THE INHIBITION OF PLASMIN, PLASMA KALLIKREIN, PLASMA PERMEABILITY FACTOR, AND THE C'1r SUBCOMPONENT OF THE FIRST COMPONENT OF COMPLEMENT BY SERUM C'1 ESTERASE INHIBITOR*

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(Received for publication 24 September 1968)

Normal human serum inhibits the activity of C'1 esterase (C'1sa), an enzyme derived from the C'1s fragment of the first component of complement (C'1) (1–3). This inhibitory property has been localized to a heat-labile α2-globulin, distinct from serum antitrypsin, designated variously as C'1 esterase inhibitor, C'1a inhibitor, or EI (4). Donaldson and Evans (5) reported that the serum of patients with hereditary angioneurotic edema behaved as if it lacked functional C'1 esterase inhibitor. Independently, Landerman and his associates (6) observed that in this disorder serum lacked inhibitory activity against a plasma kallikrein or against PF/Dil (a factor in human plasma which increases cutaneous vascular permeability), or against both of these substances. Kagan and Becker (7, 8) unified these two studies by demonstrating that preparations rich in C'1 esterase inhibitor blocked the effects of plasma kallikrein and PF/Dil. Their experiments suggested that C'1 esterase inhibitor might possess several biological functions.

The preparations of C'1 esterase inhibitor used by Kagan and Becker were relatively crude (7). The properties of this inhibitor have been reexamined, using more purified preparations. The capacity of C'1 esterase inhibitor to block the actions of a plasma kallikrein and of PF/Dil have been confirmed. Moreover, C'1 esterase inhibitor, at concentrations comparable to those in normal human plasma, has been found to inhibit plasmin and the C'1r fragment of C'1. Thus, the agent designated as C'1 esterase inhibitor appears to be a more generalized inhibitor of hydrolytic enzymes than had heretofore been appreciated.

* This study was supported in part by Grant HE 01661 from the National Heart Institute and Grants AI 06349 and AI 07353 from the National Institute of Allergy and Infectious Disease of the National Institutes of Health, United States Public Health Service, and in part by grants from the American and Northeast Ohio Heart Associations.

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Materials and Methods

C'1 esterase inhibitor was purified from human serum by a modification of a method described earlier (4). Briefly, the method consisted of adding ammonium sulfate to human serum to 40% saturation, sedimenting the insoluble protein, and dialyzing the supernatant solution exhaustively against distilled water. The dialyzed supernatant fraction was passed through a column of Dowex 2-X10 and protein containing C'1 esterase inhibitor activity was eluted by stepwise increases of sodium chloride concentration (3). Fractions rich in C'1 esterase inhibitor were pooled, concentrated by pressure dialysis, and applied to a column of DEAE cellulose (Whatman DE-52) equilibrated with 0.06 M tris (hydroxymethyl) aminomethane (Tris)–chloride buffer, pH 8.6. A concentration gradient of sodium chloride, linear from zero to 0.15 M was passed through the column and fractions richest in C'1 esterase inhibitor were again pooled, concentrated, dialyzed against 0.005 M phosphate buffer, and applied to a column of hydroxylapatite equilibrated with the same buffer. The C'1 esterase inhibitor emerged at the void volume of the column and fractions of highest specific activity were pooled and recycled through a second hydroxylapatite column. The final product had an activity of approximately 200 C'1 esterase inhibitor units per optical density unit at 280 mμ and gave a single, broad band in analytical disc gel electrophoresis (9). One unit of inhibitor corresponded to approximately 12 μg of protein. The inhibitor was dissolved in 0.005 M phosphate buffer. One unit of C'1 esterase inhibitor is that amount which neutralizes the esterolytic properties of 10 units of C'1 esterase, as assayed upon a substrate of *N*-acetyl-L-tyrosine ethyl ester (2).

Platelet-deficient citrated plasma was prepared from the venous blood of normal human subjects in such a way that it was protected from contact with glass or other clot-promoting surfaces (10). One-ninth volume of sodium citrate buffer (pH 5.0, 0.13 M with respect to citrate) was added to the blood as an anticoagulant.

Column-purified bovine thrombin, devoid of detectable Stuart factor (factor X), was prepared from Topical Thrombin (Parke, Davis and Co., Detroit, Mich.) by a modification of Rasmussen's (11) method and stored at −20°C in 1% bovine albumin in barbital saline buffer (12). Just before use, the thrombin was assayed by comparing its procoagulant activity with that of untreated Topical Thrombin. *Plasminogen-poor bovine thrombin* was prepared by treating Topical Thrombin with 2-mercaptoethanolamine (Nutritional Biochemicals Corp., Cleveland, Ohio) by the method of Markus and Ambrus (13), stored at −20°C, and diluted in barbital saline buffer to the concentration desired before use.

Partially purified plasminogen was prepared from Cohn fraction III_4 of human plasma[1] by a method described elsewhere (14). The batch used contained 8.8 Remmert and Cohen units of plasminogen per milligram of protein; about 10% was in the form of plasmin. This preparation, dissolved at a concentration of 7.2 Remmert and Cohen units per milliliter of 0.15 M sodium phosphate buffer (pH 7.5) was used as a source of *spontaneously activated human plasmin*. A second human plasminogen preparation, separated in a different way (15), provided another source of spontaneously activated plasmin.[1] This preparation was diluted in 0.15 M sodium phosphate buffer to provide a plasmin concentration of 1.3 Remmert and Cohen unit per milliliter. *Streptokinase-activated* human plasmin,[2] containing 130 Merck, Sharp and Dohme (MSD) units per milligram, was dissolved in distilled water at a concentration of 20 mg/ml and diluted as needed.

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

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[1] The gift of Cutter Laboratories, Berkeley, Calif.

[2] The gift of Merck, Sharp and Dohme, West Point, Pa.

Partially purified *human fibrinogen*, prepared as fraction I-O by the method of Blombäck and Blombäck (16), was dissolved in a solution containing 0.45 g sodium chloride and 2.0 g sodium citrate in 100 ml of water.

Fibrin plates (17) were prepared by clotting 10 ml of 0.2% human fibrinogen with 0.5 ml bovine thrombin (20 NIH units/ml) in a 9 cm disposable plastic Petri dish. The fibrinogen used to prepared these plates was separated by the technique of Bergstrom (18) in which Cohn fraction I is precipitated in the presence of epsilon aminocaproic acid.[1] The fibrinogen was dissolved in barbital saline buffer at a concentration of 200 mg/100 ml.

Urokinase,[3] containing 2200 CTA units/mg, was dissolved at a concentration of 1000 CTA units/ml of phosphate saline buffer.

C'1r and C'1s were separated from the euglobulin fraction of human serum by chromatography upon columns of diethylaminoethyl cellulose, as described earlier (3). Fractions containing C'1r and C'1s were pooled and dialyzed against sodium phosphate buffer (pH 7.4, ionic strength 0.15) and stored at 2°C until needed. To designate their concentrations, the C'1r and C'1s activities of the dialyzed pools will be described as "undiluted."

N-acetyl-L-arginine methyl ester hydrochloride (AAME; Cyclo Chemical Corp., Los Angeles, Calif.) dried by extraction with ether, and *N*-α-acetyl-L-lysine methyl ester hydrochloride (ALME, Cyclo Chemical Corp.) were dissolved at a concentration of 0.015 M in phosphate saline buffer; the *N*-acetyl-L-arginine methyl ester solution was adjusted to pH 7.4 by the addition of 0.15 M sodium hydroxide. *N-acetyl-L-tyrosine ethyl ester* (ALTEE) synthesized by Dr. F. M. Bumpus, the Cleveland Clinic, was dissolved at a concentration of 1 M in 2-methoxyethanol (Methyl Cellosolve, Eastman Organic Chemicals, Inc., Rochester, N. Y.)

Ellagic acid, synthesized by Dr. J. D. Crum (see reference 19) or purchased in cruder form from K. and K. Laboratories, Inc., Plainview, N. Y., was dissolved at a concentration of 0.0001 M in barbital saline buffer, aided by mechanical homogenization, and used without centrifugation.

Casein (Hammersten quality, Nutritional Biochemicals Corp.), was prepared as a 1.6% solution in 0.15 M sodium phosphate buffer (pH 7.5) by a modification of the method of Mullertz (20).

Kaolin (acid-washed, N.F., Fisher Scientific Co., Pittsburgh, Pa.) was suspended at a concentration of 8 mg/ml in sodium acetate buffer (pH 4.8, 0.01 M with respect to acetate) by mechanical homogenization.

Liquoid (sodium polyanetholsulfonate)[4] was dissolved at a concentration of 0.066 mg/ml in sodium phosphate buffer (pH 7.4, ionic strength 1.07).

Barbital saline buffer was composed of 0.025 M barbital and 0.125 M sodium chloride at pH 7.5 (10). *Phosphate saline buffer* was 0.067 M sodium phosphate (pH 7.4) in 0.6% sodium chloride. *Phosphate buffer*, 0.005 M, was the sodium salt at pH 6.7. Other buffers used are noted in the text.

Formaldehyde solution (38%, Fisher Scientific Co.) was rendered slightly pink to phenolphthalein by the addition of 1 N sodium hydroxide.

Celite eluate was prepared essentially by the method of Nossel (21) in which citrated plasma, at pH 7.4, was adsorbed with diatomaceous earth (Celite 512).[5] The celite was washed with twice the original plasma volume of 0.15 M sodium chloride and eluted with a volume of 10% sodium chloride in 0.05 M Tris (pH 8.0) equal to that of the original plasma. The eluate was a rich source of *plasma kallikrein* but also contained plasma thromboplastin antecedent (PTA, factor XI), small amounts of thrombin and probably kallikreinogen and Hageman factor (factor XII). It was dialyzed against phosphate saline buffer before use.

[3] The gift of Abbott Laboratories, North Chicago, Ill.

[4] The gift of Hoffmann LaRoche Laboratories, Nutley, N. J.

[5] The gift of Johns-Manville Celite Division, Cleveland, Ohio.

Crude *prokinin* was separated from normal human plasma by a modification of a previously described technique (14). Plasma, depleted of antihemophilic factor and some of its fibrinogen by cryoprecipitation, was adsorbed with one-one hundredth volume of alumina Cγ-gel (Calbiochem, Los Angeles, Calif.) to remove the vitamin K-dependent clotting factors. The fraction soluble at one-third saturation but insoluble at half-saturation with solid ammonium sulfate was dialyzed against 0.01 M sodium acetate (pH 5.2). The precipitate was separated by centrifugation and discarded. The supernatant fraction was dialyzed against barbital saline buffer, heated at 62°C for 30 min to inactivate any Hageman factor or kallikreinogen which may still have been present, recentrifuged, and the supernatant fluid, containing prokinin, stored at −20°C until needed.

Hexadimethrine bromide[6] was dissolved in barbital saline buffer at a concentration of 1 mg/ml and used at this concentration.

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

The effect of C'1 esterase inhibitor upon the clotting time of whole blood was tested by a three tube technique described earlier (22). 0.9 ml of venous blood, freshly drawn through an 18 gauge needle into a polypropylene syringe (Monoject, Roehr Products Co., Inc., De Land, Fla.) which had been rinsed with silicone oil (SF 96-200, General Electric Co., Waterford, N. Y.) was added to 0.1 ml of C'1 esterase inhibitor or 0.005 M phosphate buffer in disposable glass or polystyrene tubes (internal diameter, 11 mm). The mixtures were inverted once over parafilm, and the clotting time of successive tubes was measured at 25°C. The interval elapsing until the contents of the third tube had clotted was recorded as the clotting time.

The effect of C'1 esterase inhibitor upon the clotting time of platelet-deficient plasma was assessed by mixing 0.04 ml of inhibitor or 0.005 M phosphate buffer and 0.2 ml each of platelet-deficient citrated plasma and 0.025 M calcium chloride solution in polystyrene tubes (internal diameter, 8 mm). The mixtures were incubated at 37°C and the interval which elapsed until clotting occurred was measured, tilting the tubes once a minute.

The effect of C'1 esterase inhibitor upon bovine thrombin was tested by incubating 0.2 ml of the inhibitor or 0.005 M phosphate buffer with 0.1 ml of column-purified thrombin (40 NIH units/ml) for 5 min at room temperature. 0.1 ml of partially purified human fibrinogen was then added to each tube and the clotting time measured at 37°C, tilting the tubes continually.

The effect of C'1 esterase inhibitor upon fibrinolytic activity and its evolution from plasma was measured in several ways. To test its effect upon the generation of fibrinolytic activity, 0.2 ml of inhibitor (or 0.005 M phosphate buffer) was mixed with 1 ml of plasma and 0.5 ml kaolin suspension in Lusteroid (cellulose nitrate) tubes. The mixtures were dialyzed for 60 min against 2 liters of 0.01 N sodium acetate buffer (pH 5.2) at room temperature, centrifuged, and the supernatant solution discarded. The euglobulin precipitate, containing kaolin, was resuspended in 1 ml barbital saline buffer. To 0.2 ml. aliquots of the euglobulin-kaolin suspension in polystyrene tubes (internal diameter, 8 mm) were added 0.06 ml C'1 esterase inhibitor to tubes originally containing 0.005 M phosphate buffer, and vice versa, 0.1 ml bovine fibrinogen, and 0.1 ml plasminogen-poor thrombin (50 NIH units/ml). After mixing, the tubes were incubated at 37°C. The clot lysis time was the interval which elapsed until there was complete dissolution of the fibrin. The technique used was designed to minimize dilution of the C'1 esterase inhibitor during the initial period of incubation.

The effect of C'1 esterase inhibitor upon fibrinolytic activity generated in a plasma-kaolin mixture was tested by a variation of the method just described. 2 ml of plasma, 1 ml of kaolin suspension, and 37 ml of 0.01 M sodium acetate buffer (pH 4.8) were incubated at 37°C for 60 min in Lusteroid tubes (internal diameter 16 mm). After centrifugation at 3000 rpm for

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[6] Polybrene, the gift of Abbott Laboratories.

5 min, the supernatant solution was discarded and the precipitate suspended in 2 ml barbital saline buffer. The suspension was again centrifuged, and the supernatant euglobulin solution, free of kaolin, removed. Fibrinolysis was measured in polystyrene tubes at 37°C in a mixture of 0.2 ml of euglobulin solution, 0.1 ml of C'1 esterase inhibitor (or 0.005 M phosphate buffer), 0.1 ml bovine fibrinogen, and 0.1 ml plasminogen-poor bovine thrombin (20 NIH units/ml), and the clot lysis time measured.

The effect of C'1 esterase inhibitor upon fibrinolysis was also measured by incubating 0.1 ml of inhibitor (or 0.005 M phosphate buffer), 0.1 ml of Cutter's spontaneously activated human plasmin, 0.2 ml bovine fibrinogen solution, and 0.1 ml plasminogen-poor bovine thrombin (20 NIH units/ml) in polystyrene tubes (internal diameter 8 mm), and the interval until complete fibrinolysis took place was measured. Similar experiments were performed with a second preparation of spontaneously activated human plasmin.

In a third technique by which to assess the effect of C'1 esterase inhibitor upon the fibrinolytic activity of plasmin, 20 μl of mixtures of equal parts of Cutter's spontaneously activated human plasmin (containing 1.3 Remmert and Cohen units/ml) and C'1 esterase inhibitor, and dilutions of plasmin in 0.005 M phosphate buffer, were applied to the surface of fibrin plates 15 min after their preparation. After incubation for 20 hr at 37°C, the area of lysis (calculated as the product of two perpendicular diameters of lysis) was measured. The logarithm of the area of lysis was proportional to the logarithm of the plasmin concentration applied; the percentage inhibition of plasmin by C'1 esterase inhibitor was obtained by interpolation.

The effect of C'1 esterase inhibitor upon proteolysis by plasmin was measured by a modification of the method of Remmert and Cohen (20, 23). 0.5 ml of spontaneously activated human plasmin and 0.5 ml C'1 esterase inhibitor (or 0.005 M phosphate buffer) were incubated together at room temperature for 3 min. 1 ml of 0.15 M sodium phosphate buffer (pH 7.5) and 3.0 ml of 1.6% casein were added, and the mixture incubated at 37°C. At 2 and 62 min, 2 ml aliquots were removed and mixed with 2 ml of 10% tricloroacetic acid. After 30 min, the mixtures were centrifuged and the optical density of the clear supernatant solutions was read at 280 mμ in a Beckman model DU spectrophotometer equipped with a Gilford absorbance meter. Optical density was converted to micrograms of tyrosine per milliliter by reference to a standard tyrosine solution. Similar experiments were performed with streptokinase-activated human plasmin.

The effect of C'1 esterase inhibitor upon urokinase was measured by incubating 0.25 ml each of urokinase solution (1000 CTA units/ml) and C'1 esterase inhibitor (or 0.005 M phosphate buffer) at room temperature for 10 min. A volume of 2.5 ml of 0.015 M ALME was then added to each tube and the mixture incubated at 37°C for 60 min. At the start and after 60 min, 1 ml aliquots were removed and added to 0.5 ml 0.75 M perchloric acid in disposable glass tubes (internal diameter, 14 mm). The methyl alcohol liberated during incubation was measured by Siegelman's (24) technique.

The inhibition of C'1r by C'1 esterase inhibitor was detected in two ways. Inhibition of the capacity of C'1r to convert C'1s to C'1 esterase was tested by incubating 0.25 ml C'1r with 0.25 ml C'1 esterase inhibitor and 0.50 ml sodium phosphate buffer (pH 7.4, ionic strength 0.15) for 10 min at room temperature in disposable glass tubes (internal diameter, 11 mm). Next, 1 ml of C'1s was added, and the mixture incubated at 37°C for an additional 30 min. The activation of C'1s was halted by the addition of 0.375 ml Liquoid, and 0.125 ml of 1 M ALTEE was added as substrate for the C'1 esterase which had evolved. The mixtures were reincubated for 15 min at 37°C; 1 ml aliquots were removed at the start and after 15 min, and added to 1 ml of formaldehyde solution in the titration cup of a Radiometer model ABU 1 automatic burette (Copenhagen, Denmark). The titratable acidity in the initial and 60 min samples was measured by the addition of sufficient 0.05 N sodium hydroxide with the automatic burette to bring the hydrogen ion concentration to a predetermined end point, usually

7.4. The titrations were performed with a Radiometer TTT 11 titrator, controlled by a Radiometer model 25 pH meter.

Inhibition of the esterolytic properties of C'1r was tested by incubating a mixture of 0.5 ml C'1r, 0.25 ml C'1 esterase inhibitor, and 0.75 ml sodium phosphate buffer (pH 7.4, ionic strength 0.15) in disposable glass tubes (internal diameter, 11 mm) at room temperature for 15 min. 1 ml of 0.015 M AAME was then added as substrate and incubation continued for 60 min at 37°C. Immediately after the addition of this substrate, and 60 min later, 1 ml aliquots were removed and added to 1 ml of formaldehyde in the manner just described. The titratable acidity was then determined as in the measurement of C'1 esterase activity.

Vascular permeability was measured by the method of Miles and Wilhelm (25). 0.1 ml aliquots of the solutions to be tested were injected intracutaneously into the depilated backs of female albino guinea pigs, weighing 400–500 g which had been injected intravenously with 1.2 ml/kg of body weight of a 5% solution of pontamine sky blue 6X (E. I. du Pont de Nemours and Co., Wilmington, Del.) in 0.075 M sodium chloride solution. 15 min after the last injection, the size of the blue spots which appeared at the injected sites, a function of the vascular permeability-enhancing properties of the material injected, was estimated by averaging the largest diameter and its perpendicular.

The effect of C'1 esterase inhibitor upon the evolution of permeability-enhancing activity in diluted human plasma was tested by incubating 0.3 ml of plasma (diluted 15-fold in barbital saline buffer in silicone-coated Lusteroid tubes), 0.2 ml of C'1 esterase inhibitor (diluted serially in 0.005 M phosphate buffer) and 0.5 ml of ellagic acid solution at 37°C for 12 min. At the end of this period, 0.2 ml of C'1 esterase inhibitor or phosphate buffer was added to appropriate tubes and the solutions tested by the method just described. The effect of the inhibitor was evaluated by comparison with the permeability-increasing properties of the same plasma, diluted serially with barbital saline buffer and incubated 12 min with ellagic acid. The method used took advantage of the observation that ellagic acid, a known activator of Hageman factor (26), induced the evolution of the permeability factor designated as PF/Dil (27).

The evolution of kinin-like activity was measured by the addition of 0.2 ml aliquots of test solutions to a bath of 8 ml of de Jalon's solution (28) at 29°C, in which was suspended a segment of a horn of rat uterus. The uterus had been removed from a virgin rat (average weight about 350 g) which had been injected intraperitoneally 24 hr before with 10 μg of diethylstilbestrol/100 g of body weight. The effect of the test solution was compared with that of synthetic bradykinin,[7] diluted in 0.15 M sodium chloride solution in polystyrene tubes. The contraction of the uterus was measured essentially isotonically with a linear motion transducer, model ST-2, the transformer of which was supplied by an exciter-demodulator (Phipps and Bird, Inc., Richmond, Va.) and recorded on a Servo-Recorder, model EUW-20A (Heath Co., Benton Harbor, Mich.).

The effect of C'1 esterase inhibitor upon the evolution of kinin-like activity was measured in two ways. In one, plasma was treated with ellagic acid to activate its Hageman factor. Hageman factor is believed to activate kallikrein from its precursor in plasma, and this enzyme then releases polypeptide kinins from their precursor in plasma (29). The test mixtures consisted of 0.2 ml each of plasma and ellagic acid (or barbital saline buffer), 0.04 ml of 0.1 M trisodium ethylenediaminetetraacetic acid (Na_3HEDTA, pH 7.0), 0.16 ml of barbital saline buffer, and 0.1 ml of C'1 esterase inhibitor (or 0.005 M phosphate buffer). These mixtures were incubated in polystyrene tubes (internal diameter, 8 mm) at 37°C for 30 min, an additional 0.1 ml of C'1 esterase inhibitor (or 0.005 M phosphate buffer) was added to appropriate tubes, and aliquots were then tested for kinin-like activity by their effect upon the rat

[7] The gift of Sandoz Pharmaceuticals, Hanover, N. J.

uterus. The Na_3HEDTA served to inhibit kininases which would otherwise have inactivated the kinins as they formed (30).

The technique outlined did not distinguish clearly between an effect of C'1 esterase inhibitor upon the evolution of kallikrein from an effect upon kallikrein itself. To test the effect of C'1 esterase inhibitor upon preformed kallikrein, 0.025 ml of C'1 esterase inhibitor was incubated at room temperature for 5 min with 0.05 ml of celite eluate, 0.05 ml 0.01 M *o*-phenanthroline 0.05 ml hexadimethrine bromide (1 mg/ml) and 0.175 ml barbital saline buffer in polystyrene tubes (internal diameter, 8 mm). After 5 min, 0.15 ml of crude prokinin was added and incubation continued for 10 min. Aliquots of 0.2 ml were then tested for kinin-like activity. Control experiments were performed in which C'1 esterase inhibitor was omitted or added just before testing upon the uterus. The celite eluate was a rich source of plasma kallikrein; hexadimethrine bromide was added, in great excess of the concentration needed, to inhibit any fresh activation of kallikreinogen by Hageman factor, both of which may be present in celite eluates (31). *O*-phenanthroline was used as an inhibitor of kininase (32).

TABLE I

The Effect of C'1 Esterase Inhibitor on the Generation of Fibrinolytic Activity

Agent incubated with plasma and kaolin	Agent added to euglobulin before assay*	Clot lysis time
		min
1. C'1 esterase inhibitor (14 units/ml)	Buffer	19.5
2. Buffer	Buffer	18.5
3. Buffer	C'1 esterase inhibitor (14 units/ml)	70.0

* The generation system consisted of plasma, kaolin, and C'1 esterase inhibitor (or 0.005 M phosphate buffer) at pH 5.2, as described under Materials and Methods. After 60 min, the insoluble euglobulin was sedimented, buffer or C'1 esterase inhibitor was added, and the fibrinolytic activity of the mixture assayed. The concentrations of C'1 esterase inhibitor in tubes 1 and 3 are those in the generation and assay systems, respectively.

RESULTS

The Effect of C'1 Esterase Inhibitor upon Blood Clotting.—In an earlier study, C'1 esterase inhibitor, at a concentration of 1.6 units/ml, had no effect upon the esterolytic properties of bovine thrombin (4). Similarly, this agent did not delay the clotting of human fibrinogen by bovine thrombin. Thus, the clotting time of a mixture of 0.2 ml fibrinogen and 0.1 ml column-purified bovine thrombin (20 units/ml) was 13.8 sec in the presence of 0.1 ml 0.005 M phosphate buffer, and 10.8 sec in the presence of 0.1 ml C'1 sterase inhibitor such that its concentration in the coagulation mixture was 20 units/ml.

Thrombin is only one of many enzyme-like substances participating in blood clotting. C'1 esterase inhibitor, at a final concentration of 16.5 units/ml, did not delay the coagulation of whole blood. Measured in glass tubes, the clotting time of a mixture of blood and C'1 esterase inhibitor or 0.005 M phosphate buffer was the same, namely, 14 min. Tested in polystyrene tubes, the clotting time was 65 min in the presence of C'1 esterase inhibitor and 85 min in the presence of buffer, a difference which was probably not significant but may

have reflected contamination of the C'1 esterase inhibitor preparation with traces of a procoagulant substance.

Conceivably, any inhibitory effect of C'1 esterase inhibitor upon coagulation might have been masked by the presence of blood cells. But C'1 esterase inhibitor, at a concentration in the mixture of 16.5 units/ml, had no effect upon the recalcified clotting time of platelet-deficient plasma, measured in polystyrene tubes. Thus, the clotting time, in the presence of C'1 esterase inhibitor, was 12 min, and, in the presence of 0.005 M phosphate buffer, 11 min. Under the conditions of this test, the clotting time is sensitive to the presence of small amounts of activated Hageman factor, which was apparently not inhibited by C'1 esterase inhibitor.

The Effect of C'1 Esterase Inhibitor upon Components of the Plasma Fibrinolytic System.—When human plasma euglobulin is incubated in the presence of

TABLE II

The Effect of C'1 Esterase Inhibitor on Kaolin-Generated Fibrinolytic Activity

Concentration of C'1 esterase inhibitor*	Clot lysis time
units/ml	*min*
5.0	21.0
2.5	10.0
1.25	7.5
0	7.0

* Fibrinolytic activity was generated by incubating plasma, kaolin, and 0.01 M sodium acetate buffer for 60 min (see Materials and Methods). The euglobulin fraction of plasma was dissolved in barbital saline buffer and separated from the kaolin. The effect of the addition of C'1 esterase inhibitor (or 0.005 M phosphate buffer) upon the fibrinolytic activity of the euglobulin fraction was then tested; the concentration of C'1 esterase inhibitor in the table is that in the final assay mixture.

kaolin, fibrinolytic activity evolves (33, 34). The presence of C'1 esterase inhibitor, at a concentration of 14 units/ml, had no effect upon the generation of fibrinolytic activity (Table I). On the other hand, once fibrinolytic properties had evolved, C'1 esterase inhibitor, at a concentration of 2.5 units/ml, significantly delayed fibrinolysis (Tables I and II).

The lysis time of fibrin clots prepared in the presence of spontaneously activated human plasmin was lengthened when C'1 esterase inhibitor was mixed with the plasmin preparation before its incorporation into the clot (Table III). This inhibitory activity was greatly enhanced if the plasmin and C'1 esterase inhibitor were incubated together for 5 min before the addition of fibrinogen and thrombin. The same results were obtained with plasmin prepared by two different methods.

Assay of the residual fibrinolytic activity of mixtures of C'1 esterase inhibi-

tor and spontaneously activated human plasmin on fibrin plates revealed that C'1 esterase inhibitor at a final concentration of 2.5 units/ml inhibited 77% of the plasmin activity and at 5 units/ml inhibited 86%.

The fibrinolytic properties which evolve in kaolin-treated euglobulin frac-

TABLE III

The Effect of C'1 Esterase Inhibitor upon the Fibrinolytic Activity of Plasmin

C'1 esterase inhibitor	Clot lysis time, *sec**	
	Time of preincubation of C'1 esterase inhibitor and plasmin	
	0 min	5 min
units/ml		
0	211	220
2	237	542
5	276	—
10	318	5000
20	517	—

* Lysis time of a clot formed by the addition of bovine fibrinogen and plasminogen-free thrombin to mixtures of spontaneously activated human plasmin and C'1 esterase inhibitor (or 0.005 M phosphate buffer) either immediately after they were mixed together or after their incubation together for 5 min at room temperature. The plasmin contained 1.3 Remmert and Cohen units/ml. The concentrations of C'1 esterase inhibitor were those in the final mixture.

TABLE IV

*The Effect of C'1 Esterase Inhibitor upon Hydrolysis of Casein by Plasmin**

C'1 esterase inhibitor	Tyrosine-like material released from casein
units/ml	*μmg/ml*
0	17
2.5	10.5
5	7
15	2

* Spontaneously active human plasmin, C'1 esterase inhibitor (or 0.005 M phosphate buffer), and casein were incubated at 37°C and the tyrosine-like material released during digestion measured (see Materials and Methods). The concentration of plasmin in the inhibitor-plasmin mixtures was 0.38 Remmert and Cohen unit/ml. The table records the concentration of C'1 esterase inhibitor in the inhibitor-plasmin mixtures.

tions are presumably attributable to plasmin, a proteolytic enzyme of broad specificity. C'1 esterase inhibitor, at concentrations as low as 2.5 units/ml, decreased the rate of hydrolysis of casein by spontaneously activated human plasmin (Table IV). Essentially similar results were obtained using two sources of spontaneously activated plasmin and using streptokinase-activated human

plasmin; in the latter experiment, at a concentration of 2 units/ml, C'1 esterase inhibitor significantly inhibited hydrolysis of casein by plasmin (260 MSD units/ml).

The inhibitor had no demonstrable activity against urokinase, a hydrolytic enzyme in human urine which activates plasminogen. Under the conditions tested, urokinase released 114 μM of methyl alcohol/ml from its substrate,

TABLE V

The Effect of C'1 Esterase Inhibitor upon C'1r

Test substance	Esterase activity of acid liberated
	μEq
I. Hydrolysis of AAME*	
Buffer	4.9
C'1 esterase inhibitor, 0.5 unit/ml	2.3
C'1 esterase inhibitor, 0.25 unit/ml	2.8
C'1 esterase inhibitor, 0.125 unit/ml	4.3
II. Hydrolysis of ALTEE‡	
Buffer	5.9
C'1 esterase inhibitor added at start, 1.0 unit/ml	1.7
C'1 esterase inhibitor added at start, 0.5 unit/ml	3.7
C'1 esterase inhibitor added at start, 0.25 unit/ml	5.9
C'1 esterase inhibitor added after C'1 esterase evolved, 1.0 unit/ml	5.4
C'1 esterase inhibitor added after C'1 esterase evolved, 1.0 unit/ml§	1.7

* Hydrolysis of AAME by mixtures of C'1r and C'1 esterase inhibitor or 0.005 M phosphate buffer (see Materials and Methods). The concentration of C'1 esterase inhibitor is that in the enzyme-substrate mixture.

‡ Hydrolysis of ALTEE by C'1 esterase, evolved in a mixture of C'1r and C'1s. C'1 esterase inhibitor was added before the incubation of C'1r and C'1s, or after the addition of Liquoid, used to halt the interaction of C'1r and C'1s. The concentration of C'1 esterase inhibitor is that in the final mixture (see Materials and Methods).

§ Liquoid omitted.

N-acetyl-L-lysine methyl ester, while in the presence of C'1 esterase inhibitor (33 units/ml of enzyme-substrate mixture) it released 116 μM. Similarly, Donaldson (35), found that C'1 esterase inhibitor did not block the hydrolysis of ALTEE which was brought about by some, but not all, preparations of urokinase.

The Effect of C'1 Esterase Inhibitor upon C'1r.—The first component of complement, C'1, can be dissociated into three subcomponents, C'1q, C'1r, and C'1s (3). Preparations containing C'1r in its active state convert C'1s to C'1 esterase, probably enzymatically, and hydrolyze AAME, among other syn-

thetic substrates (36). C'1 esterase inhibitor blocked both of these reactions. The activation of C'1s was partially inhibited by as little as 0.7 units/ml, and the hydrolysis of AAME by as little as 0.25 units/ml (Table V). Measurement of the effect of C'1r upon C'1s was made possible by the fact that Liquoid, used to inactivate C'1r at the end of this reaction, appeared to block the subsequent action of C'1 esterase inhibitor upon C'1 esterase.

The Effect of C'1 Esterase Inhibitor on the Generation of Plasma Permeability

TABLE VI

The Effect of C'1 Esterase Inhibitor upon Plasma Permeability Activity

Mixture tested*			Permeability activity
Plasma	Diluent	Agent added after incubation	
			mm‡
1:50	Buffer	Buffer	6.2
1:100	"	"	4.3
1:50	Ellagic acid	"	8.3
1:200	" "	"	8.2
1:800	" "	"	6.5
1:50	C'1 esterase inhibitor (5.5 units/ml) and ellagic acid	"	4.2
1:50	Ellagic acid	C'1 esterase inhibitor (22 units/ml)	3.7
1:50	" "	C'1 esterase inhibitor (11 units/ml)	6.0
1:50	" "	C'1 esterase inhibitor (5.5 units/ml)	6.8
0	" "	Buffer	4.8
0	Buffer	"	1.3

* 1 ml mixtures, containing plasma at the dilution noted, ellagic acid, and C'1 esterase inhibitor (or buffer) were incubated at 37°C for 12 min. 0.2 ml of buffer or C'1 esterase inhibitor was then added and 0.1 ml aliquots injected into each of four guinea pigs (see Materials and Methods). The concentration of C'1 esterase inhibitor are those in the final mixture.

‡ Mean diameter of blued spots.

Activity.—Kagan and Becker (7, 8) reported that a partially purified fraction of plasma, thought to contain the permeability enhancing factor designated as PF/Dil, was inhibited by relatively small amounts of C'1 esterase inhibitor. In their studies, the PF/Dil had apparently already been activated from its precursor in plasma. In the present study, PF/Dil was generated in diluted plasma containing ellagic acid, an activator of Hageman factor. In the presence of C'1 esterase inhibitor, at a concentration of 5.5 units/ml, no detectable PF/Dil was demonstrable after the mixture of diluted plasma and ellagic acid had incubated for 12 min (Table VI); the lesion evoked was no bigger than that induced by ellagic acid alone.

C'1 esterase inhibitor was somewhat less effective in blocking the enhancement of permeability activity if it was added to the mixture of plasma and ellagic acid after these had incubated for 12 min (Table VI). In other experiments, the incubated mixture of diluted plasma and ellagic acid was injected

TABLE VII

The Effect of C'1 Esterase Inhibitor on Plasma Kallikrein Activity

Mixture tested*		Contraction of rat uterus
Initial incubation mixture	Agent added before testing	
		mm
A. Plasma + buffer	Buffer	2
Plasma + ellagic acid	"	47
Plasma + C'1 esterase inhibitor (15 units ml) + ellagic acid	"	17
Plasma + ellagic acid	C'1 esterase inhibitor (15 units ml)	43
Bradykinin, 50 ng	—	60
" 30 ng	—	40
" 10 ng	—	15
B. Celite eluate + buffer	Prokinin	28
Celite eluate + C'1 esterase inhibitor (12 units ml)	"	2
Celite eluate + C'1 esterase inhibitor (6 units ml)	"	7
Celite eluate + C'1 esterase inhibitor (3 units ml)	"	28
Celite eluate + buffer	Prokinin + C'1 esterase inhibitor (12 units ml)	38
Bradykinin, 20 ng	—	34
" 10 ng	—	22
" 5 ng	—	10

* A. A mixture of plasma, ellagic acid, Na_3HEDTA, and either C'1 esterase inhibitor or 0.005 M phosphate buffer was incubated for 30 min at 37°C (see Materials and Methods). Thereafter, 0.1 ml of C'1 esterase inhibitor or buffer was added to appropriate tubes. 0.2-ml aliquots were then added to a bath of de Jalon's solution in which was suspended a segment of rat uterus. The height of the contraction, as amplified, is recorded in the table.

B. A mixture of celite eluate, hexadimethrine bromide, *o*-phenanthroline, and either C'1 esterase inhibitor or buffer was incubated at room temperature for 5 min, after which prokinin was added as a substrate of kallikrein, and the mixture incubated at room temperature for an additional 10 min. C'1 esterase inhibitor or buffer was then added appropriately, and 0.2 ml aliquots tested for muscle contracting activity (see Materials and Methods).

into sites which had been injected immediately before with C'1 esterase inhibitor or 0.005 M phosphate buffer. Under these conditions, the inhibition of permeability activity was less impressive than when the C'1 esterase inhibitor had been mixed with the PF/Dil preparation just before injection.

These experiments suggest that C'1 esterase inhibitor may have been effective in blocking both the generation of PF/Dil and the permeability-enhancing effect of this agent.

The Effect of C'1 Esterase Inhibitor on the Evolution of Kinin-Like Activity.—Kagan and Becker (7, 8) also demonstrated that large amounts of C'1 esterase inhibitor inactivated a fraction of plasma rich in kallikrein-like activity. We have confirmed these observations by two techniques. C'1 esterase inhibitor, at a concentration of 15 units/ml, partially inhibited the evolution or activity of kallikrein generated in a mixture of human plasma and ellagic acid, while at a concentration of 12 units/ml, it blocked the action of preformed kallikrein (Table VII). When C'1 esterase inhibitor was added after kinins had been allowed to generate, it was without effect.

DISCUSSION

C'1 esterase inhibitor was first detected in human serum by its capacity to inhibit the esterolytic properties of C'1 esterase, an agent derived from the C'1s fragment of the first component of complement (1). When partially purified preparations of C'1 esterase inhibitor became available, it became apparent that it also inhibited chymotrypsin (4), plasma kallikrein, and the permeability-enhancing enzyme in human plasma known as PF/Dil (7, 8). These observations suggested that C'1 esterase inhibitor might well be an inhibitor with broader properties than had been thought originally.

Since these earlier reports, considerable progress has been made toward the purification of C'1 esterase inhibitor. The preparations used in the present study were purified 1700 times compared with the serum from which it was prepared. The inhibitory properties of C'1 esterase against PF/Dil and plasma kallikrein have been confirmed with these highly purified preparations. C'1 esterase inhibitor blocked both the generation of PF/Dil and, less effectively, its action in enhancing vascular permeability in guinea pig skin. Moreover, C'1 esterase inhibitor blocked the generation of kinin-like properties in mixtures of plasma kallikrein and kininogen.

C'1 esterase inhibitor decreased both the fibrinolytic and caseinolytic properties of human plasmin, while it had no effect upon the generation of plasmin in kaolin-treated euglobulin fractions of plasma nor upon urokinase, an activator of plasminogen (the precursor of plasmin) found in human urine. In contrast, in an earlier study, 1.6 units of C'1 esterase inhibitor did not block the hydrolysis of *p*-toluenesulfonyl-L-arginine methyl ester by 3000 MSD units of plasmin (4). The difference in results may have been due both to the higher concentrations of plasmin used in the esterolytic assay and to the lower concentration of C'1 esterase inhibitor. Alternatively, C'1 esterase inhibitor may block a site needed for the proteolytic activity of plasmin, but not for its esterolytic function.

Human plasma contains several different agents capable of inhibiting plasmin (37–40). We are unfamiliar with any definitive study clarifying the nature of these various inhibitors. Thus, inhibitory properties have been attributed to a heat-stable α2-globulin fraction of plasma (39). In another study, two heat-stable inhibitors were described, one of which was inactivated by ammonia, hydrazine, and certain primary amines, and one of which resisted these agents (38). Inhibitory properties were also found in a heat-labile α1-globulin fraction (40). Moreover, treatment of plasma with chloroform diminishes its inhibitory activity against plasmin (41), but the nature of the agent affected by this treatment is not known. Notably, plasma, extracted with another organic solvent, ether, lacks C'1 esterase inhibitory properties (42).

C'1 esterase inhibitor is rapidly inactivated at 56°C (1, 3), but it is an α2-globulin. Its identity with previously described inhibitors of plasmin is therefore not yet clear. Whether the plasmin inhibitory property of C'1 esterase inhibitor is of biologic importance is still to be learned, but the concentrations which blocked caseinolysis were less than those found in normal human plasma (5). Further experiments are needed to determine the role of C'1 esterase inhibitor in the physiologic activity of plasmin, and the identity of this inhibitor with others described in plasma.

The possibility that C'1 esterase inhibitor blocked the generation of C'1 esterase in plasma euglobulin fractions was raised in earlier studies, but the mechanisms involved were unclear (43, 44). In the test tube, C'1 esterase is generated from the C'1s fragment of the C'1 molecule through the enzymatic action of a second fragment, the C'1r subcomponent. This action of C'1r is apparently enzymatic (36). In the present experiments, the role of C'1 esterase inhibitor was studied by taking advantage of the inhibition of C'1r by Liquoid. Addition of this agent to mixtures of C'1r and C'1s allowed the measurement of any C'1 esterase which may have generated while preventing further evolution of this enzyme during the assay. Under these circumstances, the formation of C'1 esterase in mixtures of C'1r and C'1s was strikingly inhibited by the presence of C'1 esterase inhibitor. Preparations of C'1r also possess esterolytic properties (36). Proof that the esterase in preparations of C'1r is identical with this subcomponent of complement awaits its further purification, but it is noteworthy that the hydrolysis of its most unique substrate, *N*-acetyl-L-arginine methyl ester, was readily inhibited by C'1 esterase inhibitor.

SUMMARY

The fraction of human serum designated as C'1 esterase inhibitor is known to inhibit the action of C'1 esterase, a plasma kallikrein, and PF/Dil, an enzyme in plasma enhancing cutaneous vascular permeability. In the present study, C'1 esterase inhibitor has been found to block the actions of plasmin and the C'1r subcomponent of the first component of complement, and to

retard the generation of PF/Dil. No inhibition of blood clotting or of the generation of plasmin was demonstrable.

These experiments would not have been possible without the expert aid of Mrs. Edgar Stone, Mr. Paul Cox, and Mr. Stanford Williams.

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