JCI The Journal of Clinical Investigation

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J Clin Invest. 1969;48(10):1786-1801. https://doi.org/10.1172/JCI106145.

Research Article

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These studies emphasize the complex nature of the mechanisms which lead to the generation of plasmin in human plasma.

Studies on a Complex Mechanism for the Activation of Plasminogen by Kaolin and by Chloroform: the Participation of Hageman Factor and Additional Cofactors

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ABSTRACT As demonstrated by others, fibrinolytic activity was generated in diluted, acidified normal plasma exposed to kaolin, a process requiring Hageman factor (Factor XII). Generation was impaired by adsorbing plasma with glass or similar agents under conditions which did not deplete its content of Hageman factor or plasminogen. The defect could be repaired by addition of a noneuglobulin fraction of plasma or an agent or agents eluted from diatomaceous earth which had been exposed to normal plasma. The restorative agent, tentatively called Hageman factor–cofactor, was partially purified by chromatography and had an apparent molecular weight of approximately 165,000. It could be distinguished from plasma thromboplastin antecedent (Factor XI) and plasma kallikrein, other substrates of Hageman factor, and from the streptokinase-activated pro-activator of plasminogen. Evidence is presented that an additional component may be needed for the generation of fibrinolytic activity in mixtures containing Hageman factor, HF-cofactor, and plasminogen.

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Received for publication 23 December 1968 and in revised form 4 June 1969.

INTRODUCTION

Plasminogen, a normal constituent of human plasma, is the precursor of plasmin, a protease of broad specificity. The conditions which lead to its activation are still uncertain. Hypotheses suggesting the existence of a physiologic blood activator of plasminogen (1, 2), whose level is enhanced by various stresses (3–9), are supported by a considerable volume of experimental evidence (10–12). The origin of this plasminogen activator is uncertain, although vascular endothelial cells have been implicated (13).

The fibrinolytic properties of normal human plasma, presumably due to the generation of plasmin, are greatly enhanced by incubation with kaolin after acidification and lowering of the ionic strength (14, 15). Niewiarowski and Prou-Wartelle (14) and Iatridis and Ferguson (15) observed that this elaboration of fibrinolytic activity was strikingly reduced in plasma deficient in Hageman factor (Factor XII) and suggested that this clotting factor had a role in the ultimate formation of plasmin. Their hypothesis has been reinforced by the finding that the expected increase in blood fibrinolytic activity in response to physical exertion (16) or venous occlusion (17, 18) was much reduced in some Hageman factor–deficient subjects.

Despite some evidence to the contrary (19), Hageman factor does not seem to activate plasminogen directly (20, 21); it is likely that its role is to catalyze the formation of a plasminogen activator from its precursor (22). In earlier studies, this activator was identified

with that proactivator of plasminogen through which streptokinase brings about the formation of plasmin (23, 24).

In the present study, evidence is assembled that an activator of plasminogen can be derived from human plasma which is separable from the proactivator upon which streptokinase acts. This agent is dependent upon the presence of Hageman factor for its activation or functioning. Some characteristics of this agent, tentatively called Hageman factor–cofactor (HF-cofactor), and some properties of a partially purified preparation are described. The relationship between this substance and plasma thromboplastin antecedent (PTA, Factor XI) and plasma kallikreinogen, two other agents activated by Hageman factor, are explored. Data are furnished suggesting the existence of another plasma component which may be needed for the activation of plasminogen in the presence of Hageman factor.

METHODS

Venous blood was withdrawn from the antecubital veins of normal subjects or patients with specific coagulative disorders, using No. 18 gauge disposable needles and disposable polypropylene syringes (Monoject, Roehr Products Co., Inc., Deland, Fla.) rinsed with silicone oil (SF 96-200, General Electric Co., Waterford, N. Y.). The blood was immediately added to one-ninth volume of either sodium citrate buffer (pH 5.0, 0.13 mole/liter with respect to citrate), 0.13 M sodium citrate, or 0.1 M sodium oxalate solution. In experiments on the effect of calcium ions on the generation of fibrinolytic activity, blood was decalcified by the addition of one-tenth volume of 1.5% disodium ethylenediaminetetraacetic acid dihydrate (EDTA) in 0.7% NaCl solution.

Platelet-deficient plasma was separated from citrated plasma as described earlier (25); its pH was 7.4 ±0.1. *Oxalated* or *EDTA-treated plasma* was separated by centrifugation at 3500 rpm for 15 min at 2°C in silicone-coated cellulose nitrate tubes. Plasmas were used within 4 hr or frozen at —20°C until used.

Cohn fraction IV-1, prepared by the Blood Research Institute, Inc., was obtained from Nutritional Biochemicals Corporation, and was dissolved in barbital-saline buffer at a concentration of 10 mg/ml.

Aluminum hydroxide–adsorbed plasma was prepared by mixing citrated plasma with one-tenth volume of aluminum hydroxide gel (Amphojel without Flavor, Wyeth Laboratories, Philadelphia, Pa.) for 10 min at room temperature. The plasma was separated by centrifugation at 3500 rpm for 10 min at 2°C. *Calcium phosphate–adsorbed plasma* was prepared in the same way by adsorbing oxalated plasma with 10 mg of calcium phosphate per milliliter. Aluminum hydroxide–adsorbed plasma and calcium phosphate–adsorbed plasma are deficient in prothrombin (Factor II), Stuart factor (Factor X), Factor VII, and Christmas factor (Factor IX).

Crude bovine thrombin (Topical Thrombin, Parke, Davis & Co., Detroit, Mich.) was treated with 2-mercaptoethanolamine hydrochloride (Nutritional Biochemical Corporation) by the method of Markus and Ambrus (26) to reduce its plasminogen content, and stored in cellulose nitrate tubes at —20°C; after thawing, it was diluted with barbital-saline buffer to the required concentration.

Bovine fibrinogen (bovine plasma Fraction I, Armour Pharmaceutical Co., Kankakee, Ill.), containing approximately 50% sodium citrate, was dissolved in barbital-saline buffer at 37°C and filtered through Whatman No. 1 paper. The concentration used was 4 mg dry weight/ml.

Human fibrinogen was provided through the kindness of Cutter Laboratories, Berkeley, Calif. It had been prepared essentially by the technique of Bergstrom and Wallen (27) in which Cohn fraction I was precipitated in the presence of ε-aminocaproic acid. The preparation was dissolved in barbital-saline buffer at a concentration of 20 mg/ml.

Streptokinase (Varidase, Lederle Laboratories, Pearl River, N. Y.) was dissolved in barbital-saline buffer at a concentration of 1000 or 2000 Christensen U/ml. The preparation contained 25,000 U of streptococcal desoxyribonuclease for every 100,000 U of streptokinase.

Hirudin (Sigma Chemical Co., St. Louis, Mo.) was dissolved at a concentration of 100 μg/ml in 0.01 M sodium acetate buffer (pH 4.8). According to the manufacturer, it contained 270 U/mg.

Pyrex crushed glass (the gift of Corning Glass Works, Corning, N. Y.) which passed through a 100-mesh sieve but was retained by a 200-mesh sieve was used to prepare glass-adsorbed plasma. The glass was not washed and was discarded after use.

Casein (Hammersten quality, Nutritional Biochemicals Corporation) was prepared as a 1.6% solution in 0.15 M sodium phosphate buffer (pH 7.5) by a minor modification of the method of Mullertz (28). Alternatively, α-casein (Worthington Biochemical Corp., Freehold, N. J.) was dissolved at the same concentration without further preparation.

Lysine methyl ester (Mann Research Laboratories, New York) was dissolved at a concentration of 0.02 mole/liter in 0.1 M sodium phosphate buffer (pH 6.5) containing 0.15 M sodium chloride, and *p-toluenesulfonyl-L-arginine methyl ester* (Nutritional Biochemicals Corporation) was dissolved at a concentration of 0.16 mole/liter in 0.06 M sodium phosphate buffer (pH 7.4).

Hexadimethrine bromide (Polybrene, the gift of Abbott Laboratories, Chicago, Ill.), *heparin* (Sigma Chemical Corp.), and *Liquoid* (sodium polyanetholsulfonate, the gift of Roche Products Ltd., Welyn Garden City, England), were dissolved in water or in 0.01 M sodium acetate buffer (pH 5.2) at suitable concentrations. ε-Aminocaproic acid (Nutritional Biochemicals Corporation) was dissolved in water at a concentration of 0.1 mole/liter; 0.1 M NaCl solution was substituted for ε-aminocaproic acid as a control. *C'1 esterase inhibitor* was the gift of Dr. J. Pensky, Case Western Reserve University.

Phenylmethyl sulfonylfluoride (PMSF) (Calbiochem, Los Angeles, Calif.) was dissolved at a concentration of 0.05 mole/liter in isopropanol.

Carboxymethylcellulose (CM-cellulose) and *diethylaminoethyl cellulose* (DEAE-cellulose) were obtained in microgranular, preswollen form from Reeve Angel, Clifton. N. Y.

Kaolin (acid-washed, N.F., Fisher Scientific Co., Pittsburgh, Pa.), chiefly hydrated aluminum silicate, was suspended in the appropriate buffer with the aid of a mechanical homogenizer. *Bentonite,* a colloidal native hydrated aluminum silicate, was obtained from Prolabo, 12 Rue Pelie, Paris XI°, France. *Dicalite Speedex* (calcined diatomaceous earth) was a gift of Dicalite Division, Great Lakes Carbon Corp., N. Y. Native *diatomaceous earth,* in the form of *Filter Cel,* and calcined diatomaceous earth, *Celite 512,* were gifts of Johns-Manville, Celite Division, New York. *Aluminum ox-*

ide was obtained from J. T. Baker Chemical Co., Phillipsburg, N. J.

Ellagic acid (K. & K. Laboratories, Jamaica, N. Y.) was prepared as a suspension or solution in 0.01 M sodium acetate buffer (pH 4.8) with the aid of a mechanical homogenizer and used without filtration or centrifugation.

Partially purified plasminogen was prepared from Cohn fraction III_4 of human plasma (provided through the kindness of The Cutter Laboratories) by a technique described previously (29). The batch used contained 7.9 Remmert and Cohen units of plasminogen per milligram of protein; about 10% was in the form of plasmin. It was dissolved at a concentration of 1.8 Remmert and Cohen units per ml in 0.15 M sodium phosphate buffer.

Partially purified Hageman factor was prepared as described earlier (20). The relatively impure preparation used contained 2.5% protein and had been purified 175-fold.

Anti-Hageman factor globulin, adsorbed with Hageman factor–deficient or normal plasma, was prepared as reported earlier (30, 31).

O-phenanthroline (Fisher Scientific Co., Fairlawn, N. J.) was dissolved at a concentration of 0.01 M in barbital-saline buffer.

Bradykinin, the gift of Sandoz Inc., Hanover, N. J., was dissolved at a concentration of 1 μg/ml in 0.15 M NaCl solution and stored at —20°C in cellulose nitrate tubes. Before use, it was diluted appropriately in cellulose nitrate tubes; the solutions were highly unstable in containers made of other materials.

Crude *plasma kininogen* was prepared by incubating citrated plasma, which had not been allowed to come into contact with glass surfaces, at 61°C for 60 min to destroy its content of Hageman factor and plasma kallikreinogen; the plasma was centrifuged and the supernatant fluid stored at —20°C.

Glass-adsorbed plasma was prepared from citrated plasma which had been handled in silicone-coated containers. The plasma was shaken with crushed glass in silicone-coated cellulose nitrate tubes for 60 min at room temperature, using a mechanical shaker (Eberbach Corp., Ann Arbor, Mich.). The concentration of glass used was usually 200 mg/ml of plasma. After centrifugation at 3000 rpm for 10 min, the adsorbed plasma was removed with silicone-coated pipettes and stored in aliquots in silicone-coated cellulose nitrate tubes at —20°C.

"Exhausted" plasma was prepared essentially as described by Nossel (32). Citrated plasma was shaken with Celite 512 (50 mg/ml) at room temperature for 15 min in cellulose nitrate tubes. The Celite was deposited by centrifugation at 3000 rpm for 10 min; the supernatant plasma was incubated at 37°C in silicone-coated cellulose nitrate tubes for 6 hr and stored at —20°C.

Euglobulin precipitates of plasma for chloroform activation were obtained by adding 19 volumes of sodium acetate buffer (pH 4.8, 0.01 mole/liter with respect to acetate) to 1 volume of plasma; the pH of the mixture was approximately 5.2. After separation by centrifugation, the precipitate was dissolved in a volume of 0.15 M sodium phosphate buffer (pH 7.5) or barbital-saline buffer one-fourth that of the original plasma.

Crude *Celite eluates* of normal or Hageman factor–deficient plasma were prepared by shaking plasma with Celite 512 (40 mg/ml) at room temperature for 10 min. The Celite was deposited by centrifugation and washed with 0.15 M NaCl solution until the washings no longer produced a visible precipitate on the addition of an equal volume of 10% trichloroacetic acid. Elution was performed with a volume of 10% sodium chloride in 0.05 M Tris buffer (pH 8.0) equal to that of the original plasma. The eluates were dialyzed overnight at 4°C against barbital-saline buffer and stored at 4°C until used. Celite eluates from normal plasma, prepared with citrate buffer, contained traces of thrombin which was neutralized by the addition of hirudin. If 0.13 M sodium citrate or 0.1 M sodium oxalate was used as the anticoagulant, thrombin was not detectable in the eluates.

Partially purified Hageman factor–cofactor (HF-cofactor) was prepared from pools of approximately 1000 ml of citrated human plasma. The plasma was stirred for 15 min with 20 mg Celite 512/ml at room temperature, using a magnetic stirrer. The adsorbed plasma was separated by filtration through Whatman No. 1 paper, using a Büchner funnel, and the Celite was washed repeatedly with 0.15 M NaCl until the washings no longer contained material precipitable by the addition of an equal volume of 10% trichloroacetic acid. The washed Celite was eluted by the repeated addition of small quantities of 0.05 M Tris buffer (pH 8.0) containing 0.5 M NaCl. The total volume of eluate equaled the volume of the original plasma. The eluate was dialyzed overnight against 20 volumes of 0.01 M sodium acetate buffer (pH 5.2) and concentrated at 4°C to approximately one-fourth of the volume of the original plasma in an Amicon Ultrafiltration Cell (Amicon Corp., Cambridge, Mass.) with a Diaflo membrane UM10. The concentrated eluate was applied to a 2.5 × 35 cm CM-cellulose column previously equilibrated with 0.01 M sodium acetate buffer (pH 5.2) at 4°C. Sodium phosphate buffer (0.067 mole/liter, pH 6.0) was passed through the column until the effluent pH had risen to pH 6.0. The protein eluted during this stage had no HF-cofactor activity. Elution of the HF-cofactor was achieved by a linear gradient of 250 ml of 0.067 M sodium phosphate buffer (pH 6.0) and 250 ml of the same buffer in 0.5 M NaCl. Approximately 10-ml fractions were collected in cellulose nitrate tubes at a rate of 20–30 ml/hr. Individual fractions were dialyzed overnight against 40 volumes of 0.01 M sodium acetate buffer (pH 5.2), 0.01 M sodium phosphate buffer (pH 7.5), 0.025 M barbital buffer (pH 7.5), or barbital-saline buffer, as appropriate, at 4°C., and kept at that temperature until used. Typically, the protein concentrations in pools of fractions containing HF-cofactor was approximately 0.15 mg/ml. In some experiments, active fractions were pooled and concentrated in an Amicon Ultrafiltration Cell before dialysis against the appropriate buffer.

For use in some experiments, the CM-cellulose eluate, in 0.025 M barbital buffer, was applied to columns of DEAE-cellulose (0.9 × 25 cm), equilibrated with the same buffer. Active material was eluted by applying a linear gradient of 125 ml of barbital buffer and 125 ml of barbital-saline buffer.

The purification procedures outlined did not separate HF-cofactor from activated PTA or from a plasma kallikrein. Trace amounts of thrombin-like activity were present in some CM-cellulose eluates prepared from plasma containing citrate buffer. Perhaps this was because Celite contains 0.5% calcium oxide, sufficient to yield appreciable concentrations of calcium ions. This problem was circumvented in some experiments by using oxalated plasma adsorbed with calcium phosphate. In most experiments, appropriate amounts of hirudin were added to neutralize any thrombin which may have been present.

PMSF-treated HF-cofactor was prepared from HF-cofactor, separated by CM-cellulose chromatography. A

volume of 1.8 ml of HF-cofactor was incubated with 0.2 ml of 0.05 M PMSF for 15 min at room temperature and was then dialyzed overnight against 2 liters of barbital-saline buffer. As a control, 1.8 ml of HF-cofactor was incubated with 0.2 ml of isopropanol and dialyzed in a separate flask.

Acid-treated HF-cofactor, in 0.01 M sodium acetate buffer (pH 5.2), was prepared by adjusting the pH to 2.0 with 1 N hydrochloric acid. After 10 min at 25°C, the mixture was readjusted to pH 5.2 by the addition of 1 N sodium hydroxide. As a control, a volume of 0.5 M NaCl solution, equal to the combined volumes of hydrochloric acid and sodium hydroxide, was added to HF-cofactor.

Barbital-saline buffer was composed of 0.025 M sodium barbital and 0.125 M sodium chloride at pH 7.5 (33). *Sodium acetate buffers* of appropriately molarity and hydrogen ion concentration were prepared by the method of Walpole (34).

Parafilm (Marathon Div., American Can Co., Menasha, Wis.) was used as a nonwettable covering of the tubes during mixing.

Measurement of kaolin-induced generation of fibrinolytic activity in plasma. 0.5 ml plasma was incubated with 0.25 ml kaolin suspension (8 mg/ml) and 9.25 ml 0.01 M sodium acetate buffer (pH 4.8) in a 16 × 100 mm Lusteroid tube at 37°C for 60 min unless otherwise noted; the pH of this mixture was approximately 5.2. After centrifugation at 3000 rpm for 5 min, the supernatant solution was discarded and the euglobulin precipitate, containing kaolin, was resuspended in 0.5 ml barbital-saline buffer with the aid of a wooden applicator stick. 0.2 ml aliquots of the euglobulin: kaolin suspension were transferred with a plastic pipette to 10 × 75 mm polystyrene tubes in an ice bath; 0.1 ml bovine fibrinogen and 0.1 ml bovine thrombin (50 NIH U/ml) were added to each tube which, after mixing, was transferred to a waterbath at 37°C. The clot lysis time of each mixture was measured as the interval elapsing from the addition of thrombin until complete lysis of the fibrin which formed had taken place. All reported lysis times are the means of duplicate assays.

It was calculated from the results of 100 consecutive assays with lysis times ranging from 3 to 276 min that the difference between duplicates, as a percentage of the mean lysis time, had a mean value of 4.5% (SD 4.8%).

Lysine methyl esterase activity generated in kaolin-treated euglobulin mixtures was measured in the following way. Oxalated plasma was adsorbed with 100 mg barium sulfate (Baker Chemical Co.)/ml for 10 min at room temperature and separated by centrifugation at 3500 rpm for 5 min at 2°C to reduce its content of prothrombin. Kaolin-induced fibrinolytic activity was then generated by the method described. A mixture of 0.2 ml euglobulin (or buffer) and 3.0 ml lysine methyl ester was then incubated at 37°C for 121 min. 1 ml aliquots were removed at 1 and 121 min after incubation had begun, mixed with 0.5 ml 15% trichloroacetic acid, and recentrifuged. The methyl alcohol content of the samples was then estimated by the method of Siegelman, Carlson, and Robertson (35).

Chloroform activation of euglobulin precipitates was carried out as described previously (36) in 40-ml round-bottom Pyrex centrifuge tubes. After incubation of the euglobulin: chloroform mixtures at room temperatures for 18 hr and centrifugation at 2500 rpm for 5 min, the fibrinolytic and caseinolytic activities were measured in the supernatant fluids.

Fibrinolytic activity of chloroform-activated euglobulin solutions was measured by determining the lysis time at 37°C of a clot formed from 0.2 ml euglobulin dissolved in barbital-saline buffer, 0.4 ml bovine fibrinogen, and 0.1 ml bovine thrombin (20 NIH U/ml).

"Streptokinase-proactivator" activity was measured by incubating a mixture of 0.1 ml plasma diluted 1 : 30 in barbital-saline buffer, 0.2 ml bovine fibrinogen, 0.1 ml streptokinase (1000 Christensen U/ml) and 0.1 ml bovine thrombin (20 NIH U/ml) at 37°C in 10 × 75 mm polystyrene tubes. The time between the addition of thrombin and lysis of the fibrin was noted.

Plasma plasminogen concentrations were assayed by a modification of the method of Remmert and Cohen (37–39).

Assays for antihemophilic factor (Factor VIII), Christmas factor (Factor IX), plasma thromboplastin antecedent (PTA, Factor XI), Hageman factor (Factor XII), proaccelerin (Factor V), prothrombin (Factor II), Stuart factor (Factor X), and Factor VII were performed by techniques summarized earlier (40).

The fibrinolytic activity of partially purified HF-cofactor after chromatography on CM columns was assessed by adding 1 ml of each fraction to 0.5 ml glass-adsorbed plasma, 0.1 ml hirudin (100 μg/ml), 0.25 kaolin (8 mg/ml), and 8.15 ml 0.01 M sodium acetate buffer, pH 4.8, in a 16 × 100 mm cellulose nitrate tube. After incubation at 37°C for 60 min, the precipitate was deposited by centrifugation and resuspended in 0.5 ml barbital-saline buffer. The remainder of the assay was carried out as described above. Hirudin was added to prevent clotting of the glass-adsorbed plasma by coagulation factors present in the partially purified HF-cofactor preparation.

Caseinolytic activity was measured in different ways. The digestion of casein by kaolin-treated plasma, chloroform-treated plasma, and partially purified Hageman factor–cofactor was measured by a modification of the method of Remmert and Cohen (37). One part of the mixture to be tested was mixed with three parts of 1.6% casein and incubated at 37°C for 4 hr. At 2 min and again at 4 hr aliquots of the mixture were removed and mixed with an equal volume of 10% trichloroacetic acid. The tubes were centrifuged after a minimum of 15 min, the supernatant solutions filtered through glass wool, and their optical densities read at 280 mμ in a Beckman Model DU spectrophotometer equipped with a Gilford Instrument Company absorbance meter. Optical density was converted to micrograms of tyrosine per milliliter by reference to a standard tyrosine solution. To test the caseinolytic activity of kaolin-treated plasma, two parts of plasma, one part of kaolin (8 mg/ml), and 37 parts of 0.01 M sodium acetate buffer (pH 4.8) were incubated in cellulose nitrate tubes at 37°C. At intervals, aliquot tubes were centrifuged and the deposited euglobulin: kaolin precipitate suspended in 1 ml of 0.15 M sodium phosphate (pH 7.5). Incubation at 37°C was then restarted after the addition of 3 ml of 1.6% casein. Casein hydrolysis of chloroform-activated euglobulin solutions was measured by incubating 0.8 ml euglobulin, in 0.15 M sodium phosphate buffer (pH 7.5), and 3 ml 1.6% casein in the same manner. The caseinolytic activity of partially purified HF-cofactor was determined by incubating suitable amounts, in 0.01 M sodium acetate buffer (pH 5.2), glass-treated plasma (adjusted to pH 5.2 with 1.0 N hydrochloric acid just before use), kaolin suspension, and acetate buffer at 37°C for periods up to 90 min in cellulose nitrate tubes. The mixtures were then centrifuged at 2°C for 5 min at 3500 rpm, the precipitates suspended in 0.15 M sodium phosphate buffer and three volumes of casein then added. The effect of inhibitors was tested by substituting 1 ml of the agent to be tested for 1 ml of acetate buffer before the first period of incubation.

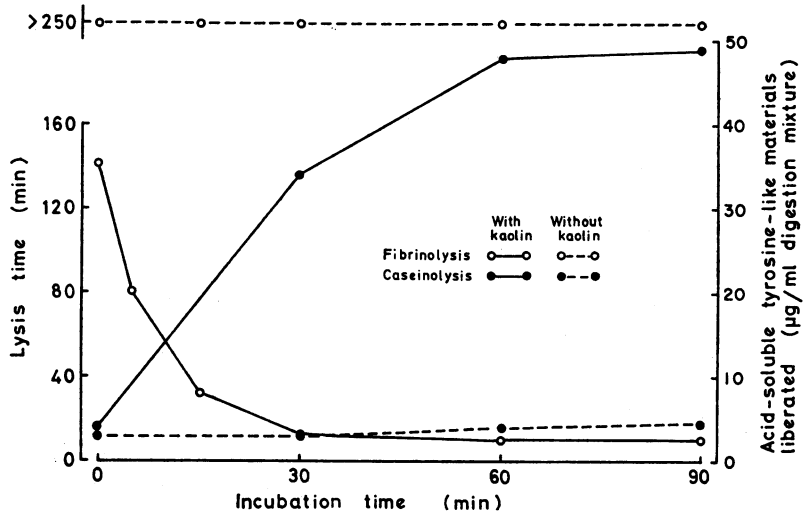

FIGURE 1 The generation of fibrinolytic and caseinolytic activity in diluted and acidified normal plasma in the presence and absence of kaolin.

The inhibitor was added to control tubes after this period and before centrifugation.

The *kallikrein-like activity* of partially purified HF-cofactor was estimated by incubation 0.1 ml of 0.01 M *o*-phenanthroline, 0.1 ml crude kininogen, 0.1-0.6 ml of the fraction to be tested, and sufficient barbital-saline buffer to bring the volume of the mixture to 1.0 ml, in polystyrene tubes for 15 min at 37°C. The effect of aliquots of this mixture was then tested upon the rat uterus by a technique used earlier (41).

Acrylamide-gel disc electrophoresis was performed as described previously (42).

The esterolytic activity of HF-cofactor was measured by incubating 2 ml of 0.16 M *p*-toluenesulfonyl-L-arginine methyl ester and 2 ml of partially purified Hageman factor–cofactor, as the CM-cellulose eluate, at pH 7.4 in disposable glass tubes (I. D. 11 mm). At the start and after 4 hr at 37°C, 1 ml aliquots were mixed with 38% formaldehyde (Fisher Scientific Co.), pink to phenolphthalein. Esterolytic activity was estimated by determining the volume of 0.05 N sodium hydroxide needed to bring the mixtures to pH 7.4.

A crude *estimate of the molecular weight* of HF-cofactor was obtained by applying 4 ml of a highly concentrated CM-cellulose eluate, dialyzed against barbital-saline buffer, to a column of Sephadex G200 (Pharmacia Fine Chemicals, Uppsala, Sweden), 2.5 × 33 cm in size. The column was eluted with the same buffer. Successive fractions were tested for protein by adsorption at 280 mμ, and for activated PTA, HF-cofactor activity, and kallikrein-like activity. The void column was determined by filtering Blue Dextran 2000 (Pharmacia Fine Chemicals, Inc.), 0.1% in 10% sucrose solution; 1% bovine gamma globulin (mol wt 160,000) and 1% bovine albumin (mol wt 67,000) (Armour Pharmaceutical Co.) in barbital-saline buffer were used as markers. The molecular weights of activated PTA, HF-cofactor, and plasma kallikrein were estimated by using the assumption that a linear relationship existed between the logarithm of the molecular weight of the markers and the elution volume at their point of maximal optical adsorption. The estimated value of the molecular weight of HF-cofactor was about the same in four determinations, but an error of approximately 20% was inherent in the method because 4-ml fractions were used, a volume needed to carry out all the needed assays.

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (43), and was estimated more crudely by adsorption at 280 mμ.

Dialyses were performed at 4°C in cellophane casings (Visking Corp., Chicago, Ill.) prepared by boiling in 0.0001 M EDTA, followed by thorough washing in distilled water.

Centrifugations were carried out in an International PR-2 refrigerated centrifuge at 2°C or in an International Universal Model UV centrifuge at room temperature.

RESULTS

I. The generation of fibrinolytic activity in normal plasma incubated with kaolin. As Niewiarowski (14) and Iatridis (15) and their associates reported, fibrinolytic activity generated when diluted, acidified normal plasma was incubated with kaolin. At the same time, the mixture acquired the capacity to hydrolyze casein and lysine methyl ester, two substrates of plasmin. These properties were present in euglobulin precipitates separated after incubation (Fig. 1).

The rate of generation of fibrinolytic activity increased with the concentration of kaolin over the range 0.5–2 mg/ml of plasma; no further increase occurred with higher concentrations. Kaolin appeared to influence the generation of fibrinolytic activity; if plasma was diluted with acetate buffer and incubated for 1 hr, no appreciable fibrinolytic activity was detected in the euglobulin precipitate even though kaolin was added before assay.

The generation of fibrinolytic and caseinolytic properties required factors in both the euglobulin precipitate and the supernatant fraction of acidified, diluted plasma. No activity evolved when the euglobulin precipitate was separated from other constituents of plasma by centrifu-

gation *before* incubation, resuspended in 19 volumes of 0.01 M sodium acetate buffer (pH 5.2) and incubated for 60 min (Fig. 2).

II. Evidence for the participation of Hageman factor in the kaolin-induced generation of fibrinolytic activity. As Iatridis and Ferguson (44) reported, the kaolin-induced generation of fibrinolytic activity was defective in plasma, deficient in Hageman factor, but was normal in plasmas deficient in other clotting factors. Thus, normal activity evolved in plasmas deficient in PTA, Christmas factor, antihemophilic factor, Stuart factor, factor VII, or proaccelerin, as well as in plasmas adsorbed with aluminum hydroxide gel or calcium phosphate to reduce their content of prothrombin, Factor VII, Christmas factor, and Stuart factor. Particular attention was paid to plasma from PTA-deficient patients because these contained 5–10% of normal PTA activity. Two such plasmas were diluted with glass-adsorbed plasma (see section IV) and the fibrinolytic activity generated compared with similarly diluted normal plasma. The PTA-deficient plasmas behaved like normal plasma when each was diluted 20-fold with glass-adsorbed plasma.

The importance of Hageman factor in the evolution of fibrinolytic activity was confirmed by demonstrating that the addition of partially purified Hagemen factor corrected the defect in plasma deficient in this agent (Table I). Similarly, specific anti–Hageman factor rabbit globulin greatly diminished the kaolin-induced generation of fibrinolytic activity in normal plasma. For example, the clot lysis time of normal plasma, treated with kaolin, was 16 min, whereas that of the same plasma, treated with specific antiserum, was 136 min. The specificity of the antiserum was assured by its absorption with Hageman factor–deficient plasma.

The small quantities of normal plasma needed to correct defective generation of fibrinolytic activity in Hageman factor–deficient plasma (Fig. 3) are consistent with the possibility that Hageman factor acts enzymatically in the kaolin-induced generation of fibrinolytic activity.

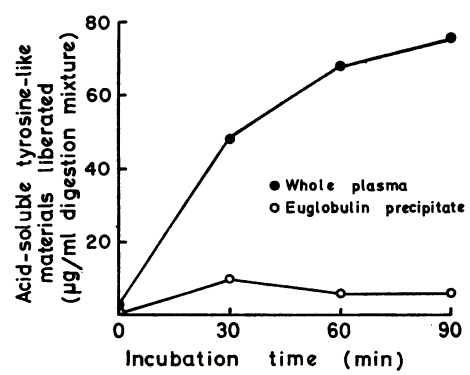

FIGURE 2 Comparison of the generation of caseinolytic activity in plasma and in its separated euglobulin precipitates.

TABLE I

Effect of Adding Purified Hageman Factor to Hageman Factor-Deficient Plasma, Glass-Adsorbed Plasma, and Unadsorbed Normal Plasma on Their Kaolin-Induced Generation of Fibrinolytic Activity

Test mixture*	Lysis time
	min
Hageman factor–deficient plasma + Hageman factor	19
Hageman factor–deficient plasma + water	127
Glass-adsorbed plasma + Hageman factor	101
Glass-adsorbed plasma + water	98
Normal plasma + Hageman factor	7
Normal plasma + water	7.5

* 0.1 ml purified Hageman factor (5 mg/ml) or 0.1 ml water added to 0.5 ml of each plasma immediately *before* the addition of kaolin, dilution with acetate buffer, and incubation at 37°C for 60 min (see Methods).

III. Factors influencing the kaolin-induced generation of fibrinolytic activity. Serum, obtained from whole blood diluted with one-ninth volume of 0.15 M NaCl and kept at 37°C for 4 hr, generated approximately the same degree of fibrinolytic activity as citrated plasma prepared from the same sample of blood.

The ability of plasma to generate fibrinolytic activity in the presence of kaolin was not diminished by incubation at 37°C for 48 hr, but was abolished by incubation at 56°C for 30 min; such treatment had no appreciable effect upon the plasma's content of Hageman factor and plasminogen. Plasmas adjusted to pH 2.0 or pH 10.0 by addition of 0.1 N hydrochloric acid and 0.1 N sodium hydroxide, respectively, and then neutralized after 10 min, had a striking loss of fibrinolytic activity, while retaining their original content of Hageman factor and

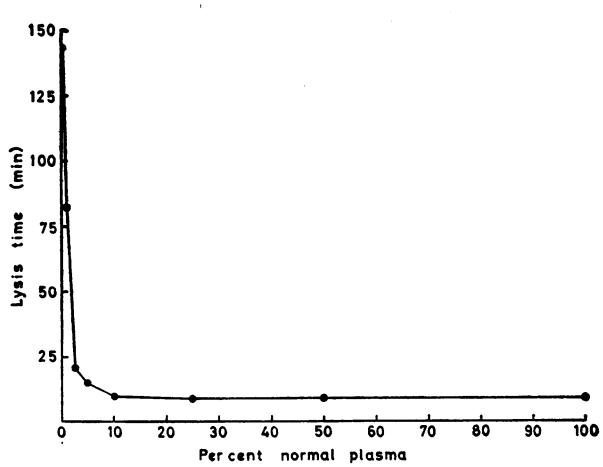

FIGURE 3 The generation of fibrinolytic activity in mixtures of Hageman factor–deficient and normal plasma.

TABLE II

Effect of Glass Adsorption of Plasma on Its Kaolin-Induced Generation of Fibrinolytic Activity, Plasminogen Concentration, and Hageman Factor Content

Sample	Untreated plasma			Glass-adsorbed plasma		
	Fibrinolytic activity, lysis time	Plasminogen*	Hageman factor assay	Fibrinolytic activity, lysis time	Plasminogen*	Hageman factor assay
	min		*sec*	*min*		*sec*
1	7.5	—	58.6	63.5	—	58.0
2	14.5	—	66.0	79	—	64.3
3	10	—	62.4	93	—	62.9
4	10.5	53	70.1	114	104	73.7
5	9	50	57.2	98	97	57.2
6	11.5	54	59.7	119	116	59.5

* Casein hydrolysis: expressed as acid-soluble tyrosine-like materials liberated in micrograms per milliliter of digestion mixture.

plasminogen. These experiments suggested that besides Hageman factor and plasminogen one or more other agents were needed to generate fibrinolytic activity.

The kaolin-induced generation of fibrinolytic activity was temperature-dependent and was maximal at 37°C. For example, in one experiment, fibrinolytic activity, measured as the clot lysis time, was 97 min when diluted, acidified plasma was incubated for 30 min with kaolin at 1°C, 26 min at 21°C, 8 min at 37°C, and 27.5 min at 45°C.

The development of fibrinolytic activity was also pH-dependent. The influence of hydrogen ion concentration was tested by measuring the generation of fibrinolytic activity in the presence of sodium acetate buffers of pH 4.8–6.0 (0.01 mole/liter with respect to acetate). To exaggerate any differences, the experiment was performed on a mixture of 5% normal and 95% glass-adsorbed plasma. Generation was maximal at pH 5.2. Visible euglobulin precipitation decreased above this pH, and, concurrent with its virtual absence at pH 6.0, generation of fibrinolytic activity was markedly reduced. The generation of fibrinolytic activity was also influenced by the molarity of the sodium acetate buffer. As the molarity was increased from 0.01 to 0.20, generation was progressively and markedly reduced. This reduction was due largely to acetate ion rather than to the increase in ionic strength per se, for there was only a minimal decrease in the evolution of fibrinolytic activity when plasma was incubated with 0.01 M sodium acetate in increasing concentrations of NaCl.

Calcium did not seem necessary for the kaolin-induced generation of fibrinolytic activity. The activity evolved during 60 min incubation of EDTA-treated plasma at 37°C was the same whether calcium ions were absent or were added to a concentration of 5 mmoles/liter. The lysis time doubled when the concentration of calcium was increased to 10 mmoles/liter. Thus, calcium ions at concentrations sufficient to overcome the chelating effect of EDTA were, in fact, inhibitory. The activity of other ions was not investigated.

IV. The effect of adsorption of plasma with crushed glass or diatomaceous earth on its capacity to generate fibrinolytic activity. The experiments described thus far suggested that factors additional to Hageman factor and plasminogen were needed for the generation of fibrinolytic activity under the circumstances described. In agreement with this, treatment of normal plasma with crushed glass at a concentration of 200 mg/ml lengthened its kaolin-induced lysis time, but did not reduce its content of Hageman factor or plasminogen (Table II). In these experiments, the assay for Hageman factor was not affected by the presence of activated PTA, resulting from treatment of plasma with glass. If such glass-treated plasma was incubated at 37°C overnight, its content of PTA was reduced to less than 1%, but the concentration of Hageman factor appeared to be unaltered.

Higher concentration of glass lengthened the lysis time further, but at the same time removed increasing quantities of Hageman factor. The generation of fibrinolytic activity in glass-treated plasma was not increased by the addition of partially purified Hageman factor (Table I). Conversely, as little as 20% Hageman factor–deficient plasma significantly increased the fibrinolytic potential of glass-treated plasma. Presumably, treatment of normal plasma with glass had removed one or more components present in Hageman factor–deficient plasma and needed for the kaolin-induced generation of fibrinolysis.

Citrated plasma, adsorbed with diatomaceous earth (Celite 512) at a concentration of 50 mg/ml and then incubated at 37°C for 6 hr, behaved as if depleted of additional factors required for fibrinolysis besides those

TABLE III

Effect of Adsorption of Plasma with Increasing Concentrations of Crushed Glass on the Generation of Fibrinolytic Activity, "Streptokinase-Proactivator" Activity, and Hageman Factor Content

Concentration of crushed glass	Fibrinolytic activity, lysis time	SK-proactivator activity	Hageman factor assay	
			Clotting time	Activity
mg/ml	*min*	*sec*	*sec*	*%*
0	14.5	260	66.0	100
200	79	265	64.3	100
400	105	260	75.0	55
600	159	255	81.5	28

SK = Streptokinase.

removed by treatment with glass. The kaolin-induced lysis time of such "exhausted" plasma consistently exceeded 250 min; this time was not shortened by the addition of partially purified Hageman factor. The residual Hageman factor activity in five samples ranged from less than 1% to 2.6%. In the four samples tested, the plasminogen content was reduced by 6 to 18% (mean 13%). Thus, exhausted plasma lacked both the factor or factors which speeded fibrinolysis and were removed by crushed glass, and all but traces of Hageman factor.

Whatever the nature of the agents removed from plasma by glass or diatomaceous earth, they were not needed for activation of plasminogen by streptokinase. Adsorption of normal plasma with crushed glass at concentrations which greatly lengthened its kaolin-induced lysis time, did not alter its streptokinase-proactivator activity (Table III). Several different adsorbents were tested for their ability to remove factors needed to speed fibrinolysis. No correlation was found between the residual streptokinase-proactivator activity and the plasma component needed for the generation of fibrinolytic activity in the presence of kaolin (Table IV). These experiments provided further evidence for their separate identity. In the experiment depicted in Table IV, Filter-Cel at a concentration of 15 mg/ml did not appear to deplete plasma of its fibrinolytic potential. This result was atypical; more usually, adsorption of plasma with as little as 5 mg/ml lengthened its kaolin-induced clot lysis time.

V. The effect of ellagic acid on the generation of fibrinolytic activity. Experiments with ellagic acid demonstrated that the lytic effects of kaolin could not be attributed solely to an action upon Hageman factor. At concentrations as low as 10^{-9} mole/liter, solutions of ellagic acid activate Hageman factor (45). The generation of fibrinolytic activity was measured in normal plasma after the addition of suspensions or solutions of ellagic acid in sodium acetate buffer (0.01 mole/liter, pH 4.8). At concentrations of ellagic acid of 10^{-8} mole/liter and 10^{-4} mole/liter, at which this substance was incompletely soluble in acetate buffer, some generation of fibrinolytic activity took place, much less than that induced by the addition of kaolin (Table V). At lower concentrations, generation of fibrinolytic activity was not enhanced by ellagic acid. Further, no caseinolytic activity developed during the incubation of diluted, acidified plasma for 90 min in the presence of ellagic

TABLE IV

The Kaolin-Induced Generation of Fibrinolytic Activity, "Streptokinase-Proactivator" Activity, and Hageman Factor Content of Plasma Adsorbed with Insoluble Substances

Adsorbent*	Concentration	Fibrinolytic activity, lysis time	SK-proactivator, activity	Hageman factor assay	
				Clotting time	Activity
	mg/ml	*min*	*sec*	*sec*	*%*
None	—	11.5	271	72.0	100
Aluminium oxide	100	11	267	—	—
Bentonite	15	1200	432	134.0	12
Dicalite Speedex	25	300	273	80.6	70
Filter Cel	15	10.5	274	99.4	38

* Adsorption performed by shaking plasma with test material for 10 min at room temperature; the adsorbent was deposited by centrifugation at 3000 rpm for 5 min.

TABLE V

Effect of Ellagic Acid on the Generation of Fibrinolytic Activity

Incubation time	Lysis time, incubation mixture*				
	Buffer	Ellagic acid, 10^{-5} M	Ellagic acid, 10^{-4} M	Ellagic acid, 10^{-3} M	Kaolin
min			*min*		
0	>450	>450	>450	>450	190
60	410	340	142	46	8
120	300	240	85	51	7
180	210	160	87	90	8
240	110	100	90	95	9

* 0.5 ml normal plasma, 0.25 ml kaolin (8 mg/ml), or 0.01 M sodium acetate buffer (pH 4.8), and 9.25 ml sodium acetate buffer containing ellagic acid or buffer alone were incubated at 37°C in 16 × 100 mm Lusteroid tubes and the fibrinolytic activity generated assayed at intervals.

acid (2.5×10^{-6} mole/liter), nor did this concentration affect the kaolin-induced generation of caseinolytic activity. Evidently, kaolin had an effect upon the development of fibrinolytic activity over and above the activation of Hageman factor.

VI. Evidence for the participation of Hageman factor in the activation of plasminogen by chloroform. For reasons not yet clear, proteolytic activity develops slowly in human plasma and serum, or their euglobulin fractions, incubated with chloroform (46, 47). Thus, the euglobulin fraction of normal plasma lysed fibrin and digested casein readily after incubation in the presence of chloroform but not in the absence of this solvent (Table VI). Similarly, normal fibrinolytic and caseinolytic activities appeared upon incubation of mixtures of chloroform and the euglobulin fractions of plasmas deficient in PTA, Christmas factor, antihemophilic factor, or proaccelerin. In contrast, similarly treated euglobulin prepa-

TABLE VI

Proteolytic Activity of Chloroform-Treated Euglobulins from Normal and Glass-Adsorbed Plasmas, and from Plasma Samples Deficient in Different Clotting Factors

Source of euglobulins		Fibrinolytic activity, lysis time	Caseinolytic activity, soluble tyrosine-like materials liberated
		hr	*μg/ml digestion mixture*
Normal plasma	(i)	1.5	100
	(ii)	4.0	74
	(iii)	—	88
Glass-adsorbed plasma	(i)	10.0	12
	(ii)	>12 <24	10
	(iii)	—	6
Hageman factor–deficient plasma	(i)	12.5	10
	(ii)	>12 <24	9
	(iii)	—	4
PTA-deficient plasma		4.5	52
Christmas factor–deficient plasma		3.5	42
Antihemophilic factor–deficient plasma		3.0	60
Proaccelerin (Factor V)–deficient plasma		2.0	69

TABLE VII

Effect of Hexadimethrine Bromide on the Development of Proteolytic Activity in Chloroform-Treated Euglobulins

Addition to euglobulins	Fibrinolytic activity, lysis time	Caseinolytic activity, soluble tyrosine-like materials liberated
	hr	*μg/ml digestion mixture*
0.1 ml water	4.0	64
0.1 ml hexadimethrine bromide,* before treatment with chloroform	>12 <22	11
0.1 ml hexadimethrine bromide,* after incubation with chloroform	5.0	58

* To give a final concentration of 20 μg/ml.

rations of glass-adsorbed plasma or plasma deficient in Hageman factor had greatly reduced fibrinolytic and caseinolytic activities.

Hexadimethrine bromide inhibits Hageman factor (48) and the generation of fibrinolytic activity in the presence of kaolin (49). The addition of hexadimethrine bromide to the euglobulin fraction of normal plasma at a final concentration of 20 μg/ml, before treatment with chloroform, markedly reduced the development of proteolytic activity (Table VII).

These experiments suggest roles for Hageman factor and for an agent or agents missing in glass-treated plasma in the evolution of plasmin in the presence of chloroform.

VII. Comparison of crude Celite eluates from normal and Hageman factor-deficient plasma. Fibrinolytic activity developed when a crude eluate of normal plasma was incubated with glass-adsorbed or Hageman factor–deficient plasma (Table VIII). The same result was obtained whether or not kaolin was present, as if the effect of surface-active agents was upon the generation of the activity found in Celite eluates. In contrast, Celite eluates of Hageman factor–deficient plasma induced fibrinolytic activity only if kaolin was added. Thus, the component in Celite eluates which was needed for the generation of fibrinolytic activity was effective only in the presence of Hageman factor. In experiments in which eluates were prepared from Hageman factor–deficient plasma, Hageman factor was furnished by the glass-adsorbed plasma to which it was added, and in this case required the addition of kaolin to function.

VIII. The partial purification and properties of Hageman factor–cofactor. In the preceding section, evidence was provided that a factor could be removed from normal or Hageman factor–deficient plasma by adsorption onto diatomaceous earth, and that this substance could

TABLE VIII

Effect of Crude Celite Eluates from Normal and Hageman Factor–Deficient Plasmas on the Generation of Fibrinolytic Activity in Glass-Adsorbed and Hageman Factor–Deficient Plasma

Incubation mixture*			
Source of Celite eluate	Plasma	Kaolin	Lysis time
			min
Normal plasma	Normal, glass-adsorbed	+	26
Normal plasma	Normal, glass-adsorbed	0	31
Normal plasma	Hageman factor–deficient	+	27
Normal plasma	Hageman factor–deficient	0	34
Hageman factor–deficient plasma	Normal, glass-adsorbed	+	33
Hageman factor–deficient plasma	Normal, glass-adsorbed	0	>250
Hageman factor–deficient plasma	Hageman factor–deficient	+	115
Hageman factor–deficient plasma	Hageman factor–deficient	0	>250
Buffer	Normal, glass-adsorbed	+	140
Buffer	Normal, glass-adsorbed	0	>250
Buffer	Hageman factor–deficient	+	130
Buffer	Hageman factor–deficient	0	>250

* 2 ml Celite eluate or 0.01 M sodium phosphate buffer (pH 7.5), 0.25 ml kaolin (8 mg/ml), or 0.01 M sodium acetate buffer (pH 4.8), 0.5 ml glass-adsorbed plasma or Hageman factor–deficient plasma, 0.1 ml hirudin (100 μg/ml), and 9 ml sodium acetate buffer (0.01 mole/liter, pH 4.8) were incubated together for 60 min before centrifugation and assay of the generated fibrinolytic activity.

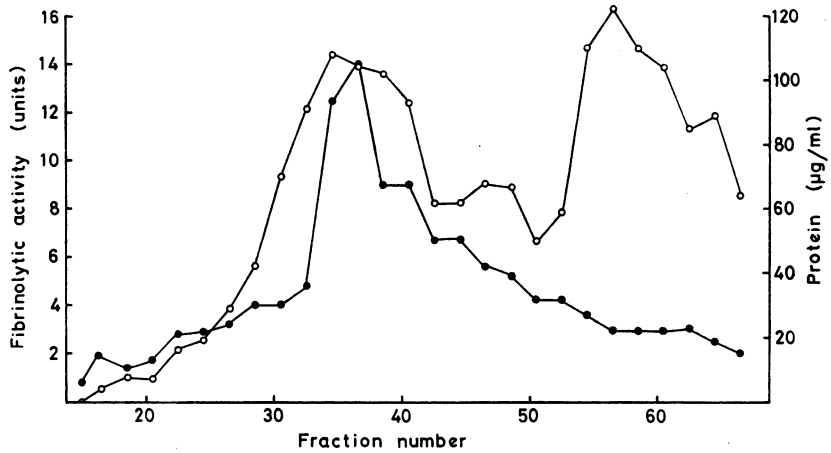

FIGURE 4 The chromatography of HF-cofactor on a CM-cellulose column. The adsorbed protein was eluted by a buffer gradient between 0.067 M sodium phosphate, pH 6, and 0.067 M sodium phosphate, pH 6, containing 0.5 M NaCl. Lysis times were converted into units of fibrinolytic activity by utilizing a double logarithmic plot of times against units, 10 U being assigned arbitrarily to a lysis time of 5 min. Fibrinolytic activity ●———●, protein ○———○.

then be eluted in active form. Further purification of the agent enhancing fibrinolysis was achieved by chromatography of Celite eluates upon columns of CM-cellulose (Fig. 4). All preparations were in an activated form and corrected the defective generation of fibrinolytic activity in glass-adsorbed or Hageman factor–deficient plasma. Such preparations, tentatively named Hageman factor–cofactor (HF-cofactor), induced caseinolytic activity in glass-adsorbed plasma (Fig. 5). Although zero order kinetics were not achieved, reducing the concentration of HF-cofactor fourfold resulted in only about a 40% reduction in the caseinolytic activity generated in 90 min. In contrast, the caseinolytic activity generated in the presence of a fixed amount of HF-cofactor was a linear function of the concentration of glass-adsorbed plasma (Fig. 6). These observations are com-

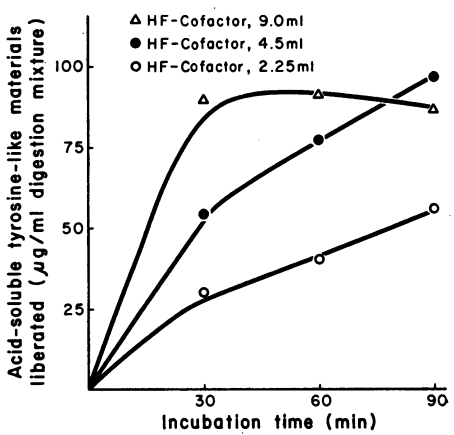

FIGURE 5 The influence of Hageman factor-cofactor upon the generation of caseinolytic activity. A mixture of 0.5 ml glass-treated plasma (adjusted to pH 5.2 just before use by addition of 1 N hydrochloric acid), 0–9 ml HF-cofactor (88 μg protein/ml) in 0.01 M sodium acetate buffer (pH 5.2), 0.5 ml kaolin (8 mg/ml), and sufficient 0.01 M sodium acetate buffer to bring the volume to 10 ml was incubated at 37°C for 90 min. At intervals, aliquot tubes were centrifuged at 2°C and the precipitate suspended in 1.25 ml 0.15 M sodium phosphate buffer (pH 7.4). Casein hydrolysis during the succeeding 4 hr was measured upon addition of 3.75 ml 1.6% casein to each tube.

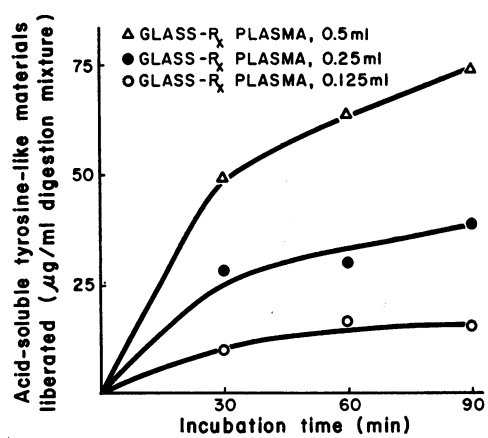

FIGURE 6 The influence of glass-treated plasma upon the generation of caseinolytic activity. A mixture of 0–0.5 ml glass-treated plasma (adjusted to pH 5.2 just before use by addition of 1 N hydrochloric acid), 9 ml HF-cofactor (114 μg protein/ml) in 0.01 M sodium acetate buffer (pH 5.2), 0.5 ml kaolin (8 μg/ml), and sufficient 0.01 M sodium acetate buffer to bring the volume to 10 ml was incubated at 37°C for 90 min. The caseinolytic activity of successive aliquot tubes was determined as in the experiment depicted in Fig. 5.

patible with the view that HF-cofactor induced fibrinolytic activity from a precursor in glass-adsorbed plasma, presumably the plasminogen which it contained.

The preparations of HF-cofactor were not pure. In different experiments, two or three faint lines could be identified by disc electrophoresis in the region comparable to that of gamma globulin in plasma. Constant contaminants of HF-cofactor, prepared by CM-cellulose chromatography, were activated PTA- and kallikrein-like activity, the latter demonstrated by its capacity to initiate the formation of kinins from a kininogen preparation. As best as could be judged, these agents were present in roughly the same concentration as HF-cofactor, a relationship maintained after further chromatography upon columns of DEAE-cellulose. The preparations were free of all other clotting factors with the possible exception of *activated* Hageman factor, the assay for which was vitiated by the presence of activated PTA. Most preparations contained traces of plasmin, demonstrable by prolonged incubation with casein but insufficient in themselves to lyse fibrin clots of bovine fibrinogen and thrombin. Plasminogen could not be detected by addition of streptokinase. Some preparations contained traces of thrombin, neutralized by the addition of hirudin and removable by DEAE-cellulose chromatography.

As might be expected from these considerations, preparations of HF-cofactor hydrolyzed *p*-toluenesulfonyl-L-arginine methyl ester, a substrate of activated PTA and plasma kallikrein. Thus, it was not possible to attribute esterolytic activity to HF-cofactor. Indirect evidence of the hydrolytic nature of HF-cofactor was obtained by exposing preparations to PMSF at a concentration of 0.005 mole/liter. Such treatment sharply decreased the generation of fibrinolytic and caseinolytic activity in a mixture of HF-cofactor and exhausted plasma. In one experiment, for example, a mixture containing untreated HF-cofactor induced fibrinolysis in 3.5 min, one containing HF-cofactor exposed to 10% isopropanol induced fibrinolysis in 5 min, while one containing HF-cofactor exposed to 0.005 M PMSF in isopropanol did not lyse a clot during a period of 5 hr. Similarly, HF-cofactor treated with PMSF generated less than one-fifth the caseinolytic activity of untreated HF-cofactor. Since PMSF is an inhibitor of many hydrolytic enzymes, these observations imply that HF-cofactor may be an enzyme of this type.

HF-cofactor was separable from activated PTA and plasma kallikrein by several means. The apparent molecular weights of activated PTA and HF-cofactor were identical, as determined by filtration of HF-cofactor through columns of Sephadex G200, approximately 165,000 in each of four experiments. But PTA-deficient plasma was as good a source of HF-cofactor as normal plasma, and a preparation of Cohn fraction IV-1 contained appreciable HF-cofactor and kallikrein-like activity, but was devoid of detectable PTA.

The distinction between HF-cofactor and plasma kallikrein rests upon several grounds. The molecular weight of plasma kallikrein, as determined by Sephadex G200 filtration, was 130,000. A second component with a molecular weight of 95,000 was detected in one of four experiments. These values are distinct from those obtained in studying activated PTA and HF-cofactor, but, within the limits of error of our technique, the same as those attributed to plasma kallikrein I and III by Colman, Mattler, and Sherry (50). Acidification of partially purified HF-cofactor at pH 2.0 did not reduce its content of plasma kallikrein, while decreasing the generation of caseinolytic activity and the concentration of activated PTA significantly. ϵ-Aminocaproic acid, at a concentration in the mixture of 0.01 mole/liter, inhibited the generation of caseinolytic activity in a mixture of HF-cofactor and glass-treated plasma, but was without effect the kallikrein-like activity of the preparation. Heparin, at a concentration of 1.25 U/ml, and Liquoid, at a concentration of 6.25 μg/ml, sharply reduced the caseinolytic activity generated in a mixture of partially purified HF-cofactor and glass-treated plasma but was without effect upon the generation of kinins; the significance of this observation is clouded by the fact that obvious precipitation occurred in the mixture of HF-cofactor and glass-treated plasma upon the addition of heparin or Liquoid, so that their effect may have been upon some other component of the test system. In an earlier study, C'1 esterase inhibitor, at concentrations inhibiting kallikrein, did not alter the rate at which fibrinolytic activity in kaolin-treated euglobulin preparations (51). The same result obtained when C'1 esterase inhibitor, at a concentration of 12 U/ml, was incubated for 10 min with HF-cofactor before incubation with glass-treated plasma and the caseinolytic activity generated thereafter was tested. Inhibition of plasmin-like activity by C'1 esterase inhibitor, noted earlier (51), was again demonstrable.

There was no significant loss of HF-cofactor activity during incubation at 37°C for 4 hr, but a substantial decline in activity had occurred in 48 hr. Its stability at higher temperatures is shown in Fig. 7.

IX. Evidence for the existence of an additional plasma factor which participates in the generation of fibrinolytic activity. To localize the components needed to generate fibrinolytic activity, glass-adsorbed plasma was mixed with buffer or with HF-cofactor. Each mixture was then diluted and acidified, and the euglobulin fraction separated from the supernatant solution by centrifugation. Experiments in which the euglobulin and supernatant fractions of the two plasmas were interchanged

demonstrated that the activated form of partially purified HF-cofactor was in the euglobulin precipitate (Table IX). In contrast, a component present in the supernatant fraction was necessary for kaolin-induced generation of fibrinolytic activity. Since euglobulin fractions of plasma contain both Hageman factor and plasminogen, the possibility was raised by these experiments that still another agent was needed for the generation of fibrinolytic activity. In agreement with this, virtually no caseinolytic activity evolved when preparations of HF-cofactor were incubated with plasminogen; in contrast, HF-cofactor readily induced caseinolytic activity in glass-adsorbed plasma.

Several explanations were explored for the poor yield of plasmin when HF-cofactor was incubated with plasminogen. This did not appear to be related to the Hageman factor content of glass-adsorbed plasma, for the addition of purified Hageman factor did not influence the yield of plasmin in a mixture of plasminogen and HF-cofactor. Two possible further explanations are reduced sensitivity of the purified plasminogen preparation or that a further plasma factor was required for the generation of plasminogen activator, as the experiments described in the last paragraph suggested. Evidence for the latter suggestion is provided by a study of the effect of the noneuglobulin, supernatant fraction of exhausted plasma upon fibrinolysis. Exhausted plasma, lacking Hageman factor and HF-cofactor, was acidified and diluted and the euglobulin fraction separated by centrifugation (Table X). The supernatant fraction was then incubated with HF-cofactor and the euglobulin precipitate of the exhausted plasma. Thus, the exhausted plasma was fractionated and its components combined in different proportions. Fibrinolysis was slow in the absence of the supernatant solution, although HF-cofactor and plasminogen were present. The addition of increasing amounts of the supernatant fraction speeded lysis, as if it provided some additional agent.

TABLE IX

Localization of Partially Purified Hageman Factor–Cofactor Added to Plasma in the Precipitate after Euglobulin Fractionation

Incubation mixture*	Lysis time
	min
1. HF-cofactor + glass-adsorbed plasma	9.5
2. Buffer + glass-adsorbed plasma	>250
3. Euglobulin supernatant of (1) + precipitate of (2)	159
4. Euglobulin precipitate of (1) + supernatant of (2)	11

* 1 ml HF-cofactor (68 μg protein/ml) or 0.01 M sodium phosphate buffer (pH 7.5), 0.5 ml glass-adsorbed plasma, 0.25 ml 0.01 M sodium acetate buffer (pH 4.8), 0.1 ml hirudin (100 μg/ml), and 8.5 ml 0.01 M sodium acetate buffer (pH 4.8) incubated at 37°C for 60 min either together or the euglobulin precipitates and supernatants interchanged before the 60 min incubation period.

DISCUSSION

The mechanisms through which plasmin forms under physiologic conditions are not yet delineated. Observations of Niewiarowski and Prou-Wartelle (14) and

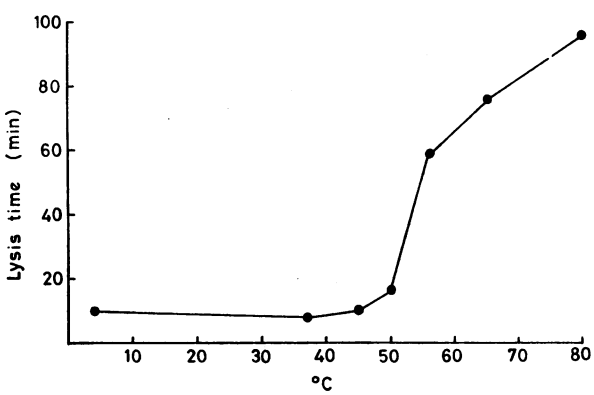

FIGURE 7 The heat stability of partially purified HF-cofactor. The HF-cofactor preparation (68 μg protein/ml) was kept at the indicated temperature for 30 min. 0.75 ml HF-cofactor or 0.01 M sodium phosphate buffer (pH 7.5), 0.5 ml glass-adsorbed plasma, 0.25 ml kaolin (8 mg/ml), 0.1 ml hirudin (100 μg/ml), and 8.5 ml 0.01 M sodium acetate buffer (pH 4.8) were incubated for 60 min at 37°C before assay of the general fibrinolytic activity. In the absence of HF-cofactor the lysis time was 128 min.

TABLE X

Effect of the Euglobulin Supernatant of Exhausted Plasma on the Production of Fibrinolytic Activity by HF-Cofactor

Volume of supernatant in incubation mixture	Lysis time
ml	*min*
0	41
1	32
2	23.5
5	13.5
10	9.5

9.5 ml sodium acetate buffer (0.01 mole/liter, pH 4.8) was added to 0.5 ml exhausted plasma; the formed precipitate was deposited by centrifugation and the supernatant decanted off and retained. 0.2 ml HF-cofactor (260 μg protein/ml) and 0.1 ml hirudin (100 μg/ml) were mixed with each euglobulin precipitate. Varying quantities of supernatant were added and the volume made up to 10 ml with 0.01 M sodium acetate buffer, pH 4.8. The fibrinolytic activity generated was assayed after incubation for 60 min at 37°C.

Iatridis and Ferguson (15) suggest that one pathway for plasmin formation involves the participation of Hageman factor. They based their views upon the observation that fibrinolytic activity develops in normal, but not in Hageman factor–deficient plasma exposed to surface-active substances. Using purified Hageman factor and a specific anti–Hageman factor rabbit serum, we have established that Hageman factor itself is indeed required for the generation of fibrinolytic activity under these circumstances. Plasmin formation in diluted, acidified normal plasma, incubated with kaolin, was demonstrated by the hydrolysis of casein and lysine methyl ester by the euglobulins separated from this mixture.

Earlier, we were unable to demonstrate that activated Hageman factor directly converted plasminogen to plasmin (20), an observation agreed to by Schoenmakers, Kurstjens, Haanen, and Zilliken (21). These observations have been confirmed; fibrinolytic activity did not develop upon the addition of kaolin to plasma rich in Hageman factor and plasminogen, but depleted of certain other agents by adsorption with glass. These experiments imply that treatment of plasma with crushed glass and other insoluble materials removes one or more components needed for the generation of fibrinolytic activity in the presence of a surface. This property of crushed glass is contrary to a previous report (52); it is likely that this discrepancy results from the use of glass of different particle size for adsorption.

Solutions of ellagic acid activate Hageman factor in the absence of *surface contact* (45); its addition to normal plasma might be expected to initiate the reaction leading to plasmin formation, and supporting experimental data have been reported (53). We found that ellagic acid induced some generation of fibrinolytic activity in acidified, diluted plasma, but only when present in such high concentrations that much insoluble material was present. It is likely, therefore, that its effect was mediated through its surface properties rather than by its specific activation of Hageman factor alone. The implication is that activation of Hageman factor *and* the presence of a suitable surface are required for the generation of fibrinolytic activity.

The mechanism by which proteolytic activity develops in the euglobulin fraction of plasma during incubation with chloroform has been the subject of debate for a number of decades. We have found that the development of proteolytic activity in chloroform-treated euglobulins prepared from glass-adsorbed plasma or Hageman factor–deficient plasma was greatly diminished in comparison to similarly treated euglobulins prepared from normal plasma or from plasmas deficient in other factors which participate in the intrinsic coagulation mechanism. In addition, hexadimethrine bromide, an inhibitor of Hageman factor (49), reduced the ability of plasma euglobulins to develop proteolytic activity after chloroform treatment. These findings suggest that the formation of proteolytic activity in euglobulins shaken in glass with chloroform is mediated through the activation of Hageman factor rather than through autocatalysis. Such an explanation is in keeping with the finding that preparations of partially purified plasminogen cannot be activated by chloroform (54). The role of chloroform itself remains uncertain, but it seems likely that it destroys the inhibitory activity remaining in the euglobulin fraction (55).

The experiments described have suggested the hypothesis that one or more additional agents in plasma are required for the activation of plasminogen by activated Hageman factor. No fibrinolytic or caseinolytic activity appeared in plasma which had previously been treated with glass in a specific way. This defect in glass-treated plasma could be corrected by small amounts of Hageman factor–deficient plasma, or by a fraction of such plasma prepared by adsorption of the active principle upon Celite and its subsequent elution. But Hageman factor–deficient eluates were effective only if kaolin was added to the mixture; presumably, the kaolin served to activate Hageman factor in the glass-treated plasma. In contrast, incubation of Hageman factor–deficient plasma with a Celite eluate prepared from normal plasma induced the generation of fibrinolytic activity. Under these conditions we presume that the effective principle in the Celite eluate was activated during its preparation by Hageman factor in the normal plasma, itself activated by the Celite. These experiments resemble those reported by Iatridis and Ferguson (22), Onchi (24), and Mackay, Maycock, and Combridge (56) although their interpretations differed from ours.

The existence of an agent in normal plasma, activated by contact with Celite, was further demonstrated by the separation of an activator of plasminogen by chromatography of Celite eluates upon columns of CM-cellulose. This agent, when added to diluted, acidified Hageman factor–deficient plasma, induced the generation of plasmin even in the absence of a surface. Tentatively, the substance present in Hageman factor–deficient plasma and absent from glass-adsorbed plasma and needed for the formation of plasminogen by Hageman factor has been designated as Hageman factor–cofactor; this nonspecific name underscores our uncertainty of its physiologic role. While the agent separated by chromatography is presumably the same as that present in crude Celite eluates, the possibility cannot be excluded that it is yet another plasminogen activator. HF-cofactor, prepared by CM-cellulose chromatography, sometimes contained traces of thrombin; these traces could be removed by further chromatography upon DEAE-cellulose.

CM-cellulose eluates containing HF-cofactor were rich in activated PTA and in a plasma kallikrein, both of which are agents activated from their respective precursors by activated Hageman factor. Experiments are described which appeared to separate HF-cofactor from the other two agents. Hageman factor–cofactor could be prepared from plasma congenitally deficient in PTA and from a preparation of Cohn fraction IV-1 devoid of PTA-like activity. Its apparent molecular weight was about 165,000, in contrast to that of plasma kallikrein in preparations of HF-cofactor, which was 130,000, with a second component in one of four experiments with a mol wt of 95,000.

Further, acidification to pH 2.0 or the addition of ε-aminocaproic acid, heparin, or Liquoid inhibited the formation of caseinolytic activity in mixtures of HF-cofactor and glass-adsorbed plasma without affecting the kallikrein-like activity of HF-cofactor preparations under the conditions used. Conversely, C'1 esterase inhibitor was without effect upon the generation of fibrinolytic or caseinolytic activity at concentrations inhibiting plasma kallikrein (51). These observations suggest that HF-cofactor, PTA, and plasma kallikrein are functionally separate entities. They do not prove that these substances do not exist in nature as a single entity, altered by our separation procedures. This assumption requires, however, that each property is altered in a different manner by pH and the various inhibitors tested.

To further complicate this system, evidence was obtained that another substance required for the activation of plasminogen by Hageman factor may be present in plasma. Plasma was "exhausted" of Hageman factor and HF-cofactor, and its euglobulin fraction separated by acidification and dilution. The supernatant fraction, after centrifugation, increased the potential of HF-cofactor to generate fibrinolytic activity. Our observations would be compatible with the hypothesis that we have isolated in the CM-cellulose fraction a plasma component which has been activated under the influence of Hageman factor during its preparation. This plasma component requires a further factor, present in the euglobulin supernatant, for the formation of a plasminogen activator.

The presence of small amounts of an activator of plasminogen can be detected in blood withdrawn from the circulation and the level is greatly enhanced in a variety of circumstances (12). The origin of this plasminogen activator is unproven although there is evidence that the vascular endothelium may be a source under certain circumstances. From the data of previous investigators (22) and the results of the present study we conclude that the circulating blood contains components which, on activation of Hageman factor, interact to form an activator of plasminogen. Further investigation is required, however, to unravel the complexities of this system and to elucidate the relationship of this plasminogen activator to that which is present in normal human plasma.

The studies presented serve once again to remind us that the body's defense mechanism is complicated. However appealing the view that the plasma contains separate pathways leading to clotting fibrinolysis, inflammation, or the activation of complement, our experiments emphasize instead the interwoven reactions among these systems.

ACKNOWLEDGMENTS

Dr. Doris Ménaché participated in some of the preliminary experiments leading to the present study.

This study was supported in part by U. S. Public Health Service Grant HE 01661 from the National Heart Institute, the National Institutes of Health, and in part by a grant from the American Heart Association.

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