STUDIES ON A PROTEOLYTIC ENZYME IN HUMAN PLASMA

V. The Relationship between the Proteolytic Activity of Plasma and Blood Coagulation*

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Much confusion exists concerning the relationship between the proteolytic activity of plasma and the coagulation of blood. Evidence has been offered both to support and deny the hypothesis that the activated proteolytic enzyme of plasma accelerates blood clotting. The data relating the proteolytic activity of plasma to clotting were reviewed by Nolf (1), by Ferguson (2), and by Macfarlane and Biggs (3). Delezenne and Pozerski (4) in 1903 reported that serum became proteolytic following the addition of chloroform. Nolf (5) observed that chloroform-treated serum was capable of clotting solutions of fibrinogen, and he suggested that proteolysis was a necessary step in the clotting process (1). The chloroform-activated proteolytic property of plasma was shown by Tagnon (6) to be present in its acid-insoluble globulin fraction. This fraction, too, was reported to clot fibrinogen solutions as well as oxalated plasma (7).

The proteolytic properties of plasma globulin can be activated not only by chloroform, but also by bacteria-free filtrates of cultures of beta hemolytic streptococci (8–11). The active principle present in these filtrates has been called streptococcal fibrinolysin (12) or streptokinase (13). Ferguson (14) observed that a fraction of plasma globulin, activated by streptococcal fibrinolysin, was thromboplastic, and concluded that the proteolytic enzyme of plasma plays a rôle in blood clotting.

Further evidence suggesting a relationship between proteolysis and coagulation has been provided by a study of trypsin inhibitors. The inhibitors of trypsin derived from pancreas and soy bean were observed to inhibit the activity of the proteolytic enzyme of plasma (15, 16). These substances also inhibited blood coagulation (17–19), lending support to the belief that the proteolytic enzyme of plasma participates in the clotting mechanism.

On the other hand, certain data cast doubt that proteolysis plays any physiologic rôle in clotting. In particular, it was reported that purified preparations of the proteolytic enzyme of plasma did not convert prothrombin to thrombin, nor clot fibrinogen (20, 21). Furthermore, preparations of plasma globulin were said to be separable into two fractions. One of these fractions promoted clotting, while the other exhibited proteolytic activity (22).

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In the experiments to be reported, an attempt was made to determine whether any relationship exists between the plasma proteolytic activity and the clotting of the blood. A globulin fraction of plasma, rich in proteolytic activity, was prepared. This fraction was apparently not thrombic, since it did not clot decalcified plasma, and it had little or no thromboplastic activity. It failed to clot platelet-deficient hemophilic plasma, or clotted it only after great delay. This globulin fraction accelerated the clotting of the platelet-deficient plasma of normal individuals, but no relationship was demonstrable between its clot-accelerating and its proteolytic activities. The evidence to be presented indicates that no obvious relationship exists between the proteolytic activity of plasma and the physiologic clotting of blood.

Methods and Materials

Nomenclature.—The nomenclature to be used was discussed in an earlier publication (23). The enzyme present in the acid-insoluble globulin of plasma will be called the plasma proteolytic enzyme, a term synonymous with plasmin (13) or fibrinolysin (21). This enzyme is ordinarily present in plasma as an inactive *precursor*. The filterable principle of cultures of hemolytic streptococci which activates the precursor will be called *streptococcal fibrinolysin* (12) although the newer term streptokinase (13) is frequently used in the current literature. As in previous studies, the term *enzyme* will be used to designate the crude enzyme preparation used, and does not imply a purified product or even a single substance. The name *thromboplastin* will be applied to describe a substance or substances which initiate the conversion of prothrombin to thrombin in the presence of calcium.

Preparation of Enzyme.—The general methods employed in the preparation of precursor were detailed elsewhere (23, 24). Briefly, a globulin fraction was separated from oxalated plasma in the following manner. Oxalated plasma was diluted with 19 volumes of distilled water and the solution was acidified to pH 5.2, as measured with a Beckman laboratory model G pH meter. The resultant precipitate was separated by centrifugation and dissolved in a buffer solution. In the experiments to be described, the *buffer* solution consisted of 7.30 gm. of sodium chloride, 2.76 gm. of barbital, and 2.06 gm. of sodium barbital, diluted to a volume of 1 liter with distilled water. The buffer, then, was composed of 0.125 M sodium chloride, buffered by 0.025 M barbital, at pH 7.5.

Preparation of Streptococcal Filtrate.—In most experiments, the sterile filtrate of a 24 hour culture of the H46A strain of group C hemolytic streptococcus in trypticase soy broth was used as a crude fibrinolysin (23). In some experiments, purified streptococcal fibrinolysin (streptokinase), dissolved in barbital buffer, was used[1]. This preparation, containing 36 units per microgram (25), was dissolved in buffer in a concentration of 10,000 units per ml. of solution, and was stored in a refrigerator at −10°C. until use.

Measurement of Proteolysis.—Enzymatic activity was determined by methods previously described (23, 24). The enzyme-substrate mixture was incubated at 37°C. and the degree of proteolysis was determined by assaying the precipitable protein turbidimetrically before and after digestion. The substrate used was a 0.3 per cent solution of casein (vitamin-test, Lot No. 17218, General Biochemicals, Inc.). Three gm. of casein was dissolved at 56°C. in 900 ml. of water containing 6 ml. of 1 N sodium hydroxide. To this was added 3 ml. of 1 N hydrochloric acid, and the volume brought to 1 liter with water. Thus the final solution contained 3 mg. of casein per ml., in 0.003 N sodium hydroxide and 0.003 N sodium chloride.

[1]Purified streptokinase was obtained through the courtesy of Dr. L. R. Christensen.

To measure the precipitable protein, 2 ml. of 12.5 per cent hydrochloric acid and 0.5 ml. of 20 per cent sulfosalicylic acid were added in rapid succession to a 1 ml. aliquot of the solution to be tested. Maximal turbidity appeared in about 5 minutes, and was read in a cuvette of 8 mm. internal diameter with a Coleman, Jr. spectrophotometer, at a wave length of 420 mμ. With the tube, instrument, and lot of casein used, an optical density of 0.265 was equivalent to a concentration of 1.0 mg. of casein per ml. of enzyme-substrate mixture.

The proteolytic enzyme of plasma digested the preparation of casein used in direct proportion to the concentration of the enzyme preparation until approximately 0.8 mg. of casein per ml. remained. Beyond this point, digestion was not linear. For this reason it was convenient to express the activity of proteolytic enzyme preparations in terms of arbitrary digestion units, such that 50 digestion units of enzyme digested 0.50 mg. of casein per ml. of enzyme-substrate mixture in 1 hour. It should be emphasized that the casein used as substrate differed from that used in experiments reported earlier.

Preparation of Prothrombin-Deficient Plasma.—Oxalated (23) human plasma was shaken with approximately one-fifth its volume of powdered barium sulfate, and incubated at 37°C. for 10 minutes. The barium sulfate was then removed by two successive centrifugations. The supernatant plasma was tested for the presence of prothrombin by the addition of suitable amounts of calcium and thromboplastin derived from rabbit brain (26). In the barium-sulfate–treated plasmas used for the preparation of proteolytic enzyme, no prothrombin was detectable by this method.

Preparation of Platelet-Deficient Plasma.—Blood was collected from the antecubital veins of fasting human subjects, using needles and syringes coated with silicone (27). The blood was transferred immediately to lusteroid centrifuge tubes lined with silicone. Then, without the addition of anticoagulants, it was centrifuged for 10 minutes at 2°C. in an angle centrifuge (rim diameter 10 inches) at 12,500 R.P.M. The upper two thirds of the plasma was transferred with a silicone-lined pipette to a silicone-treated test tube, and stored in an ice bath until used. Platelet counts were performed on this plasma, using methods previously described (27).

Measurement of Clotting Time.—Clotting times were determined in uniform, acid-washed, pyrex Wassermann tubes of 11 mm. internal diameter. In most experiments, the tubes were lined with silicone. The tubes were submerged in an ice bath. Suitable amounts of the solution to be tested and of platelet-deficient plasma were added in succession to each tube. After thorough mixing, the tubes were transferred to a water bath at 37°C., and the clotting times determined to the nearest half minute. Each clotting time was determined in duplicate.

Measurement of Lysis Time.—The tubes containing clotting mixtures were closed with rubber stoppers and incubated at 37°C. in a water bath. After clotting occurred, the tubes were inspected at frequent intervals for evidences of fibrinolysis. The clot lysis time was the interval which elapsed from the time of mixing of platelet-deficient plasma and the test solution until the clot was completely dissolved (28). It was not practical to maintain sterility during these experiments, but there was no evidence that bacterial contamination influenced the results of clot lysis tests.

Measurement of Thromboplastic Activity.—The thromboplastic activity of preparations of globulin was measured by determining their clot-promoting effects on hemophilic plasma. Platelet-deficient hemophilic plasma failed to clot in silicone-treated tubes when incubated for indefinite periods at 37°C. (27). However, tissue thromboplastin clotted hemophilic plasma and platelet-deficient normal plasma equally rapidly. For this reason, platelet-deficient hemophilic plasma was used to detect the presence of thromboplastin in the globulin preparations. Three-tenths ml. of platelet-deficient hemophilic plasma was added to 0.6 ml. of the solution to be tested, and the mixture was then incubated at 37°C. in a water bath. Even highly diluted thromboplastin, derived from rabbit brain, brought about the clotting of this

hemophilic plasma within a few minutes. Therefore, failure of hemophilic plasma to clot within a few minutes after the addition of a globulin preparation was believed to be evidence that this preparation was deficient in thromboplastic activity.

EXPERIMENTAL

1. The Relationship between the Proteolytic Activity of a Plasma Globulin Fraction, and Its Clot-Accelerating Properties.—In preliminary experiments, a study was made of the clot-accelerating properties of proteolytic enzyme prepared from fresh oxalated normal human plasma and activated with chloroform (23, 24). Such chloroform-activated enzyme clotted decalcified plasma. This thrombic property was not present in preparations of proteolytic enzyme made from plasma from which the prothrombin had first been removed. Therefore, in subsequent experiments the proteolytic enzyme was prepared from prothrombin-deficient plasma.

The globulin fraction of normal human plasma has been said to contain an accelerator globulin (29–32) and a plasma thromboplastin (33), both of which have been reported to accelerate the conversion of prothrombin to thrombin. In preliminary experiments, it became evident that plasma globulin containing these substances accelerated the clotting of normal plasma independently of the proteolytic activity of the preparation. Various expedients were used to minimize the effects of these known clot accelerators, so that any clot-promoting activity of plasma proteolytic enzyme might be detected. Platelet-deficient hemophilic plasma has been said to be deficient in plasma thromboplastic activity (33). For this reason, in some experiments platelet-deficient hemophilic plasma was used as a source of enzyme. Prolonged storage of plasma, or heating plasma to 56°C. for 30 minutes, has been said to diminish the activity of both accelerator globulin and plasma thromboplastin (34, 35). In different experiments with hemophilic plasma, both storage and heating were used for this purpose. When normal plasma was used as a source of enzyme, it was necessary to heat the plasma to 58°C. for 30 minutes in order to decrease materially the thromboplastic activity of its globulin fraction. Fibrinogen was precipitated when the plasma was incubated at 56 or 58°C.

In a typical experiment, platelet-deficient plasma, low in thromboplastic activity, was obtained from a patient with hemophilia. This plasma was oxalated and stored at 4°C. for 67 days, in order to decrease the activity of its thromboplastin and accelerator globulin. The plasma was then shaken with one-fifth its volume of powdered barium sulfate and incubated at 37°C. for 10 minutes, to remove prothrombin. The barium sulfate was separated by centrifugation. The supernatant, prothrombin-deficient plasma was heated in a water bath at 56°C. for 30 minutes, to reduce further the activity of its thromboplastin and accelerator globulin, and to precipitate fibrinogen. The heated plasma was centrifuged, and a 6 ml. portion of the supernatant fluid was diluted with 19 volumes of distilled water in a 200 ml. round bottomed centrifuge tube. Sufficient 1 per cent acetic acid was added to bring the pH to 5.2, and the precipitate which appeared was separated by centrifugation. The supernatant fluid was discarded. The precipitate, an acid-insoluble globulin fraction of the plasma, was dissolved in 4 ml. of buffer.

Two ml. of the solution of globulin was pipetted into each of two 50 ml. round bottomed centrifuge tubes. To the first was added 1 ml. of a solution of purified streptococcal fibrinolysin (streptokinase) containing 10,000 units per ml. To the second tube was added 1 ml. of buffer, as a control. The tubes were stoppered and allowed to stand for 5 minutes at room temperature (32°C.). The contents of each of the tubes was then diluted to 40 ml. with distilled water, acidified to pH 5.2 with one-tenth per cent acetic acid, and the resultant precipitate was redissolved in 3 ml. of buffer. The first of these precipitates, then, was a solution of globulin containing fibrinolysin-activated enzyme in the same volume of fluid as the original sample of plasma. The second preparation was a solution of globulin containing an equal concentration of unactivated precursor of plasma proteolytic enzyme.

The clot-accelerating properties of the two globulin preparations were compared. Each globulin was diluted serially in fourfold and twofold steps with buffer, and 0.4 ml. of each dilution was pipetted into a silicone-treated clotting tube. Two-tenths ml. of normal platelet-deficient plasma was then added to each tube, and the clotting time at 37°C. recorded. Each tube was closed with a rubber stopper, and the clots were observed for evidence of fibrinolysis.

Simultaneously, the proteolytic activity of the two globulin preparations was assayed by their ability to hydrolyze casein.

In the experiment just described, the preparation of fibrinolysin-activated plasma proteolytic enzyme contained 440 digestion units per ml. In contrast, the control preparation of globulin, containing the unactivated precursor, did not possess any caseinolytic activity measurable by the technique used.

The clotting times of mixtures of platelet-deficient plasma and each of the globulin preparations are recorded in Table I. In the tubes containing control, unactivated globulin, clot formation was accelerated in proportion to the concentration of globulin. In those tubes with the highest concentrations of fibrinolysin-activated enzyme, no clotting occurred at all. Presumably, proteolysis was sufficiently rapid so that the prothrombin or fibrinogen was digested before clotting could occur. In tubes with lesser amounts of active enzyme, the clotting times were the same as in those containing corresponding amounts of the control, unactivated globulin. It was evident that proteolysis took place in all the tubes with activated plasma proteolytic enzyme, since the clots in all these tubes lysed more rapidly than the control clots.

Plasma proteolytic enzyme, then, was prepared by techniques designed to minimize the activity of such interfering substances as prothrombin, thrombin, fibrinogen, plasma thromboplastin, and accelerator globulin. This proteolytic preparation of globulin accelerated the clotting of normal platelet-deficient plasma. However, its clot-accelerating effect was identical with that of the same fraction of globulin in which the enzyme had not been activated.

2. The Influence of Plasma Proteolytic Enzyme on the Clotting of Hemophilic Platelet-Deficient Plasma.—Hemophilic platelet-deficient plasma was observed to be spontaneously incoagulable when incubated at 37°C. (27). Presumably this was because hemophilic plasma is deficient in plasma thromboplastic activity (33). If plasma proteolytic enzyme had thromboplastic properties, it should be capable of clotting platelet-deficient hemophilic plasma.

The thromboplastic activity of the preparations of globulin used in the experiments described in section 1 was tested in the following manner. Three-tenths ml. of hemophilic platelet-deficient plasma was added to 0.6 ml. of each solution of globulin. The tubes were closed with rubber stoppers, incubated at 37°C. in a water bath, and the clotting times recorded. In many tubes, no clots formed. Conceivably, this may have been due to the digestion of prothrombin or fibrinogen by plasma proteolytic enzyme. For this reason the following test was performed for the presence of prothrombin and fibrinogen. Ninety minutes after the platelet-deficient plasma and the globulin solutions were mixed, an aliquot of 0.2 ml. was transferred to a soft glass test tube containing 0.1 ml. of thromboplastin derived from rabbit brain. Coagulation of this mixture indicated the presence of prothrombin and fibrinogen.

TABLE I

Effect of Proteolytic Enzyme of Plasma on the Rate of Clotting of Normal Platelet-Deficient Plasma

Concentration of globulin	Streptococcal fibrinolysin-activated globulin (440 digestion units/ml.)		Control globulin (0 digestion units/ml.)	
	Clotting time in duplicate	Clot lysis time in duplicate	Clotting time in duplicate	Clot lysis time in duplicate
	min.	*hrs.*	*min.*	*hrs.*
Original plasma concentration	∞, ∞	—	4.0, 4.5	>72, >72
$\frac{1}{4}$ original plasma concentration	∞, ∞	—	6.0, 5.5	>72, >72
$\frac{1}{8}$ " " "	7.5*, 7.5*	0.2, 0.2	6.0, 6.0	>72, >72
$\frac{1}{16}$ " " "	7.5, 7.5	2.5, 2.5	8.0, 7.0	72, 72
$\frac{1}{32}$ " " "	8.5, 8.5	20, 20	8.0, 8.5	72, 72
$\frac{1}{64}$ " " "	10.0, 10.0	44, 44	10.0, 10.0	72 72
$\frac{1}{128}$ " " "	12.5, 12.5	44, 44	10.5, 10.0	72, 72
Buffer control	15.0, 14.0	52, 52	15.0, 14.0	52, 52

0.2 ml. portions of normal platelet-deficient plasma (17 platelets/mm.^{3}) were added to 0.4 ml. of each globulin solution. The clotting times of the mixtures were determined in silicone-treated tubes at 37°C.

* Incomplete clots.

The thromboplastic activity of the preparations of globulin is recorded in Table II. In high concentration, the preparations of plasma globulin were weakly thromboplastic. The same preparations, diluted 16 times, failed to promote the clotting of hemophilic plasma, although an identical amount appreciably accelerated the clotting of normal platelet-deficient plasma (Table I). In repeated experiments, no differences were noted between the clot-promoting effects of preparations containing fibrinolysin-activated enzyme and the same globulin fractions containing the inactive precursor of plasma proteolytic enzyme. Furthermore, the failure of plasma proteolytic enzyme to clot hemophilic plasma was not due to the digestion of prothrombin or fibrinogen. The subsequent addition of thromboplastin derived from rabbit brain promptly clotted plasma which had been mixed with the preparations of globulin (Table II).

3. The Effect of Purified Streptococcal Fibrinolysin on the Clotting Time of Normal and Hemophilic Platelet-Deficient Plasma.—The globulin preparations used in the experiments described in the preceding sections accelerated the clotting of normal platelet-deficient plasma. It is possible that this property of the globulin fraction may have masked a clot-accelerating action of plasma proteolytic enzyme. A method of activating the proteolytic enzyme in the platelet-deficient plasma, without the addition of globulin, would help to clarify the role of proteolysis in blood coagulation. It has long been known that the proteolytic activity of plasma can be activated directly by the addition of

TABLE II

Effect of Proteolytic Enzyme of Plasma on the Rate of Clotting of Hemophilic Platelet-Deficient Plasma

Concentration of globulin	Streptococcal fibrinolysin-activated globulin (440 digestion units/ml.)			Control globulin (0 digestion units/ml.)		
	Clotting time in duplicate	Clot lysis time in duplicate	Clottng time with thrombo-plastin after 90 min.	Clotting time in duplicate	Clot lysis time in duplicate	Clotting time with thrombo-plastin after 90 min.
	min.	*hrs.*	*sec.*	*min.*	*hrs.*	*sec.*
Original plasma concentration	80*, 80*	72, 72	—	43, 43	>72	—
$\frac{1}{16}$ " " "	∞, ∞	—	20, 21	∞, ∞	—	20, 19
Buffer control	∞, ∞	—	20, 22	∞, ∞	—	20, 22

0.2 ml. portions of hemophilic platelet-deficient plasma (80 platelets per c.mm.) were added to 0.4 ml. of each globulin solution. The clotting times of the mixtures were determined in silicone-treated tubes at 37°C. After 90 min. 0.2 ml. portions of the unclotted mixtures were added to 0.1 ml. of rabbit brain thromboplastin and the clotting times recorded.

* Incomplete clots.

potent preparations of streptococcal fibrinolysin (8). Therefore, the effect of a purified preparation of streptococcal fibrinolysin upon the clotting time of normal and hemophilic platelet-deficient plasma was studied.

A solution of 10,000 units per ml. of purified streptococcal fibrinolysin in buffer was serially diluted with buffer. Six-tenths ml. of each dilution of streptococcal fibrinolysin was pipetted into silicone-treated test tubes. Three-tenths ml. of platelet-deficient plasma was then added to each tube. The tubes were closed with rubber stoppers, the mixtures incubated at 37°C in a water bath, and the clotting and lysis times recorded.

The solutions of streptococcal fibrinolysin failed to shorten the clotting time of normal platelet-deficient plasma (Table III). The amount of streptococcal fibrinolysin added was apparently adequate to activate the proteolytic enzyme present in the plasma. As evidence of this, fibrinolysis occurred most rapidly in those tubes with the highest concentrations of streptococcal fibrinolysin.

TABLE III

The Effect of Purified Streptococcal Fibrinolysin on the Rate of Clotting of Normal Platelet-Deficient Plasma

Final concentration of streptococcal fibrinolysin	Clotting time in duplicate	Lysis time in duplicate
units/ml.	*min.*	*hrs.*
6,666	17*, 17*	16, 16
1,667	∞, ∞‡	—
416	19, 17	40, 40
208	12.5, 19	40, 40
104	16.5, 16	48, 48
52	16.5, 15	48, 48
26	16.5, 19	48, 48
13	16.5, 16.5	48, 48
7	16.5, 16.5	48, 48
0	19, 12	48, 48

0.6 ml. portions of solutions of purified streptococcal fibrinolysin in buffer were added to 0.3 ml. of platelet-deficient plasma (17 platelets/mm.^{3}) and the clotting times at 37°C. determined.

* Incomplete clots.

‡ This experiment suggested that purified streptococcal fibrinolysin was less effective in high than in lower concentrations. This phenomenon was described by Christensen and MacLeod (13). Experiments on the caseinolytic activity of globulin activated with the purified streptococcal fibrinolysin used in this study confirmed this.

TABLE IV

Effect of Purified Streptococcal Fibrinolysin on the Rate of Clotting of Hemophilic Platelet-Deficient Plasma

Final concentration of streptococcal fibrinolysin	Clotting time in duplicate	Clotting time with thromboplastin after 24 hrs.*
units/ml.	*min.*	*sec.*
6,666	∞, ∞	*ca.* 1500, *ca.* 1500
1,667	∞, ∞	113, 111
416	∞, ∞	82, 85
208	∞, ∞	43, 42
104	∞, ∞	30, 31
52	∞, ∞	22, 23
26	∞, ∞	17, 17
13	∞, ∞	17, 17
7	∞, ∞	18, 18
0	∞, ∞	17, 17

0.3 ml. portions of hemophilic platelet-deficient plasma (45 platelets per c.mm.) were added to 0.6 ml. of solution of streptococcal fibrinolysin in buffer. The mixtures were observed for clotting for 24 hours at 37°C. After 24 hours 0.2 ml. portions of the unclotted mixtures were added to 0.1 ml. of rabbit brain thromboplastin and the clotting times recorded.

* The clots formed on the addition of thromboplastin were incomplete in the mixtures containing the highest concentrations of streptococcal fibrinolysin.

Platelet-deficient hemophilic plasma, incubated in silicone-treated tubes, failed to clot upon the addition of streptococcal fibrinolysin (Table IV). This suggests that neither activated plasma proteolytic enzyme, nor the preparation of streptococcal fibrinolysin itself was thromboplastic. Twenty-four hours after the platelet-deficient hemophilic plasma and streptococcal fibrinolysin were mixed, an aliquot of each unclotted mixture was tested for the presence of prothrombin and fibrinogen in the manner described in section 2. The data of this experiment are recorded in Table IV. It will be noted that the clotting time of the mixture of hemophilic plasma, streptococcal fibrinolysin, and thromboplastin increased in proportion to the amount of streptococcal fibrinolysin present. In other words, the hemophilic plasma failed to clot even though sufficient streptococcal fibrinolysin was present to result in the hydrolysis of some of the proteins of the plasma.

DISCUSSION

From the teleological point of view, no satisfactory hypothesis has been offered to explain the presence in plasma of a potentially highly active proteolytic enzyme system. That proteolysis is an essential step in the clotting of blood seemed an attractive hypothesis (1). The observation that pancreatic trypsin clotted oxalated plasma (36) seemed to support this view. However, none of the experiments recorded in the literature conclusively demonstrated that proteolysis is essential in the physiologic clotting of the blood. The stumbling block to the solution of the problem has been the difficulty of preparing plasma proteolytic enzyme free from other clot-promoting substances. For example, the proteolytic property of plasma can be activated by chloroform, and such chloroform-activated preparations were observed to clot oxalated plasma (5). However, preparations of enzyme obtained from prothrombin-deficient plasma did not possess this thrombic property (section 1). Presumably, chloroform not only activates precursor, but converts prothrombin to thrombin.

Others have suggested that proteolytic enzyme activated by streptococcal fibrinolysin is thromboplastic. In Ferguson's (14) experiments, the enzyme present in Cohn's fraction I was activated by preparations of streptococcal fibrinolysin. However, fraction I is a complex mixture of proteins, containing not only the precursor of plasma proteolytic enzyme, but also fibrinogen and antihemophilic thromboplastin (35). Proteolytic enzyme prepared from plasma which was deficient in the known clot-promoting substances did not possess thromboplastic activity (section 2).

Finally, it has been observed that various substances which inhibited the activity of plasma proteolytic enzyme also inhibited blood coagulation (16–18, 37). The data reported do not exclude the possibility that these two effects were unrelated.

In the present study, a fraction of globulin was prepared from plasma in such a way as to minimize the activity of certain known substances participating in blood clotting. These included prothrombin, thrombin, fibrinogen, plasma thromboplastin, and accelerator globulin. This preparation of globulin contained considerable potential proteolytic activity, which could be activated by streptococcal fibrinolysin. This fraction of globulin accelerated the clotting of normal platelet-deficient plasma. However, the clot-accelerating effect of the globulin fraction was the same whether or not its proteolytic property had been activated (section 1). This suggested that the clot-accelerating property of the globulin fraction was not related to its proteolytic activity. Furthermore, streptococcal fibrinolysin, added directly to platelet-deficient plasma, activated the proteolytic enzyme present in the plasma without accelerating clotting (section 3).

The nature of the clot-accelerating property in the globulin fraction is not clear. This globulin fraction had little or no thromboplastic activity (section 2). Perhaps the clot-accelerating factor was an artefact of preparation. Or the globulin fraction may have had some non-specific effect on the clotting of plasma. Or perhaps the globulin contained a relatively heat-resistant accelerator of clotting, not identified with plasma thromboplastin or accelerator globulin. Attempts at separation of the clot accelerator in the globulin from the precursor of plasma proteolytic enzyme have, as yet, been unsuccessful.

Furthermore, purified precursor (fibrinolysin) of bovine origin, prepared by Dr. E. C. Loomis,[2] accelerated the clotting of normal platelet-deficient plasma. The presence of this clot-accelerating factor has led to confusion in previous attempts to relate proteolysis to blood clotting.

The possibility cannot be excluded that the *precursor* of plasma proteolytic enzyme may promote blood clotting even though it is apparently proteolytically inactive in respect to fibrinogen, prothrombin, and casein. However, the data presented, suggest that proteolysis by *activated* proteolytic enzyme is not an essential step in the clotting of blood.

SUMMARY

A fraction of globulin was prepared from human plasma which was deficient in prothrombin, thrombin, fibrinogen, plasma thromboplastin, and accelerator globulin. The preparation of globulin contained considerable potential proteolytic activity which could be activated by streptococcal fibrinolysin. This fraction of globulin accelerated the clotting of normal platelet-deficient plasma. However, the clot-accelerating effect of the globulin fraction was the same whether or not its proteolytic property had been activated.

The addition of streptococcal fibrinolysin to normal platelet-deficient plasma

[2]Purified bovine fibrinolysin was obtained through the courtesy of Dr. E. C. Loomis.

did not accelerate coagulation. Nor did the addition of streptococcal fibrinolysin to hemophilic platelet-deficient plasma promote its coagulation.

The data presented suggest that proteolysis by activated plasma proteolytic enzyme is not an essential stage in the coagulation of the blood.

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