thrombin. Origin of the D and E Fragments

In 1965,[444] 1966,[445] and 1967,[441] we

showed that thrombin splits TCA soluble fragments from the clottable (0 to 25% ammonium sulfate saturation fraction) and the unclottable residues (25 to 50% fraction) of fibrinogen. The amount of peptides removed within an hour from samples which are isolated at the end of the clottable period is equal on the average to 1.5% of the protein of the derivatives.[441,445] This indicates that one pair of the fibrinopeptides has already been removed by this stage. Shainoff et al. in 1968[469] and Mills and Karpatkin in 1972[450] showed that this is fibrinopeptide B and that it is removed very early during the degradation. The authors also showed that thrombin splits acid soluble peptides from early fragment E (Small Peak of AFIF isolated at 100 to 150% of the clottable period) but not from late fragment E or from fragment D (the small amounts which were detected as split from the latter were due to contamination with fragment E).[445] The transient susceptibility of fragment E to the action of thrombin has been confirmed recently by Mills.[451] The amount of peptides which are split from fragment E at the beginning is high (5 to 23% of the protein of this fragment), but it decreases to zero quickly at about 200% of the clottable period.[445] The amount that is split at the beginning is higher than the difference in size between early fragment E (mol wt, 55,000 to 60,000) and fibrinogen could justify. This suggests that plasmin, in all probability, uncovers additional bonds which are sensitive to the action of thrombin but which were less accessible or were inaccessible to this enzyme before. Chromatographed thrombin has been shown to solubilize fibrin completely in the presence of high concentrations of Trasylol and ϵ-ACA.[154,470]

The susceptibility of fragment E to the action of thrombin suggests that this fragment originates from the *N* terminal portion of fibrinogen, the *N* terminal dissulfide knot, which was described by Blombäck et al. in 1968[292,293] after the authors' results had been published.[445] This conclusion has been supported by other findings: (a) work in the authors' laboratory has demonstrated that fragment E has a very high cystine content (Table 1[277,309]) and that its glycopeptide resembles the glycopeptide of the γ chain.[309] Both of these properties are characteristic of the disulfide knot;[292] (b) Sanchez-Avalos and Miller[424] have shown by immunoelectrophoresis that fragment E obtained from lysed fibrin exhibits a greater mobility than fragment E obtained from lysed fibrinogen. Fragments D under the same conditions show the same mobility regardless of their origin; (c) Marder et al.[471] have found that the *N* terminal disulfide knot is antigenetically similar to fragment E but not to fragment D.

Recent immunological studies indicate that the D and E fragments come from continuous parts of fibrinogen.[464,472] Budzynski[473] studied the conformations of these fragments by hydrogen exchange with deuterium and tritium, and by spectropolarimetry and compared them to the conformation of fibrinogen. He found that fragment E is a loosely assembled molecule with hydrophilic character and penetrable conformation, while fragment D has a compact structure with hydrophobic character retaining a great part of the native conformation of fibrinogen. He concluded that plasmin splits off from fibrinogen a considerable portion of disorderly structure.

Sequence in the Fragmentation of the Chains

Recently Mills and Karpatkin,[450] Mills,[451] Pizzo et al.,[468] and Furlan and Beck[454] subjected fibrinogen digests from various stages of incubation to SDS polyacrylamide gel electrophoresis in the presence of mercaptoethanol (to reduce the S-S bonds) and estimated the size of the constituent polypeptide chains. There is remarkably little difference between the results of the first two groups. Mills and Karpatkin and Mills found that the chains are degraded as follows (values multiplied by 10^{-3}):

α(A) chains	β (B) chains	γ chains
73(72) → 27.5 → 26.5 > 15;	59 → 53 → 52 → 42 37 → 30 → 25*	49 → 42

*Mills could not distinguish whether these fragments came from the β or the γ chains.

Pizzo et al. obtained the following values:

$$70(68) \rightarrow 25 \rightarrow < 15 \qquad 56 \rightarrow 52 \rightarrow 44.5 \qquad 47.5 \rightarrow 42 \rightarrow 37.$$

The results of both of these groups indicate that plasmin splits first, a 45,000 molecular weight fragment from each of the α (A) chains, then a 4,000 to 6,000 fragment from the β (B) chains and that it attacks the γ chains last, after it has converted most of the α (A) chains into fragments smaller than 15,000 and the β (B) chains into 52,000 and 44,500 weight fragments. By subjecting the digests to the action of thrombin, Mills[451] found that the first fragment is split from the C terminal portion of the α (A) chains while the second fragment is split from the *N* terminal of the β (B) chains, thus removing the B fibrinopeptide with it very early during the digestion.

Furlan and Beck[454] found that plasmin removes small peptides from the *N* terminal portion of the β (B) chains and the C terminals of the γ chains before it attacks the α (A) chains at the C terminals, removing large polypeptide fragments successively. The differences between the results of these groups are obviously due to the fact that small differences in the weights of the chain remnants cannot be detected easily, especially when the digestion of fibrinogen proceeds at a fast rate. It is characteristic that in an earlier work Mills and Karpatkin[474] had found that the first fragment which plasmin removes from the α (A) chains of human fibrinogen has a molecular weight of 3,000 instead of 45,000 as their recent work indicated.[450,451]

Chains of Isolated Fragments

When purified derivatives were subjected to the SDS polyacrylamide gel electrophoresis after reduction with mercaptoethanol, chain remnants having the apparent molecular weights (values multiplied by 10^{-3}) indicated below were obtained:

Degradation products	Apparent molecular weight of chains
Fragment X	53,43,30,28,14[454]
Fragment Y	53,43,14[454]
Fragment D (mol wt 80,000–90,000)	50,46,14;[454] 40,35,30,23,10[464]
Fragment D (mol wt 45,000–50,000)	31,25,21,18,14;[454] 18,*4*[309]
Fragment E	14;[454] 9.5,8.0,7.2;[464] ~6.0*[309]

*Obtained by sulfitolysis and gel filtration on Sephadex G-100.

It is characteristic that the values for the molecular weights of the chains of fragments D and E, which were reported by each laboratory, are different. The difference in the values emphasizes the variability of these fragments. It also indicates that determination of molecular weights by SDS polyacrylamide gel electrophoresis involves a considerable degree of experimental error.

Yield in Final Degradation Products. Their Implication on the Fragmentation of Fibrinogen

Nussenzweig et al.[434] reported in 1961 that component D represents 50% and component E, 15 to 20% of the protein of fibrinogen without explaining how they reached this conclusion. Calculation of the relative concentrations of the components from the area under the peaks of Figure 2 of their paper, which shows the separation of the fragments by chromatography on DEAE-cellulose and which was used for the identification of the fragments, gives the following values: D:35%, E:50%, A:6% and B+C:9%. These values do not take into account the dialyzable fragments, since the samples were dialyzed before they were applied on the column. The results show a ratio of 1.4: 1 between E and D. A similar ratio of 1.5: 1 between E and D was obtained by calculating the results of Furlan and Beck[454] from their Figure 2 data (chromatography on carboxymethylcellulose).

The concentration of the protein in both cases was estimated by measuring the absorption of 280 mμ. The authors have found[277] that fragment E (Small Peak of AFIF) at 200% of the clottable period contains fewer ultraviolet absorbing residues (try: 17.4, tyr: 30.3 residues/10^5 g) than fragment D (27.7 and 37.1 residues, respectively). The differences may be greater during the final stages. Marder et al.[453] have shown that fragment D has an extinction coefficient at 280 mμ ($E^{1\%}_{1cm}$) of 20.8, fragment E has a coefficient of only 10.2, and fibrinogen has a coefficient of 15.1. If an allowance of 20% is made for the acid soluble fragments[466] and corrections for the differences in the absorption coefficients of fragments D and E from the absorption coefficient of fibrinogen are applied, the values which were obtained from the results of Nussenzweig et al.[434] become A = 4%

(14,000), B+C = 7% (25,000), D = 17% (58,000), and E = 52% (177,000) of the protein of fibrinogen. These values suggest that (a) fragment E was eluted during the chromatography together with fragment F and possibly with other relatively large fragments (Nussenzweig et al. had detected two impurities in this fraction by immunochemical techniques), and (b) the final yield in fragment D cannot be greater than 60,000 and this value allows only for one molecule of fragment D from one molecule of fibrinogen.

Results obtained in the authors' laboratory indicate similarly that one, rather than two, molecules of fragment D can be derived from one molecule of fibrinogen. In 1965[437] the authors isolated the breakdown products of fibrinogen sequentially from the beginning through relatively advanced stages of incubation by ammonium sulfate fractionation and continuous flow paper electrophoresis. They found that during the advanced stages of the digestion only 42% of human fibrinogen (biuret method) precipitated between 25 to 50% ammonium sulfate saturation. Calculation of the concentrations of the components of this fraction from the area under the peaks of Figure 2 (last pattern) of their 1965 paper, which represents the separation of the fragments by the continuous flow electrophoresis technique, gave the following values: 54% of the protein separated as fragment D (Large Peak of AFIF), 40% as fragment E (Small Peak of AFIF), and 10% as an electropositive component (peak at the beginning of the graph). The protein concentration in the effluent fractions was determined by the method of Lowry et al.[475] These findings suggest that approximately 78,000 (340,000 x 0.42 x 0.54) of the mass of fibrinogen became fragment D, approximately 58,000 became fragment E (this was probably contaminated with other fragments) and 14,000 of it became the electropositive component. These values fit well with the fact that these products were isolated earlier in the digestion (500% CP) than the products which were studied by Nussenzweig et al.,[434] which were isolated after a digestion of seven hr (2,100 to 4,200% of clottable period) from a solution which had become uncoagulable within 10 to 20 min.

Competitive inhibition radioimmunoassays reported recently[472] suggest similarly that fragments D and E are formed during the degradation in a 1:1 molar ratio. The conclusion of the authors,[472] however, that two molecules of each of these fragments are formed from each molecule of fibrinogen cannot be supported by the yield data. Secondly, it has been shown that the two halves of fibrinogen at their *N*-terminal positions are joined together by three disulfide bridges.[300] There is no formation of free SH groups during the plasminolytic degradation of fibrinogen[476] and this suggests that the two *N*-terminal disulfide knots of each molecule remain bound together. Consequently, only one molecule of fragment E can be formed/molecule of fibrinogen. If the 1:1 molar ratio between D and E is correct, there should also be only one fragment D per molecule of fibrinogen.

If only one molecule of fragment D is formed from each molecule of fibrinogen, this fragment should have three pairs of chains. As mentioned earlier, SDS polyacrylamide gel electrophoresis of reduced samples of purified fragment D having molecular weights of 80,000 to 90,000 separated chains of apparent molecular weights of (values multiplied by 10^{-3}): 50, 46, 14;[454] and 40, 35, 30, 23, and 10.[464] Reduced samples of fragment D having molecular weights of 46,000 to 50,000 had chains of: 31, 25, 21, 18, and 14.[454] Combination of these chains in three pairs gives values higher (106,000 to 220,000) than the estimated molecular weights of the respective fragments and makes the conclusion of one molecule of fragment D/molecule of fibrinogen seem unrealistic. However, if we take into consideration that fragment D is heterogeneous[461-463,459] and that by the end of the digestion 50% of the original protein has been split into fragments smaller than 20,000,[437] the possibility exists that by this time the α (A) and the β (B) chains have been degraded so much that only traces of the former and a little of the latter have been left for fragment D. The larger polypeptide chains which were detected could then represent successive steps in the degradation of the γ chains and fragment D could have essentially two pairs of chains. We should also take into consideration that the estimated values for the molecular weights of the chains may be higher than their actual size. It has been shown that reduced proteins have a slower mobility on SDS polyacrylamide gels than nonreduced proteins of comparable molecular weight.[451] Fragments produced by cleavage of peptide bonds may similarly show a slower mobility on these gels than intact proteins.[451] In favor of this hypothesis are the findings of Mills

and Triantaphyllopoulos.[309] These investigators determined the molecular weights of the chains of fragment D of 45,000 mol wt, by gel filtration on Sephadex G-100 after sulfitolysis. They found values of 18,000 and 4,000 which suggest that fragment D may have two pairs of chains per molecule.

Marder[453,477] has advanced the theory that two molecules of fragment D and one molecule of fragment E are formed from one molecule of fibrinogen. He has postulated that plasmin removes the A, B, and C fragments all at once and that the remainder of the molecule forms fragment X, which has a molecular weight of 240,000 and is the only clottable derivative of fibrinogen. This fragment is then split into one molecule of fragment Y (molecular weight, 155,000) and one molecule of fragment D (molecular weight, 83,000). Fragment Y is finally split into another molecule of fragment D and one molecule of fragment E (molecular weight, 50,000).

This theory does not take into consideration the large number of acid soluble or dialyzable fragments[446-448] and cannot account for the variability and the heterogeneity of the large fragments. The theory was based on the observation that fragment D is detected earlier during the degradation than fragment E by agar gel electrophoresis[452,453] and on the reports of Nussenzweig et al.[434] and Larrieu et al.[452] that fragment D represents 50% of the protein of fibrinogen while fragment E accounts for only 15 to 20% of it.

Early fragment D has a molecular weight almost twice as large as the molecular weight of early fragment E. Consequently, twice as many molecules of fragment E as molecules of fragment D need to be accumulated in order to form a detectable protein band on the gels. In addition, judging from the photographs, fragment D seems to stain very well with the dye (Amido black) which was used to detect the protein on the agar gels of Marder[453] after the electrophoresis, while fragment E stained only faintly. These properties by themselves could explain the delay in the detection of fragment E on the electrophoretic gels.

The yields reported by Nussenzweig et al.[434] were discussed at the beginning of this section. Their data actually suggest that only one molecule of fragment D can be produced from each molecule of fibrinogen. Larrieu et al.[452] determined the relative concentrations of the final degradation products by chromoscan analysis of agar gel electrophoretic patterns without making allowance for the acid soluble fragments, which constitute approximately 20% of the final products. In addition, they based their calculation on the assumption that the relationship between color intensity and protein concentration is the same for all fragments. Even so, they found a relationship of 24:54 between the yields in fragment E and fragment D, i.e., the protein of fragment E was equal to 45% of the protein of fragment D. Marder's theory postulates a yield in fragment E equal to 30% of the yield in fragment D [50,000 x 100/(2 x 83,000)].

Pizzo et al.[468] calculated the relative concentrations of the derivatives from the intensities of the colored bands on SDS polyacrylamide gels after staining them with Coomassie blue. Their Figure 2 indicates that the relative concentration of fragment E is equal to approximately 58% of the concentration of fragment D. This fits well with a 1:1 molar ratio between these two fragments (50,000 x 100/ 83,000 = 60%). It should be noted that with this dye the derivatives stain better than fibrinogen (Figure 2) and the concentration of fragment D alone seems greater than the concentration of the parent protein.

Fragmentation of Fibrinogen by Trypsin, Chymotrypsin and Papain

As mentioned earlier, Garner and Tillett in 1934[411,412] found that the degradation of fibrinogen by trypsin is more extensive than its degradation by the streptococcal fibrinolysin. Similarly, Christensen and MacLeod[426] showed in 1945 that casein previously digested with trypsin is resistant to further digestion with plasmin, while casein extensively digested with plasmin can be further degraded by trypsin.

Mihalyi and Godfrey[421-423,427] have studied the breakdown of fibrinogen by trypsin and chymotrypsin by automatic titration, ultracentrifugation, optical rotary dispersion, and measurement of viscosity changes. They found that these enzymes break fibrinogen in a series of three reaction classes. During the first class of reactions, the fast reaction, trypsin splits 12 and chymotrypsin 7 peptide bonds; during the second class or slow reaction, these enzymes split 80 and 96 bonds, respectively, and during the third class they attack the remaining bonds which are susceptible

to them at even slower rates. Mihalyi and Godfrey found that when trypsin is the enzyme, the ratio of the three reaction rates is 1,050:70:1.[427] Trypsin splits the thrombin sensitive bonds very early at the beginning of the fast reaction and the clottability disappears quickly. Chymotrypsin does not attack the thrombin sensitive bonds. Nevertheless, the clottability of the fibrinogen deteriorates progressively during the chymotryptic digestion and disappears at approximately 25% of the slow reaction.[423,427]

According to Mihalyi[427] the first two stages of the tryptic and chymotryptic digestion of fibrinogen are similar to its digestion by plasmin and a fragment similar to fragment D is produced with a molecular weight of 83,000. The digestion by the pancreatic enzymes, however, continues further and during the stage of the very slow reaction, the large protein fragment is degraded to smaller heterogeneous fragments. Sanchez-Avalos and Miller,[424] on the other hand, on the basis of immunoelectrophoretic and acrylamide gel electrophoretic observations, concluded that the degradation of fibrinogen by trypsin is all the way similar to its digestion by plasmin, resulting in the formation of two final macromolecular derivatives which are immunologically similar to fragments D and E, respectively. They further found that only chymotrypsin and papain degrade fibrinogen more extensively than plasmin, but each of these enzymes acts in a different way: chymotrypsin degrades only fragment D, while papain degrades only fragment E.

Fragmentation of Fibrinogen by Thrombin

Guest and Ware in 1950,[153] using semipurified preparations of thrombin in the presence of high concentrations of soybean trypsin inhibitor, showed that this enzyme at high concentrations can solubilize fibrin in vitro. Seegers and his colleagues[151] confirmed these findings using purified, chromatographed thrombin and showed that the fibrinolytic activity of thrombin is independent of its ability to clot fibrinogen. Procedures which reduce or destroy the ability of thrombin to clot the fibrinogen (acetylation[148] storage at room temperature,[118,147] acetylation of the parent prothrombin,[150] or activation of the prothrombin in saline containing 0.05 M $CaCl_2$[149]) do not destroy its ability to lyse fibrin or to hydrolyze TAMe. If anything, they seem to potentiate these properties.

Muirhead and Triantaphyllopoulos[478] and Triantaphyllopoulos et al.,[470] using gel filtration through Sephadex G-100, polyacrylamide gel electrophoresis, and DEAE-cellulose chromatography, found that the degradation products of thrombin-lysed fibrin are heterogeneous. Using SDS polyacrylamide gel electrophoresis in the presence of mercaptoethanol, Mills and Karpatkin[474] found that after the removal of the fibrinopeptides, thrombin attacks the α chains further. It first removes a 5,000 molecular weight fragment and on prolonged digestion, it degrades these chains completely into small polypeptides, which can no longer be detected on the gels.

Biological Activity

The breakdown products of fibrinogen and fibrin are known mainly for their effect on blood coagulation.[415-419,456,465,479-483] Their activity, however, is not confined to this system. The information available so far indicates that these products can also affect the function of the platelets[484-491,509] and fibrinolysis;[492,493] they can also potentiate the effect of biologically active peptides (angiotensin, bradykinin, etc.) and amines on the blood pressure, the contractility of the intestine and of the uterus, and on the permeability of the capillaries.[494,495]

Effects on Blood Coagulation

It was shown from the very beginning that the fibrinogen derivatives can affect several phases of blood coagulation: They can inhibit the clotting of fibrinogen by thrombin,[415-419] the generation of plasma thromboplastin,[416,482] and the consumption of prothrombin.[416,499] Recent work has demonstrated that they can also inhibit the consumption of factor XIII in the plasma.[496,497]

Contrary to general belief, the fibrinogen derivatives do not act only as inhibitors on blood coagulation. Under certain conditions they can promote clotting; they can potentiate the procoagulant effect of factor VIII on the prothrombin complex,[498] protect purified prothrombin complex from inactivation by thrombin,[499] protect thrombin from spontaneous inactivation,[500,501] and, at relatively low concentrations, they can promote the consumption of prothrombin in plasma.[188]

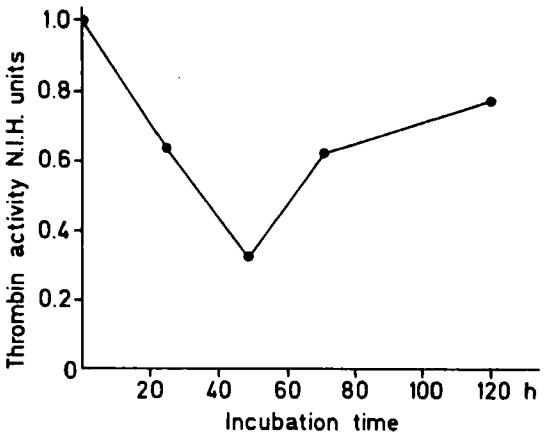


FIGURE 5. Effect of fibrinogen incubated for varying lengths of time on the thrombin time of fresh plasma. Sodium chloride precipitated fibrinogen was incubated under sterile conditions for five days. Each day samples were removed, tested for coagulability, and then frozen at -20°C. Clottability disappeared at 48 hours. On the sixth day of incubation all samples were mixed 1:2 with the same fresh citrated plasma and the thrombin times of the mixtures were determined. The results were converted to units of thrombin activity by means of a thrombin dilution curve. (From reference 416.)

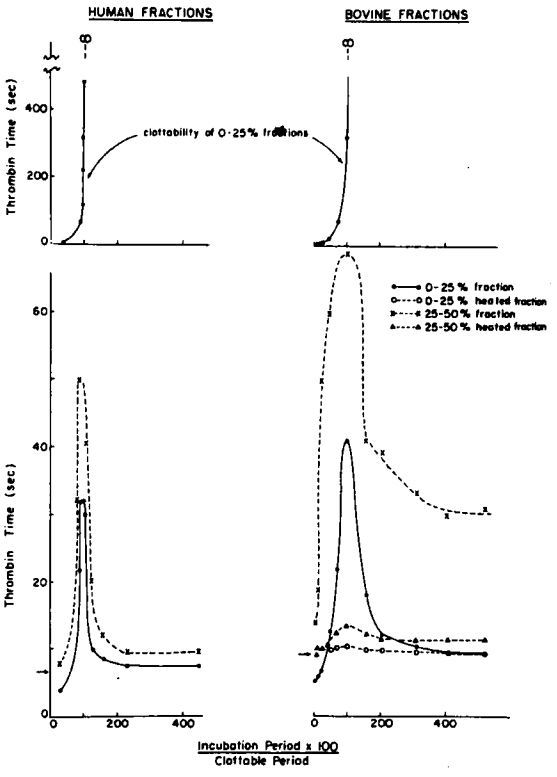


FIGURE 6. (upper section) Disappearance of coagulability of the 0 to 25 percent ammonium sulfate saturation fraction from lysed fibrinogen solutions with increasing incubation. (Thrombin times determined without addition of fibrinogen.) (lower section) Effect of the 0 to 25% and 25 to 50% (AFIF) ammonium sulfate saturation fractions of lysed fibrinogen solutions (2 mg/ml) on the thrombin time of intact fibrinogen (1 mg/ml) as a function of the incubation stage. Arrows indicate the thrombin time of the controls where saline was added instead of the fractions. (From reference 441.)

Anticoagulant Effects

Inhibition of the Clotting of Fibrinogen by Thrombin Variability of the Anticoagulant Effect

The original reports from the authors' laboratory[415,416] as well as from Poland[418,419] demonstrated that the ability of the fibrinogen digests to prolong the thrombin time of intact fibrinogen varies with the degree of fibrinogenolysis. The inhibitory effect is low at the beginning, increases more or less sharply to a peak when the incubated solution becomes uncoagulable by thrombin, and then declines, quickly at first but at a slower rate afterwards (Figure 5). This pattern of variability has been confirmed by subsequent studies[436,437,458,502] and seems to be characteristic of these products, since their action on similarly acting enzymes, reptilase, thrombin, coagulase, and arvin, varied more or less in a similar manner.[502]

In 1967[441] the authors showed that the specific activity of both the clottable (0 to 25% ammonium sulfate saturation fraction) and the unclottable (25 to 50% fraction) fibrinogen derivatives followed the same pattern of variability as the activity of the original digest (Figures 5 and 6). These activities differed only in the degree of inhibition: At all times the activity of the unclottable derivatives was higher than the activity of their clottable contemporaries. These results suggested, together with changes in electrophoretic mobility and precipitability by ether and heat,[441] that both types of derivatives changed as the digestion progressed. Fractionation with continuous flow paper and column electrophoresis indicated that responsible for the peak of the inhibitory effect were the final forms of both the clottable (Large Peak of the 0 to 25% fraction) and the unclottable (Large Peak of AFIF) residues[440,441] CR and UR, respectively, and that the latter were the most inhibitory (Figure 7). The sudden fall in the specific activity (at the end of the clottable period) coincided with the fragmentation of the molecule into the early D and the early E fragments.[440] Both of these fragments

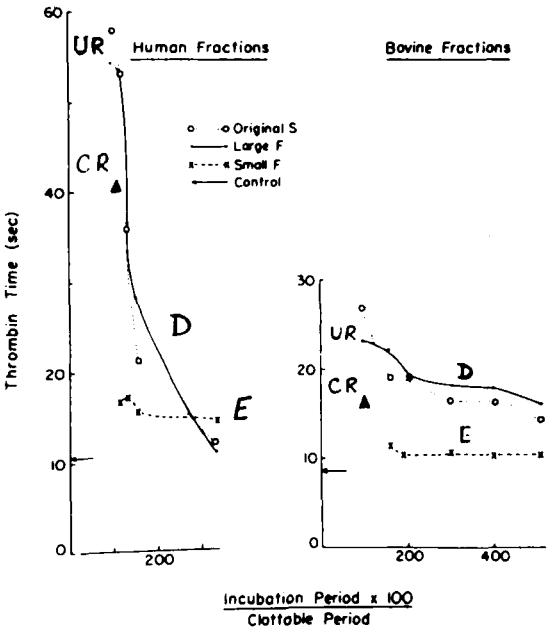


FIGURE 7. Thrombin time prolonging effect of the major anticoagulant derivatives of lysed fibrinogen, isolated at various stages of incubation by ammonium sulfate fractionation and column electrophoresis. Original S = original sample = 25 to 50% ammonium sulfate saturation fraction before column electrophoresis. The large fraction (Large F) represented fragments D, except at 100% of the clottable period, when it contained unclottable residues (bovine samples) or mainly unclottable residues and some early fragments D (human samples). The small fraction (Small F) represented E fragments throughout. CR, clottable residues or fragment X; UR, unclottable residues or fragment Y. (Adapted from reference 440.)

were found to be able to inhibit the clotting of fibrinogen by thrombin, but the activity of the former was greater than the activity of the latter (Figure 7). As the digestion progressed the activity of the D fragments decreased and at the terminal stages both types of human fragments exerted more or less tha same low degree of inhibition.[440]

Similar findings concerning the antithrombic activity of the main derivatives were reported by Marder and Shulman in 1969,[483] although these investigators thought that each of these derivatives occurred as a single molecular entity. They found that fragment Y (a form of unclottable residue) was the most inhibitory of all the fibrinogen derivatives, fragment X (a form of clottable residue) was second in the inhibitory activity, fragment D third, and fragment E the least inhibitory.

Nature of the Inhibition

Neither thrombin nor fibrinogen is destroyed by the fibrinogen derivatives.[416] Kinetic studies indicated that the overall inhibitory effect is competitive in nature[465,479,502] and in purified solutions is proportional to the pH of the reaction mixture while in plasma it levels off after pH 7.5.[479] Calcium and magnesium ions do not affect the inhibition.[479]

In 1961, by measuring the absorption of fibrinogen solutions at 600 nm after addition of thrombin in the presence and in the absence of fibrinogen derivatives, Triantaphyllopoulos[481] found that mixtures of unclottable residues (AFIF) delayed both the onset and the general course of polymerization. The delay in the onset of polymerization suggested that, in addition to inhibiting the rate of the polymerization, the fibrinogen derivatives could either inhibit the proteolytic action of thrombin on fibrinogen and thus delay the formation of the fibrin monomers or alternatively they could be particularly effective in retarding the initial association of the monomers or oligomers to form visible polymers. Finally, they could inhibit both of these steps.

Similar findings concerning the inhibition of the polymerization of the fibrin monomer were obtained by the St. Louis group.[503] In addition, Alkjaersig et al.[465] subjected washed clots formed in the presence and in the absence of final fibrinogen derivatives to ultracentrifugation after solubilization in 5 M urea, while in other experiments they labeled the degradation products with ^{131}I and studied the distribution of the radioactivity in the clots and in the supernatants. Bang et al.[456] in the same laboratory examined the clots under the electron microscope. They found that fragment D (a) inhibited the polymerization, (b) became incorporated into the clots, and (c) altered the structure of the fibrin network in a striking way. On the basis of these and later findings[449] Fletcher and co-workers postulated that the coagulation defect which develops in the presence of the fibrinogen derivatives is due solely to defective polymerization of the fibrin monomers because of the incorporation of fragment D, which they called polymerization inhibitor (or fragment 5.27S), into the fibrin network and the formation of a structurally disorganized and weak clot.

In a series of 26 experiments published in 1965,[444] 1966,[445] and 1967[441] the authors

showed that (a) thrombin splits trichloroacetic acid soluble fragments from the clottable (0 to 25% ammonium sulfate saturation fraction) and the unclottable residues (AFIF of bovine origin) and from purified early E fragments (Small Peak of AFIF), and (b) the peptides which are split from mixtures of fibrinogen and the unclottable intermediate derivatives are considerably fewer than the sum of peptides which are split from individual solutions of fibrinogen and the anticoagulant derivatives of the same concentrations.*[441,445] These findings supported and extended the kinetic studies referred to earlier and implied that the intermediate derivatives compete with fibrinogen for thrombin as both are substrates for this enzyme. In contradistinction no peptides were liberated from fragments D (the small amount which was detected was due to contamination with early E fragments) or from late fragments E and this can explain the negative results which were obtained by Alkjaersig et al.[465] regarding the antithrombic activity of the fibrinogen derivatives, since these investigators were working with final degradation products only.

Lipinski et al.[505] in 1967 added increasing amounts of fibrinogen derivatives ("early" and "late" FDP) into samples of blood or plasma containing ^{131}I labeled fibrinogen or into solutions of purified ^{131}I labeled fibrinogen (or labeled fibrin monomer) and clotted the mixtures with thrombin (or by diluting the monomers with buffer). They found that increasing amounts of radioactivity remained unclotted in the supernatants, expecially when "early" FDP (intermediate derivatives) were used. Most or all of the radioactivity could be precipitated by addition of protamine sulfate (paracoagulation). Extending further the original observation of Shainoff and Page[383] that fibrin monomer has the ability to form complexes with intact fibrinogen and remain soluble instead of polymerizing, Lipinski et al. postulated that the fibrinogen derivatives, like fibrinogen itself, form soluble complexes with the fibrin monomers and keep them from polymerizing. Marder and Shulman[483] reached similar conclusions after subjecting to ultracentrifugation mixtures of fibrinogen and purified degradation products X, Y, or D or mixtures of purified fragment X and fragments Y or D. They found that after the addition of thrombin to these mixtures, peaks with sedimentation constants of 13S and 23S or of 17 to 18S were formed and concluded that these were complexes of fibrin monomer with each of the degradation products on the one hand, or complexes of fragment X with fragment Y or D on the other.

The formation of complexes between the unclottable fibrinogen derivatives and fibrin monomers has been challenged, however, by the work of other investigators. Shainoff et al.[469] found that only the clottable derivatives can complex with the fibrin monomers and form peaks with high sedimentation constants during ultracentrifugation. An analogous conclusion was reached recently by Smith and Bang.[506] These investigators incubated mixtures of fibrinogen (I-2 or I-8) and final degradation products or mixtures of fibrinogen and purified fragments D with small amounts of thrombin. At various intervals they removed samples from the incubation mixtures and chromatographed them on a column of agarose (Bio-Gel A-5m). The elution patterns (absorption at 280 nm) indicated that complexes of molecular weights of 690,000, 990,000, and 1,400,000 were formed slowly

*Latallo et al. reported in 1964[504] two experiments where they added 0.8 mg of unfractionated fibrinogen digests at the peak of their anticoagulant effect, "early FDP," to 16 mg and 4 mg of fibrinogen, respectively. They incubated these mixtures with thrombin and found that the presence of this small amount of fibrinogen derivatives reduced the amount of the TCA soluble peptides which were split by the enzyme from 17.2 (in the absence of the derivatives) to 14.7 μg in the first experiment and from 6.3 to 4.3 μg in the second experiment. It is hard to understand how these results were obtained. The fibrinogen in the two experiments was used in considerable excess over the concentration of the anticoagulant derivatives and since the inhibition is competitive in nature, it should have overcome the inhibitory effect of the derivatives at least in the first experiment. Secondly, plasmin itself splits TCA soluble peptides from fibrinogen,[419,432,446-448,465] which amount to 4% of the protein at the peak of the inhibitory effect.[442] Consequently, the 0.8 mg of unfractionated, undialyzed "early FDP" contained about 32 μg of TCA soluble peptides before they were added to the fibrinogen solutions. Since zero time controls were not carried out by these investigators, the amount of peptides which should have been found in the mixtures after the action of thrombin should have been greater, not smaller, than 32 μg. Thirdly, as mentioned earlier, thrombin splits TCA soluble peptides (fibrinopeptide A) from "early FDP." As a rule, the peptides which are split from mixtures of fibrinogen and these derivatives are fewer than expected but they are more than the peptides which are split from frbrinogen (of the same concentration) alone.[445]

during the incubation while the concentration of the fibrinogen decreased proportionally. In contrast, the concentration of the breakdown products remained unaltered. The products were eluted at their usual volume as if they had been chromatographed alone. Identical complexes were formed when the fibrinogen was incubated alone with thrombin (no degradation products present). In contradistinction, complexes were not formed when thrombin was omitted or when its action was inhibited by hirudin. Conclusive evidence for the nonparticipation of the fibrinogen derivatives in the formation of the complexes was provided when ^{125}I labeled degradation products were incubated with fibrinogen and thrombin. The polymers which were formed contained no radioactivity. All the radioactivity was eluted separately as one peak at the usual volume where the degradation products were eluted. *N*-terminal analysis of the polymers indicated that they were composed of fibrin monomers from which the fibrinopeptides A and B had not been completely removed. The molecular weights of the polymers indicated that they were dimers, trimers, and tetramers of fibrin monomer. Smith and Bang suggested that the earlier studies should be reinterpreted. Neither Lipinski et al.[505] nor Marder and Shulman[483] had determined the composition of their polymers; they had *assumed* that they represented complexes of the degradation products with fibrin monomer since they had not seen them in the absence of the fibrinogen derivatives. Presumably, the incorporation of fragment D into clots, which was observed by Alkjaersig et al.[465] and Bang et al.,[456] should be considered as the result of passive entrapment within the fibrin network and not the result of active copolymerization. Smith and Bang[506] concluded that the anticoagulant activity of fragment D and of the other nonclottable degradation products resided in their ability to competitively bind thrombin. This explanation seems logical since binding would reduce the effective concentration of thrombin in the mixture and would result in delayed formation of fibrin monomers and consequently in delayed polymerization. It should be pointed out, however, that early fragment E, which is a good substrate for thrombin,[445] has a very weak antithrombic activity[440] and does not inhibit the polymerization.[502] Early fragment D is not a substrate for thrombin;[445] nevertheless, its anticoagulant activity is greater than the anticoagulant activity of fragment E.

Experiments performed in the authors' laboratory in 1959[479] and by Aljaersig et al. in 1962[465] and recently by Larrieu et al.[502] indicated that neither the intermediates[479] nor the final fibrinogen derivatives[465,502] interfere with the hydrolysis of TAMe or BAMe by thrombin. The earlier experiments were used as evidence by Fletcher and co-workers that the fibrinogen derivatives cannot interfere with the enzymatic action of thrombin. It must, however, be considered that the molar concentrations of TAMe or BAMe which were used in these experiments (0.02 to 0.2 M) were 100 to 1,000 times greater than the molar concentrations of the fibrinogen derivatives. As mentioned earlier, the inhibitory effect of these derivatives on the action of thrombin is competitive in nature[465,479,502] and such an overwhelming excess of substrate could have counteracted the inhibition very easily.

Inhibition of the Generation of Plasma Thromboplastin

Triantaphyllopoulos reported in 1958[416] that when plasma anticoagulated with fibrinogen derivatives is used as supplier of factors V and VIII in the thromboplastin generation test instead of oxalated plasma, there is no generation of intrinsic prothrombin activator. A normal generation is observed, however, when the fibrinogen derivatives are added to the serum or when the plasma has been oxalated before the addition of the fibrinogen derivatives. The activities of factors V[416] and VIII[416,511] were found to be reduced in the plasmas which were anticoagulated with fibrinogen derivatives, but they were found normal in oxalated or citrated plasmas to which the same amount of the derivatives had been added, thus explaining the lack of generation of intrinsic activator when the first plasmas were used and the normal results with the others.

The inactivation of factor VIII in the nondecalcified plasmas could be prevented if the prothrombin complex was removed before the addition of the fibrinogen derivatives[500] indicating that a derivative of this complex was responsible or acted as a co-factor for the inactivation. Further experiments showed that traces of thrombin generated in this plasma upon standing were the inactivating agent and that in the defibrinated plasma or in purified systems both

fibrinogen and its derivatives could protect factor VIII from this inactivation, fibrinogen being the most effective.[498] In normal plasma, on the other hand, the addition of the fibrinogen derivatives seemed to potentiate the inactivating effect of thrombin on factor VIII.[498,500] This seemingly paradoxical result can be explained if it is considered that both fibrinogen and its derivatives are substrates for thrombin as is factor VIII[265-269] and compete with this factor for the enzyme. Fibrinogen has the advantage of forming fibrin, which adsorbs thrombin and thus reduces the amount of the enzyme which remains available for the inactivation of factor VIII even more. In the complete plasma, fibrinogen is already there ready to exert its protective effect. When its derivatives are added they inhibit the formation of fibrin due to their anticoagulant effect and thus eliminate part of the protective mechanism. Consequently, more thrombin is left to act on factor VIII and the net result appears as a potentiation of the inactivating effect of thrombin. These studies suggested that when blood or plasma is anticoagulated with fibrinogen derivatives some thrombin generation takes place. Traces of this enzyme are sufficient to inactivate factor V and factor VIII on standing.[416,498]

Niewiarowski et al. in 1959[482] showed that addition of fibrinogen derivatives directly into the reaction mixture of the thromboplastin generation test inhibits the generation of the intrinsic activator of prothrombin. They found that responsible for the inhibition was a thermoresistant component which they called IGT (inhibitor of the generation of thromboplastin). In 1962 the authors performed similar experiments with purified fragments D (Large Peak of AFIF) and E (Small Peak of AFIF) and confirmed the findings of Niewiarowski et al.[455] The authors found that fragment E, which is thermoresistant, inhibited the generation while fragment D, which is thermosensitvie, was without effect. Larrieu et al. obtained similar results recently.[502]

The mechanism of this inhibition does not seem to thermosensitive, the one described above where the fibrinogen derivatives were added to native blood or plasma because (a) fragment D which is a much stronger inhibitor of clotting[440,483] than fragment E does not inhibit the generation of the intrinsic activator when it is added into the generating mixture, (b) fragment E inhibits the generation at concentrations which are not sufficient to inhibit clotting (0.32 mg/ml), and (c) the inhibition is temporary, affecting only the rate of generation, not the final amount of intrinsic activator. The inhibition which is observed when the fibrinogen derivatives are added to the native blood or plasma is permanent.[416]

Inhibition of the Consumption of Prothrombin

It was shown in 1958[416] that when blood is anticoagulated with high concentrations of fibrinogen derivatives, the consumption of prothrombin is inhibited. In later work the two authors found that more thrombin units are generated during the two-stage determination of prothrombin with plasmas containing large concentrations of fibrinogen derivatives than are generated with controls to which oxalated saline instead of fibrinogen derivatives is added.[499]

Disruption of the platelets by freezing and thawing or by sonication of platelet-rich plasma did not improve the consumption. The inhibition was counteracted only when excess of platelet factor 3 or of platelet substitutes[499] or putified factor VIII[500] were added. These findings suggest that the fibrinogen derivatives in all likelihood interfere with the formation or with the activity of the factor VIII-phospholipid-Ca^{++}-factor IXa complex.

The inhibition of the prothrombin consumption was found to be a general property of the larger fibrinogen fragments (intermediate or final D and E), i.e., it is independent of the degree of degradation of fibrinogen in contradistinction to the antithrombic effect, which, as it was mentioned earlier, is very much dependent on this parameter.[415,416,418,419,437,458,502]

Inhibition of the Consumption of Factor XIII in Plasma

Buluk et al.[353] and Lorand and Konishi[507] have shown that thrombin activates factor XIII. Work in the authors' laboratory[496,497] demonstrated that thrombin can also inactivate this factor in the plasma and that an exponential relationship exists between the amount of thrombin which is added and the residual factor XIII activity (C^{14} putrescine incorporation assay). Five units of thrombin are sufficient to inactivate 95% of this factor, while 10 units inactivate 97% of it. Related to this effect of thrombin are the findings of Mandalaki and Schizas[508] who noted that the concentration of factor XIII is decreased

in disseminated intravascular coagulation, both clinical and experimental. The fall is proportional to the severity of the condition and has diagnostic value.

Addition of fibrinogen derivatives to native blood in quantities sufficient to inhibit both clotting and the consumption of prothrombin (final concentration greater than 20 mg/ml) inhibited completely the inactivation of factor XIII. The amount of the factor in this plasma was the same as in the plasma obtained from control mixtures of citrated blood. When, however, a smaller concentration of the anticoagulant derivatives (16 mg/ml) was used, an amount which inhibited clotting completely but allowed partial activation of prothrombin, factor XIII was inactivated within one hour. Its level in the plasma was equal to its level in the serum of control mixtures which were clotted in the absence of the fibrinogen derivatives.[496,497] These results suggest that as far as factor XIII is concerned, the important function of the degradation products is to inhibit the generation of thrombin and not to inhibit the formation of fibrin strands.

Procoagulant Effects

Potentiation of the Procoagulant Effect of Factor VIII

In experiments published in 1967[22] and 1970[188] the authors had studied the effect of fibrinogen derivatives on mixtures containing preparations of partially purified prothrombin complex, platelet factor 3 or Bell-Alton reagent, adsorbed bovine serum, and calcium chloride. They had found that the presence of the fibrinogen derivatives increased the number of units of thrombin which were generated in the mixture by one half – four times over the control values. It appeared from these results that the fibrinogen derivatives had a factor VIII-like activity.

These experiments were repeated recently with more purified preparations of prothrombin complex.[498] The addition of the fibrinogen derivatives to the new mixtures induced the generation of only a few units of thrombin. When, however, 1 to 2 units of factor VIII were also added to the incubation mixture, the amount of thrombin which was generated was many times greater than the additive amounts which were generated in the presence of factor VIII or the fibrinogen derivatives alone (Figure 8).

These results suggest that the effect of the

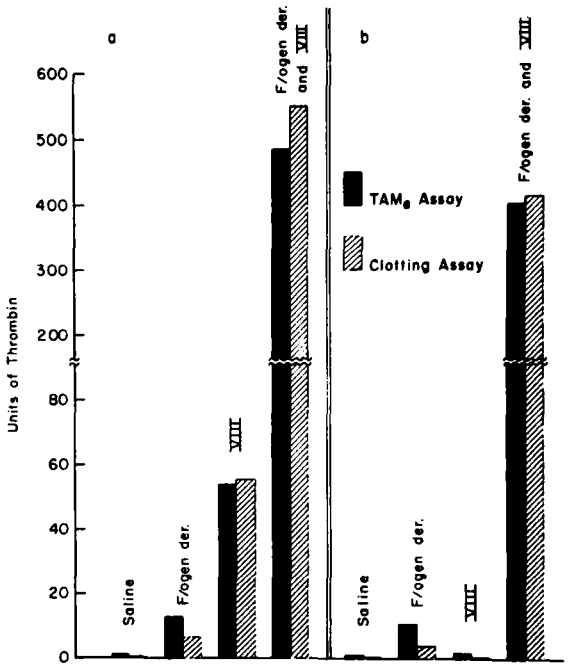


FIGURE 8. Potentiation of the procoagulant effect of factor VIII by fibrinogen derivatives. To a mixture of bovine prothrombin complex, inosithin, calcium chloride, and adsorbed bovine serum, one of the following was added: (1) saline, (2) factor VIII (native in "a" heat, defibrinated in "b"), (3) plasmin-free fibrinogen derivatives, (4) a mixture of factor VIII (native or heat defibrinated) and fibrinogen derivatives. The final concentrations/ml were prothrombin, 1,292 NIH units; inosithin, 17 μg; fibrinogen derivatives, 12.3 mg; factor VIII, two units when native, one unit when defibrinated. Adsorbed bovine serum was present at a final dilution of 1:75, calcium chloride 0.01 M, and imidazole buffer 0.025 M. At frequent intervals and for a period of two hours, aliquots were removed and tested both for their clotting and esterolytic (TAMe) activities. The latter was converted into NIH units of thrombin activity by means of a dilution curve of thrombin of known potency. (Triantaphyllopoulos, D.C., 1972, unpublished results.)

fibrinogen derivatives on the activation of the prothrombin complex, under these conditions, is indirect through the potentiation of the effect of factor VIII and that the preparations of prothrombin complex which were used in the early experiments contained some factor VIII. It was mentioned earlier that the fibrinogen derivatives protect factor VIII from inactivation by thrombin. The potentiation of its activity may well be the result of this protective effect.

Promotion of the Consumption of Prothrombin

In contradistinction to high concentrations, low

concentrations of fibrinogen derivatives (3.5 to 8 mg/ml) promote the consumption of prothrombin.[188] This was best demonstrated with hemophilia A plasma, where the consumption was increased by 6 to 45%. The best results were observed when the plasma was enriched with platelet factor 3 rather than with the Bell-Alton reagent.

Protection of Prothrombin Complex from Inactivation by Thrombin

In 1939, Mertz, Seegers and Smith[234] made the observation that when thrombin is allowed to act upon purified preparations of prothrombin complex, the proenzyme rapidly loses its ability to produce thrombin with the two-stage reagents (tissue thromboplastin, factor V, phospholipids and calcium). The authors found that addition of fibrinogen derivatives to the prothrombin complex before the addition of thrombin reduces significantly the inactivation.[499,467]

Protection of Thrombin from Spontaneous Inactivation

Fibrinogen derivatives rendered free of plasmin by affinity chromatography[501] were found to protect purified (chromatographed) thrombin from spontaneous inactivation. The inactivation of thrombin by antithrombin III, however, was not affected by the fibrinogen derivatives.[500,501]

Effect on the Platelets

In vitro Effect

Kowalski and co-workers[484,487] found that unfractionated fibrinogen digests are potent inhibitors of the aggregation and viscous metamorphosis (release reaction) of the platelets induced by ADP, thrombin, connective tissue contact factor, and kaolin and that they can also interfere with the adhesiveness of these cells to glass and to connective tissue. Jerushalmy and Zucker[486] found similar but weaker effects concerning the aggregation of the platelets by ADP and by connective tissue and the release of serotonin by thrombin. Hirsh et al.,[510] on the other hand, reported that the degradation products do not affect platelet functions (clot retraction, thrombin, and ADP induced aggregation), unless they are removed early during digestion when they can only inhibit the aggregation induced by thrombin. Kowalski et al.[484] and Jerushalmy and Zucker[486] noted that the degree of inhibition paralleled the changes in the antithrombin activity. The digests which exerted the highest antithrombic effect were the most inhibitory. Samples obtained before and after this stage were less active,[486] while very late products were ineffective.[486] Stachurska et al.,[491] on the other hand, found that early and late degradation products inhibited the aggregation of the platelets (by thrombin, ADP, adrenaline, and noradrenaline) more or less to the same extent but the very late products were the most inhibitory. Similarly, Niewiarowski et al.[509] found insignificant differences between early and late degradation products concerning their inhibitory effect on the aggregation of the platelets by ADP.

The potent inhibitory effects which were observed by Kowalski et al.[484] were obtained with digests, which had been dialyzed exhaustively. At variance to these results Larrieu et al.[452,489] noted that most of the ability of the fibrinogen digests to inhibit the aggregation of the platelets by ADP, collagen and adrenaline was abolished by dialysis and that none of the macromolecular fragments (A,B,C,D,X, and Y) were inhibitory. The D and E fragments inhibited thrombin induced aggregation only. They concluded that the dialyzable fragments must be responsible for the inhibition. Similar results concerning the effect of the large fibrinogen fragments were obtained by Gronberg[490] while Barnhart et al.[488] found that late fragment D could induce platelet aggregation by itself and that far from inhibiting the aggregation by ADP, it could actually potentiate it. Niewiarowski et al.[509] confirmed that most, but not all, of the inhibitory activity was abolished by dialysis.

Based on the findings of Larrieu et al.,[452,489] Stachurska et al.[491] studied the effect of concentrated dialyzates of fibrinogen digests or of fractions of them obtained by gel filtration through Sephadex G-25 and found that these preparations inhibited the aggregation of human platelets by ADP, adrenaline, and noradrenaline.

Controversial findings have also been reported regarding the effect of streptokinase and plasminogen or plasmin on the platelet functions. Kowalski et al.[484] found that addition of streptokinase and plasminogen to platelet-rich plasma or to platelet suspensions diminished or completely abolished the ability of the platelets to aggregate and undergo viscous metamorphosis under the influence of ADP, thrombin, or connective tissue

extracts. Cronberg[490] and Niewiarowski et al.,[509] on the other hand, found that addition of streptokinase to platelet-rich plasma[490] or addition of plasmin to washed platelets[509] caused a release reaction and aggregated the platelets.

In vivo Effects

Kowalski et al.[485] injected fibrinogen digests (270 mg/kg body weight) into dogs. They found that the number of circulating platelets decreased, the bleeding time became prolonged, and the ability of the platelets to adhere and also to aggregate and undergo viscous metamorphosis under the influence of thrombin and connective tissue extracts was impaired. The effects were more pronounced when digests with peak antithrombic activity ("early FDP") were injected when profuse bleeding from all operation wounds was observed. Similar results were obtained when streptokinase, together with plasminogen, was injected (3,000 to 3,800 units and 7.5 mg/kg body weight, respectively) or infused (620 units SK + 3 mg Pl/kg/hr, in three consecutive doses) into other dogs.

The authors had found in 1960[480] that infusion into rabbits of large doses (540 to 1,240 mg/kg body weight) of fibrinogen derivatives (AFIF), obtained at the peak of the antithrombic effect, although it rendered the blood of the animals uncoagulable and prolonged their bleeding time, did not cause bleeding from the surgical wounds. Similarly, Barnhart et al.,[488] Cronberg,[490] and Niewiarowski et al.[509] did not report any hemorrhages after infusion of large doses of streptokinase, or urokinase for periods of 22 hr to 4 days or of fibrinogen derivatives (17% to 211% of the fibrinogen of the dog plasma) into dogs, patients, or cats. Cronberg and Niewiarowski et al. found that the infusions affected the reactivity of the platelets to ADP in a variable way: In some cases they inhibited, while in others they potentiated the aggregation. Barnhart and her co-workers, on the other hand, found that each of these agents induced platelet aggregation by itself and decreased the number of circulating single platelets due to formation of aggregates. Cronberg found no decrease in the number of circulating platelets.

Few, if any, of the differences in the results seem to be due to species differences. Most of the in vitro work was performed with platelet-rich human plasma and some with washed human platelets. Similarly, the most controversial of the in vivo findings were obtained in the same species, the dog.[485,488]

Differences in the relative concentrations of the inhibitor and the inducer may explain part of the controversy. Kowalski et al.[484] used small concentrations of inducers (0.25 or 0.5 μg ADP and 0.45 or 0.9 units thrombin/ml) and high concentrations of fibrinogen derivatives (2 to 8 mg/ml). Hirsh et al.[510] used high concentrations of fibrinogen derivatives (12 mg/ml) but their ADP concentration was 20 to 40 or 600 to 1,400 times higher than the concentrations used by the Polish investigators. (It is not clear whether the values reported by Hirsh et al. refer to concentrations in working solution or in the final mixture.) The effect seems to be competitive in nature.[484]

The technique which was used to assess the results may account for one of the controversies. Barnhart et al.[488] studied the effect of fragment D mainly in whole blood; they found that washed platelets and platelet-rich plasma were less suitable for their study. The other investigators used platelet-rich plasma or washed platelets; in addition, they shook the mixtures more or less well before assessing the results. Barnhart and her co-workers counted the number of aggregates in respect to single platelets after gentle agitation or observed the platelets on a formvar coated slide which had been incubated with the platelet-inhibitor mixture without shaking. Formed aggregates were not disturbed before evaluation of the results by this technique, while shaking was found to disaggregate the platelets.

Effect on the Fibrinolytic System

Nanninga and Guest reported in 1968[492] that the "anticoagulant product of fibrinogen" (AC product), most likely fragment D of final stages, inhibited the lysis of fibrinogen and fibrin by fibrinolysin in plasma and in a purified system. Significant inhibition (doubling of the lysis time) of fibrinolysis was observed at concentrations insufficient to cause any antithrombic effect (0.15 mg Ac product/mg of fibrinogen).

Pharmacological Action

Buluk and Malofiejew[494] and Malofiejew[495] studied the action of the small fibrinogen fragments in anesthetized rats and on isolated organs. They found that although concentrated dialyzates of fibrinogen digests do not affect the blood

pressure of the rat or the contractility of the guinea pig ileum and rat uterus when they are tested alone, they can nevertheless influence these functions and the functions of other smooth muscles indirectly by potentiating the effect of biologically active peptides (angiotensin, bradykinin, kallidin, oxytocin) and amines (adrenaline, histamine, acetylcholine, 5-hydroxytryptamine).

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