MECHANISMS OF ACTIVATION OF C'1 ESTERASE IN HEREDITARY ANGIONEUROTIC EDEMA PLASMA IN VITRO

THE ROLE OF HAGEMAN FACTOR, A CLOT-PROMOTING AGENT*

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(Received for publication 15 August 1967)

Absence of inhibition of C'1 esterase from plasma of persons with hereditary angioneurotic edema (1) has been ascribed to at least two inherited abnormalities of biosynthesis of a specific alpha globulin which inhibits C'1 esterase (2). These defects have permitted studies of the activation of the first component of complement (C'1) and of the action of C'1 esterase in unfractionated serum and plasma. In the absence of serum inhibition of C'1 esterase, C'1 tends to be converted to C'1 esterase (3). This tendency to spontaneous activation of C'1 esterase in plasma from persons with hereditary angioneurotic edema is notably increased during the bouts of swelling which these individuals sustain (3, 4). Such variations in activation of C'1 esterase cannot be due merely to the lack of normal serum inhibition, for this deficiency is lifelong, and the bouts of edema and associated enhanced activation of C'1 esterase are sporadic.

In experiments designed to measure the in vitro development of C'1 esterase activity in plasma from persons with hereditary angioneurotic edema, esterolytic activity attributable to this enzyme was increased when glass rather than silicone-coated vessels were used. This suggested that one or more enzymes reactive to contact with foreign surfaces, such as glass, and effective in the generation of clot-promoting activity might influence the action of C'1. Lister (5) observed that contact with glass surfaces seemed to initiate the clotting of blood, a phenomenon also recognized by Bordet and Gengou (6). Ratnoff and his associates (7, 8) have provided firm evidence that a specific plasma protein, now called Hageman factor (9), is required for the generation of clot-promoting activity which occurs when plasma comes in contact with a surface such as glass. Hageman factor is markedly deficient in the plasma of persons with Hageman trait.

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* This work was supported by grant No. HE-08865 from The National Institutes of Health, United States Public Health Service, and by grants from the American Heart Association, The Fulton, Paulding, Williams, Henry and Defiance County branches of the Northwest Ohio Heart Association and The Northern Ohio chapter of The National Hemophilia Foundation.

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When partially purified preparations of Hageman factor were activated, in the experiments to be described, besides acquiring clot-promoting activity, they developed the capacity to enhance the generation of C'1 esterase in plasma, prepared with silicone-coated equipment, obtained from persons with hereditary angioneurotic edema in remission. Purified serum inhibitor of C'1 esterase functionally obscured the activation of C'1 in this plasma. Certain other inhibitors which do not impair the action of C'1 esterase blocked the generation of this enzyme in the plasma of persons with hereditary angioneurotic edema treated with activated Hageman factor.

Materials and Methods

Plasma samples from normal persons and persons with hereditary angioneurotic edema, inherited deficiencies of plasma thromboplastin antecedent (PTA or factor XI), or Hageman factor (factor XII) were separated from blood drawn with silicone-coated syringes (SC-87, G-E Dri-Film) into Na_2H_2EDTA already dissolved in 0.13 M sodium citrate, pH 5.2, to give a final Na_2H_2EDTA concentration of 6×10^{-3} M.[1] Plasma samples were rendered platelet-deficient by centrifugation at 20,000 *g* for 15 min at 2°C in an International PR-2 centrifuge. The plasma at no time came in contact with glass, and samples were divided and stored at −20°C or −65°C in silicone-coated containers (Lusteroid Container Co., Inc., Maplewood, N.J.) until used.

Partially purified Hageman factor was prepared from siliconed plasma obtained from normal persons, an individual with Hageman trait, and an individual with an inherited deficiency of PTA. The method of purification of Hageman factor was modified from that used by Ratnoff, Davie, and Mallet (10) in that the capacitance of the carboxymethyl cellulose used was 0.6 meq of hydrogen ion per gram. These solutions of Hageman factor were stored in silicone-coated Lusteroid containers at −65°C and used within 1 wk. The Hageman factor was dissolved in sodium phosphate buffer in a volume equal to that of the plasma from which they were derived. Thus, its concentration in experimental mixtures was less than in plasma.

A preparation of Hageman factor representing a 2000–5000-fold purification with respect to starting plasma was used in some experiments employing "highly purified Hageman factor." This Hageman factor, prepared as described by Ratnoff and Davie (11) and provided by Dr. O. D. Ratnoff, was largely in the form of an inactive precursor. It was dissolved in 0.067 M sodium phosphate buffer (pH 7.4) at 10 times the concentrations desired in experimental mixtures.

The amounts of Hageman factor (the inactive precursor) and *activated Hageman factor* in the preparations used were measured by testing the ability of the solutions of Hageman factor to shorten the clotting time of plasma obtained from an individual with an inherited deficiency of this factor. This plasma, from Mr. John Hageman, was also provided by Dr. O. D. Ratnoff. The shortening of the clotting time of this plasma in the presence of "cephalin" (12) and calcium in silicone-coated test tubes reflected the action of activated Hageman factor. The shortening of the clotting time induced by Hageman factor preparations in the presence of kaolin and a phospholipid (Gliddex-p, Glidden Paint Co., Chicago, Ill.) and calcium was a measure of both the activated Hageman factor and its precursor in the preparation. The methods used and preparation of the reagents have been described elsewhere (12–14).

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[1] Donaldson, V. H. Mechanisms of activation of C'1-esterase in hereditary angioneurotic edema plasma in vitro. The role of plasminogen activators and trypsin and the effect of protease inhibitors. Submitted for publication.

Crude C'1 esterase was prepared from serum of a normal person and serum of a person with Hageman trait (severe inherited deficiency of Hageman factor). A euglobulin precipitate was prepared by mixing 1 volume of serum with 8 volumes of 0.02 M acetate buffer at a pH of 5.5. The precipitate was dissolved in a volume of 0.067 M sodium phosphate buffer, pH 7.4, equal to $\frac{1}{10}$ that of starting serum. After dialysis for 48 hr against pH 7.4 sodium phosphate buffer at 4°C, these preparations of C'1 esterase were rendered active by incubation at 37°C for 30 min and tested for properties attributable to C'1 esterase.

A preparation of human serum inhibitor of C'1 esterase, representing at least a 2000-fold purification with respect to starting serum, was generously provided by Dr. Jack Pensky of Western Reserve University.

Ellagic acid (4,4',5,5',6,6'-hexahydroxydiphenic acid 2,6,2',6'-dilactone) was obtained from K and K Laboratories, Inc., Plainview, N. Y. It was mixed with buffer to provide a concentration of 10^{-3} M. After mechanical homogenization of this somewhat insoluble material, the mixture was centrifuged at 37,000 *g* for 15 min in a Servall RC-2 centrifuge to remove any remaining insoluble substance. The supernatant ellagic acid solution, freshly prepared, was diluted in experimental procedures as noted.

Trasylol (compound A-128, proteinase inactivator), a kallikrein inhibitor, was used in a concentration of 625 units/ml in incubation mixtures and diluted to 125 units/ml in titration mixtures. *Heparin* (The Upjohn Co., Kalamazoo, Mich.), in a concentration of 125 USP units/ml of incubation mixtures, was diluted to 25 USP units/ml in titration mixtures. *Epsilon aminocaproic acid* (Mann Research Laboratories, New York, N.Y.) was dissolved in 0.15 M sodium chloride in a concentration of 1.0 M; it was used in a concentration of 0.125 M in incubation mixtures and, in titration mixtures, diluted to 0.025 M. *Soybean trypsin inhibitor* (5 × crystallized, Nutritional Biochemicals Corp., Cleveland, Ohio) was used at a concentration of 1.25 mg/ml in incubation mixtures and 0.250 mg/ml during titrations.

N-acetyl-L-tyrosine ethyl ester monohydrate (*ALTEE*),[1] prepared in the laboratories of The Department of Chemistry, Western Reserve University by Mr. Albert Arters, was dissolved in 2-methoxyethanol (Methyl Cellosolve, Matheson Co., Norwood, Ohio) in a concentration of 1.6 M for use as substrate in assays measuring C'1 esterase activity; R4, a serum reagent markedly deficient in the fourth component of complement (C'4) and used to titrate C'4 in experimental mixtures, was prepared by treating normal serum with hydrazine (15, 16). Normal serum was heated to 56°C for 30 min to provide a source of C'4 free of C'1 and C'2. To prepare sensitized erythrocytes for use in titrating hemolytic complement, sheep erythrocytes were washed 4 or more times in 10 times their volume of cold 0.15 M sodium chloride, and incubated with a $\frac{1}{1000}$ dilution of rabbit antiserum to sheep erythrocytes (Baltimore Biological Laboratories, Baltimore, Md).[1]

Triethanolamine-buffered saline containing calcium, magnesium, and gelatin was prepared according to the method of Kent et al. (17) at a pH of 7.4 and ionic strength of 0.15, and was used in assays titrating hemolytic C'4 activity. Sodium phosphate buffer, at a pH of 7.4 and ionic strength of 0.15, was used in assays measuring ALTEE hydrolysis. Barbital-saline buffer, pH 7.4 and ionic strength 0.15, was used in coagulation studies and contained 7.8 g of sodium chloride, 2.76 g of barbital, and 2.06 g of sodium barbital in 1 liter of distilled water.

Continuous monitoring of ALTEE hydrolysis by plasma mixtures was carried out with a Radiometer pH meter (Radiometer, Copenhagen, Denmark, model PHM 27 with expanded scale), automatic titrator (model TTT 11), and a 2.5 ml syringe burette (ABU 19). To maintain a constant temperature of 37°C and constant pH, jacketed electrode vessels connected to a continuously circulating water bath at 37°C were used with a magnetic stirrer. Electrodes and vessels were coated with silicone (Stopcock Grease, Dow Corning Corp., Midland, Mich.). Plasma samples of 0.5 ml each, and 1.875 ml of phosphate buffer were warmed to

37°C for 10 min in a titration vessel. The pH of this mixture was adjusted to 7.45–7.47, if not at this point, and 0.125 ml of 1.6 M ALTEE rapidly injected via syringe and needle as a stop clock was started. Readings of the volume of 0.05 N sodium hydroxide titrated to maintain the pH of the mixture at 7.4 were taken at 2 min intervals. If the pH of the enzyme-substrate mixture rose to 7.5 before esterolysis began, it was lowered to 7.45–7.47 with 0.1 N HCl.

To test the effect of Hageman factor upon the generation of ALTEE esterase activity in hereditary angioneurotic edema plasma samples obtained during remissions, 0.18 ml of the Hageman factor preparation in solution was mixed with 0.02 ml of 10^{-3} M ellagic acid at room temperature. Ellagic acid in solution has been shown to activate Hageman factor (14). The ellagic acid–activated Hageman factor was then mixed with 0.5 ml of the plasma to be tested for activation of its C'1 esterase. These mixtures were incubated in silicone-coated Lusteroid test tubes at 37°C for the time intervals noted and 0.5 ml of each was then used to measure esterolysis in the incubation and titration mixtures described. Hereditary angioneurotic edema plasma was incubated with buffer, ellagic acid, or ellagic acid–activated Hageman factor and the differences in ALTEE-esterolytic activity generated were then measured during continuous acidimetric titrations. Esterolysis generated was also compared with that formed in mixtures of hereditary angioneurotic edema plasma and "unactivated" Hageman factor and "activated" Hageman factor prepared from plasma deficient in this factor.

The ability of preparations of Hageman factor and of mixtures of Hageman factor and hereditary angioneurotic edema plasma to inactivate the fourth component of complement (C'4) was tested by the method of Haines and Lepow (18).

RESULTS

Effect of Activated Hageman Factor on the Generation of ALTEE Esterolytic Activity in Hereditary Angioneurotic Edema Plasma.—Freshly prepared plasma from normal individuals or persons with hereditary angioneurotic edema in remission did not hydrolyze *N*-acetyl-L-tyrosine ethyl ester (ALTEE). While normal plasma did not digest ALTEE even after prolonged incubation with 10^{-4} M ellagic acid, plasma from a person with hereditary angioneurotic edema in remission developed this esterolytic activity during incubation with 10^{-4} M ellagic acid. Usually, both the rate at which ALTEE-hydrolytic activity appeared and the quantity of ester hydrolyzed were increased when plasma obtained during remission was incubated with ellagic acid instead of with buffer. Fig. 1 illustrates the effect of prolonged incubation of ellagic acid with hereditary angioneurotic edema plasma at 4°C.

Ellagic acid converts Hageman factor to an active clot-promoting substance (14). To determine whether the ALTEE esterase activity in hereditary angioneurotic edema plasma might have resulted from the activation of the Hageman factor in this plasma, a highly purified preparation of human Hageman factor was treated with 10^{-4} M ellagic acid and then incubated at 37°C with hereditary angioneurotic edema plasma which had not developed ALTEE-esterase activity. The highly purified activated Hageman factor induced ALTEE-esterase activity in the plasma, and both the rate of generation of esterolysis and amount of ester digested were increased in relative proportion to the quantity of activated Hageman factor which was originally incubated with the plasma (Fig. 2). In this experiment, very little esterolytic activity was generated during

incubation of the plasma with buffer or with ellagic acid alone for 1 hr at 37°C prior to titration of its effect upon ALTEE. The quantity of Hageman factor used in this experiment far exceeded the concentrations which might occur in plasma.

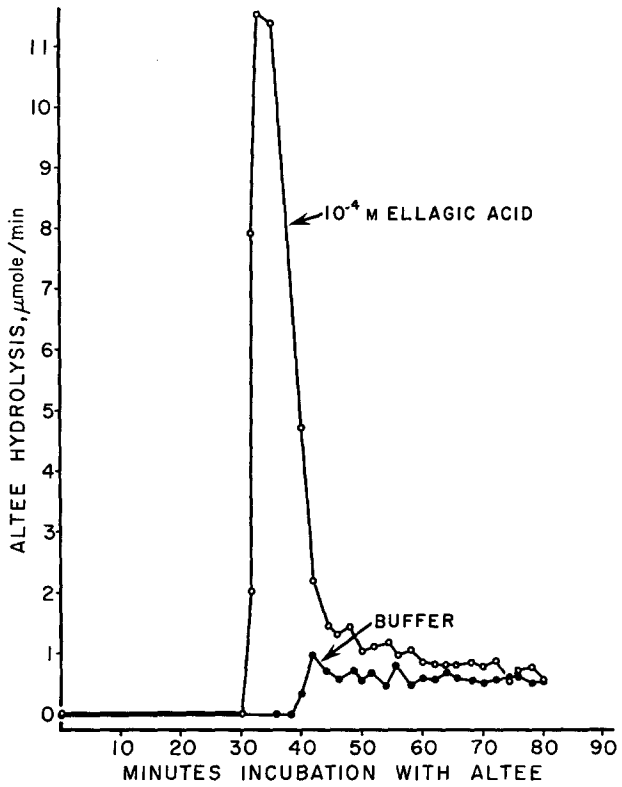

FIG. 1. Samples of plasma from a person with hereditary angioneurotic edema were mixed with ellagic acid or buffer and stored in silicone-coated vessels at 4°C for 48 hr. Then, each sample was tested for its ability to hydrolyze ALTEE during constant automatic titration at 37°C in a Radiometer pH-stat. Open circles designate the rate of ALTEE hydrolysis by the sample stored with ellagic acid; closed circles describe the rate of hydrolysis by the same sample stored with buffer.

To determine if the enhanced formation of ALTEE-esterase activity in mixtures of activated Hageman factor and hereditary angioneurotic edema plasma was due to the activated Hageman factor rather than to a contaminating substance, partially purified Hageman factor was prepared from the plasma of Mr. Hageman, severely deficient in this plasma protein (8, 9). The preparation was less pure than the material used in the previous experiments, but was used in concentrations approximating those more likely to occur in plasma. Hageman

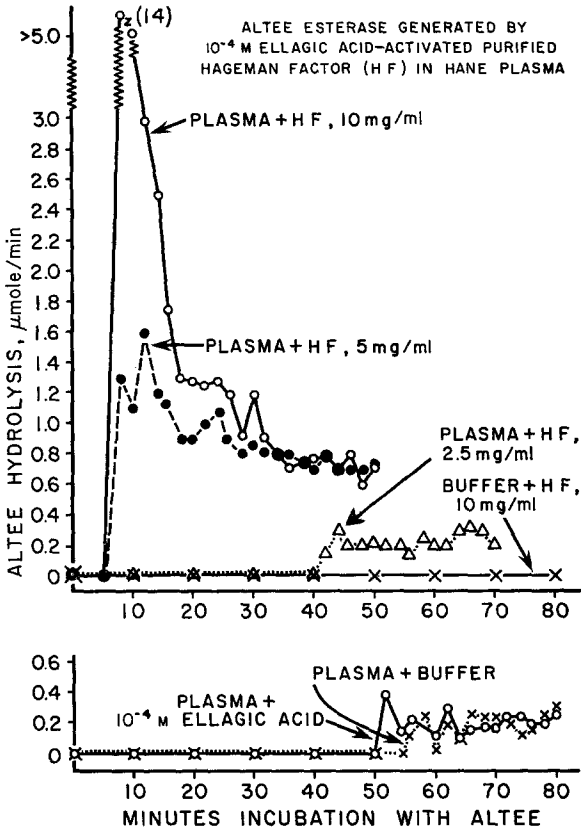

FIG. 2. Curves in the upper panel describe rates of ALTEE hydrolysis by hereditary angioneurotic edema (HANE) plasma which had been treated with varying concentrations of ellagic acid-activated highly purified Hageman factor (HF), and lack of activity in ellagic acid activated Hageman factor alone. Curves in the lower panel represent rates of ALTEE hydrolysis by the same plasma treated with buffer or ellagic acid without added Hageman factor. Before assay, 0.5 ml samples of the plasma had been incubated with 0.05 ml of the additive for 2 hr at 37°C in silicone-coated vessels. In the assays, 0.5 ml of these incubation mixtures were tested for ALTEE hydrolytic activity.

factor prepared from normal plasma and plasma deficient in plasma thromboplastin antecedent was also tested. None of the preparations of Hageman factor hydrolyzed ALTEE by themselves even after activation with ellagic acid. Nor could an increase in the generation of ALTEE-esterase in the mixture be demonstrated after incubation of mixtures of ellagic acid-activated "Hageman-deficient" Hageman factor with hereditary angioneurotic edema plasma (Fig. 3). However, ellagic acid-activated Hageman factor prepared from plasma deficient in PTA generated a larger amount of ALTEE-hydrolytic activity in

hereditary angioneurotic edema plasma more rapidly than did PTA-deficient Hageman factor not treated with ellagic acid. Activated Hageman factor prepared from normal plasma and hereditary angioneurotic edema plasma also readily induced ALTEE-esterolytic activity in hereditary angioneurotic edema plasma. Apparently activated Hageman factor was required to hasten the formation of ALTEE-esterase activity.

Since activated Hageman factor is a potent clot-promoting agent, the ability of these preparations to shorten the clotting time of Hageman factor–deficient plasma was measured to define any correlation between their capacity to induce

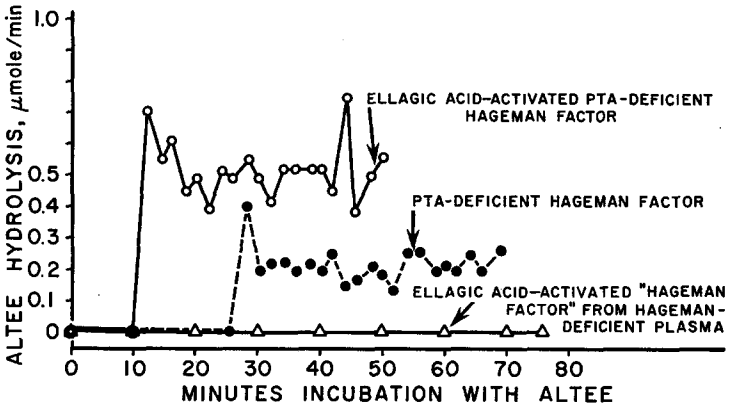

FIG. 3. Crude Hageman factor prepared from PTA-deficient plasma and from Hageman factor–deficient plasma was mixed with 10^{-4} M ellagic acid, or with buffer, and 0.2 ml of each of these mixtures, then incubated with 0.5 ml of hereditary angioneurotic edema plasma at 37°C for 1 hr in silicone-coated vessels. The rates of ALTEE hydrolysis by 0.5 ml samples of each mixture were compared during continuous automatic titration to pH 7.4 at 37°C. Open circles, plasma treated with ellagic acid–activated PTA-deficient Hageman factor. Closed circles, plasma treated with the PTA-deficient Hageman factor (unactivated). Triangles, plasma treated with ellagic acid–activated "Hageman-deficient" Hageman factor.

esterase activity and to promote clotting. The Hageman-deficient Hageman factor preparation failed to shorten the clotting time of this plasma in the presence of calcium, kaolin, and phospholipid, a mixture known to activate Hageman factor and to permit the subsequent events leading to thrombin generation to occur (19, 20) (Table I). The Hageman factor from PTA-deficient plasma significantly shortened the clotting time of an otherwise identical mixture (Table I). When preparations of Hageman factor were tested for their ability to shorten the clotting time of Hageman factor–deficient plasma in the presence of only cephalin and calcium, Hageman factor from normal plasma and PTA-deficient plasma significantly shortened the clotting time of the mixture (Table I). Again, preparations of Hageman factor from plasma deficient in this factor were ineffective. Since this assay measures the clot-promoting activity of any

Hageman factor already activated in the preparations tested (13), these experiments indicate that some of the Hageman factor had become activated during its preparation. From 5–10% of the Hageman factor in more purified preparations was activated during fractionation (14). All of the Hageman factor preparations which had clot-promoting activity could increase the rate of generation of ALTEE-esterase activity to some degree in hereditary angioneurotic

TABLE I

Clot-Promoting Activity of Partially Purified Preparations of Hageman Factor

Sample	Clotting time	
	Unactivated and activated Hageman factor	Already activated Hageman factor
	sec	*sec*
Hageman factor from Hageman factor–deficient plasma		
Undiluted	over 1740	over 1700
Hageman factor from PTA-deficient plasma		
Undiluted	190	485
1/10 dilution	210	
1/40 dilution	350	
Buffer control	2880	
Hageman factor from normal plasma		
Undiluted	—	466
Buffer control	1740	over 3000

To measure the total Hageman factor activity in a partially purified fraction of this substance, 0.1 ml of this preparation was mixed with 0.1 ml of a Gliddex-kaolin mixture and 0.1 ml of plasma deficient in Hageman factor in Pyrex test tubes. After incubation at 37°C for 8 min to allow activation of the unactivated Hageman factor and its interaction with the plasma thromboplastin antecedent in the plasma, 0.1 ml of 0.025 M calcium chloride was added, mixed, and the time required for visible fibrin formation measured. To measure the already activated Hageman factor, 0.1 ml of the Hageman factor preparation was incubated with 0.1 ml of a solution of 0.06 mg of cephalin/ml of barbital-saline buffer, pH 7.4, and 0.1 ml of Hageman factor–deficient plasma in silicone-coated Pyrex test tubes. After incubation for 8 min at 37°C, to allow the activated Hageman factor to interact with the plasma, 0.1 ml of the calcium chloride was added, mixed, and the time needed for clotting measured.

edema plasma even without treatment with ellagic acid. Thus, the capacity of the Hageman factor preparations to enhance the clotting of plasma deficient in Hageman factor, bore a relationship to their ability to increase the generation of ALTEE-esterase activity in hereditary angioneurotic edema plasma. Activated partially purified Hageman factor did not induce ALTEE-esterase activity in normal plasma.

Identity of the Esterase Activity Generated with C'1 Esterase.—Although it has

been shown that the digestion of ALTEE by plasma under the conditions of pH and ionic strength of the assays employed in these experiments apparently reflects the action of C'1 esterase (1, 3), further confirmation was sought. When two units of purified human serum inhibitor of C'1 esterase were added to a mixture of activated Hageman factor and hereditary angioneurotic edema plasma which had already begun to hydrolyze ALTEE, the rate of esterolysis dropped from 0.9 μmole/min to 0 within 1 min. No further esterolysis occurred in the following 10 min. The observed inhibition supports the likelihood that the esterase measured was C'1 esterase.

Since C'1 esterase in solution can inactivate the fourth (C'4) component of complement (18), hereditary angioneurotic edema plasma which had been incubated with activated Hageman factor was tested for its ability to inactivate the hemolytic C'4 in serum depleted of C'1 and C'2 by heating to 56°C for 30 min. Activated Hageman factor, hereditary angioneurotic edema plasma, buffer, and ALTEE were mixed and observed in the pH-stat for any drop in pH. At intervals noted (Table II), 0.2 ml samples were removed from the titration vessel, mixed with 0.4 ml of heated serum, incubated at 0°C for 30 min, and the residual C'4 was then measured in a hemolytic assay.

Activated Hageman factor derived from normal plasma, PTA-deficient plasma and Hageman factor–deficient plasma did not directly inactivate C'4. However, during incubation of either normal or PTA-deficient activated Hageman factor with hereditary angioneurotic edema plasma, C'4-inactivating properties appeared immediately (Table II). In the absence of added Hageman factor, hereditary angioneurotic edema plasma less readily inactivated C'4 after its incubation with ellagic acid or with buffer. Ellagic acid–activated Hageman factor from Hageman factor–deficient plasma increased the rate of formation of C'4-inactivating property somewhat, but slightly more rapidly than did ellagic acid alone. None of the activated Hageman factor preparations induced the normal plasma to destroy C'4. The normal plasma contained a normal amount of inhibitor of C'1 esterase.

C'4 is a natural substrate for C'1 esterase, and its inactivation is a much more sensitive way of measuring small amounts of C'1 esterase than is the digestion of ALTEE. The fact that the generation of ALTEE-esterase activity in hereditary angioneurotic edema plasma treated with activated Hageman factor was closely associated with the formation of a property which inactivates C'4 supports the liklihood that the ALTEE-esterolytic activity was identical with that of C'1 esterase.

Since these studies indicated a role for Hageman factor in the activation of C'1 esterase in plasma from persons with hereditary angioneurotic edema, it was possible that activated Hageman factor was a necessary prerequisite. To explore this possibility, a crude preparation of C'1 esterase derived from serum of an individual with Hageman trait was tested for its C'1 esterase–like activity. After activation, this Hageman factor–deficient C'1 esterase hydrolyzed

ALTEE and inactivated C'4 in solution in a manner quantitatively parallel to the action of C'1 esterase derived from normal serum.

The Effect of Some Inhibitors upon the Generation of C'1 Esterase in Hereditary Angioneurotic Edema Plasma by Activated Hageman Factor.—The activation of C'1 in mixtures of activated Hageman factor and hereditary angioneurotic edema plasma obtained during remissions was impaired by some inhibitors which block some other plasma enzymes, but not C'1 esterase. Soy bean trypsin

TABLE II

Inactivation of C'4 in Solution by Mixtures of Hageman Factor and Hereditary Angioneurotic Edema (HAE) Plasma

Time	Per cent of C'4 inactivated by:				
	Activated Hageman factor + HAE plasma	Activated Hageman-deficient "Hageman factor" + HAE plasma	Ellagic acid + HAE plasma	Buffer + HAE plasma	Activated Hageman factor from normal plasma + buffer (no HAE plasma)
Min					
0	100	25	0	0	0
10	"	—	—	—	0
15	"	—	—	—	0
25	—	81	50	—	0
46	—	88	75	—	0
66	—	over 90	94	81	0
76	—	—	—	over 90	0
86	—	—	—	—	0

Plasma from an individual with hereditary angioneurotic edema in remission was incubated either with ellagic acid–activated Hageman factor from PTA-deficient plasma, ellagic acid–activated Hageman factor from Hageman-deficient plasma, 10^{-4} M ellagic acid, or buffer for 60 min at 37°C in silicone-coated Lusteroid test tubes. Then, 0.5 ml of the incubation mixture to be tested was added to 1.875 ml of sodium phosphate buffer, pH 7.4, in a siliconed constant temperature titration vessel and, after warming the mixture to 37°C for 10 min, 0.125 ml of 1.6 M ALTEE was added. At the times noted, 0.2 ml samples were removed from the continuous-titration mixture and tested for their capacity to inactivate C'4 in heated serum. Aliquots of the same sample of hereditary angioneurotic edema plasma were used in each mixture in this experiment.

inhibitor can inhibit plasmin (21, 22), plasma kallikrein (23), and one or more clot-promoting substances effective in the conversion of prothrombin to thrombin (24, 25). It does not inhibit the action of C'1 esterase upon ALTEE (26). When this inhibitor was added to hereditary angioneurotic edema plasma prior to its incubation with activated Hageman factor, the generation of ALTEE-esterase activity in the mixture was inhibited (Fig. 4).

Heparin did not inhibit the hydrolysis of ALTEE by hereditary angioneurotic edema plasma during continuous titration. It did not change the rate of hydrolysis of this ester during such a titration beyond the temporary effect of dilution,

which was also noted if buffer were added to the titration mixture once esterolysis had begun. However, when heparin in identical concentrations (125 USP units/ml) was added to hereditary angioneurotic edema plasma before the activated Hageman factor, it did block the generation of this esterolytic property (Fig. 4).

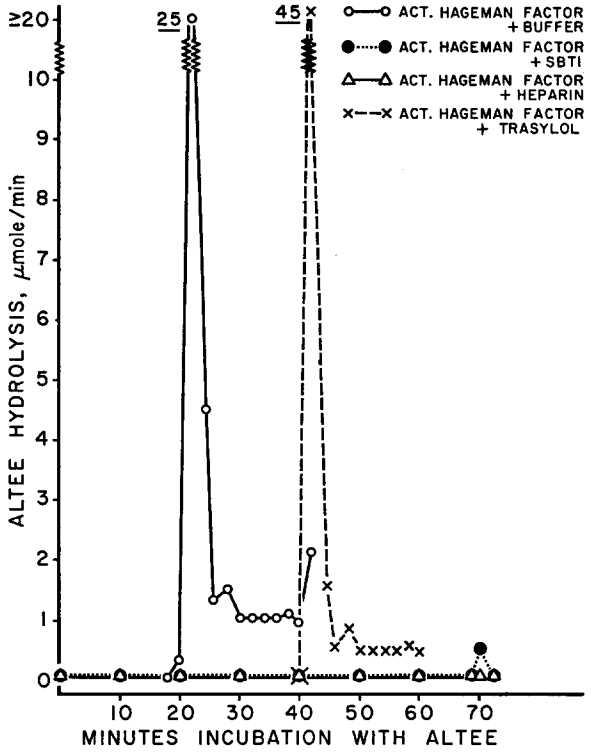

FIG. 4. In this experiment, 0.18 ml of ellagic acid–activated crude Hageman factor was mixed with 0.02 ml of buffer (open circles), 0.02 ml of soy bean trypsin inhibitor, 10 mg/ml (closed circles), 0.02 ml of heparin, 1000 U/ml (triangles) or 0.02 ml of Trasylol, 5000 U/ml (×). Then, 0.5 ml of hereditary angioneurotic edema plasma was added to each, mixed well, and these mixtures incubated at 37°C for 40 min before being tested for ALTEE esterase activity using the method of continuous titration described.

Trasylol in high concentrations (625 units/ml) delayed the generation of ALTEE-esterase activity in mixtures of hereditary angioneurotic edema plasma and activated Hageman factor, but to a smaller degree than did either heparin or soy bean trypsin inhibitor. Once esterolysis began, the mixture containing Trasylol hydrolyzed even more ALTEE within the ensuing 20 min than did a control mixture containing buffer instead of Trasylol (Fig. 4). Trasylol did not inhibit the esterolytic activity of preparations of activated C'1 esterase.[1]

Epsilon aminocaproic acid can inhibit several proteolytic ezymes (27), but in a concentration of 0.1 M does not, to our knowledge, delay the clotting or normal plasma or inhibit esterolysis by C'1 esterase.[2] It can, however, impair the activation of C'1 on sensitized erythrocytes (28). The effect of 0.1 M epsilon aminocaproic acid on the generation of C'1 esterase in mixtures of hereditary angioneurotic edema plasma and activated Hageman factor was quite unpredictable, as noted in other experiments testing the effect of urokinase upon the activation of C'1 in these plasma samples.[1]

DISCUSSION

The ALTEE-esterase activity which can be generated in plasma obtained from persons with hereditary angioneurotic edema can be identified with C'1 esterase because its appearance was associated with the development of a property which inactivated C'4 (18, 29), and because a preparation of inhibitor of C'1 esterase representing at least a 2000-fold purification with respect to serum, inhibited this esterolytic activity (30). In the present experiments, highly purified preparations of activated Hageman factor enhanced the generation of C'1 esterase activity in hereditary angioneurotic edema plasma. The rate of generation and the amount of C'1 esterase formed were proportional to the amount of purified activated Hageman factor used (Fig. 2). If derived from plasma deficient in Hageman factor, a fraction normally rich in this activity failed to develop clot-promoting activity when activated, and similarly failed to induce C'1 esterase activity in hereditary angioneurotic edema plasma (Fig. 3). Hageman factor appeared to be necessary for both. The initial bursts of esterolytic activity were unexplained (Fig. 1–4).

While activated Hageman factor did not induce formation of free C'1 esterase in normal plasma, it was possible that any C'1 esterase formed was obscured by the normal inhibitor, but might have been detected indirectly by measuring consumption of normal inhibition in the plasma after its incubation with activated Hageman factor. However, it was not possible to show conclusively a significant decrease in normal plasma inhibition after prolonged incubation of the mixture, perhaps reflecting the relative insensitivity of the method for measuring inhibition.

Some substances which do not themselves inhibit C'1 esterase, impaired the effect of activated Hageman factor upon the activation of C'1 in hereditary angioneurotic edema plasma. Soy bean trypsin inhibitor does not block the esterolytic activity of C'1 esterase (26), but does inhibit some other hydrolytic enzymes which may appear in plasma (21–25). Heparin also fails to block esterolysis by C'1 esterase, but inhibits some of the plasma enzymes participating in coagulation (31). Both of these substances markedly inhibited the gen-

[2] Donaldson, V. H. Unpublished observations.

eration of C'1 esterase in hereditary angioneurotic edema plasma during its incubation with activated Hageman factor. Therefore, C'1 must have been activated after some intermediate steps initiated by activated Hageman factor and susceptible to inhibition by soy bean trypsin inhibitor and heparin.

Other enzymes active in coagulation might have been involved. Soy bean trypsin inhibitor in a concentration of 500 μg/ml did not prevent the interaction of activated Hageman factor with plasma thromboplastin antecedent in the generation of clot-promoting activity in the studies of Ratnoff, Davie, and Mallett (10). In the present experiments, 250 μg of soy bean trypsin inhibitor per ml of titration mixture blocked the ability of activated Hageman factor to generate C'1 esterase. It is therefore unlikely that the activated Hageman factor affected C'1 directly after interacting with plasma thromboplastin antecedent in hereditary angioneurotic edema plasma, for inhibition should not have occurred. A later enzymatic reaction effective in coagulation is very sensitive to soy bean trypsin inhibitor (24). Heparin can block the next step in coagulation in which there is an interaction of plasma thromboplastin antecedent and Christmas factor, initiated by activated Hageman factor (32). Thus, later acting coagulation enzymes might participate in the formation of C'1 esterase in hereditary angioneurotic edema plasma treated with activated Hageman factor.

The activation of C'1 esterase in the presence of activated Hageman factor results more likely from some of the enzymatic events which can lead to the formation of vasoactive polypeptides, called kinins, as illustrated in Fig. 5. Margolis (33) and Ratnoff and Miles (34) found that activated Hageman factor was required for the generation of permeability factor activity in normal plasma diluted and shaken with glass. The development of the permeability property could be inhibited by soy bean trypsin inhibitor, and heparin, and was not dependent upon the interaction of activated Hageman factor with plasma thromboplastin antecedent or other later acting coagulation factors (34). Neither heparin nor soy bean trypsin inhibitor impaired this permeability factor activity once it was formed. These inhibitors behaved in like manner toward the activation of C'1 by activated Hageman factor in the present experiments.

It has been proposed that a globulin permeability factor of plasma which requires activated Hageman factor for its formation does not directly release kinins, but can activate a plasma kinin-forming enzyme, called kallikrein (35, 36). The studies of Margolis (37), as well as those of Armstrong and her associates (38), Spector (39), Webster and Ratnoff (40), and Ratnoff and Miles (34) provide evidence suggesting that Hageman factor, once activated, may in some way induce the formation of kinins which cause increased vascular permeability, pain, vasodilitation, and can induce the isolated rat uterus to contract. The mechanism of kinin formation seems not to involve the other clot-promoting enzymes in plasma, but rather, the interaction of activated

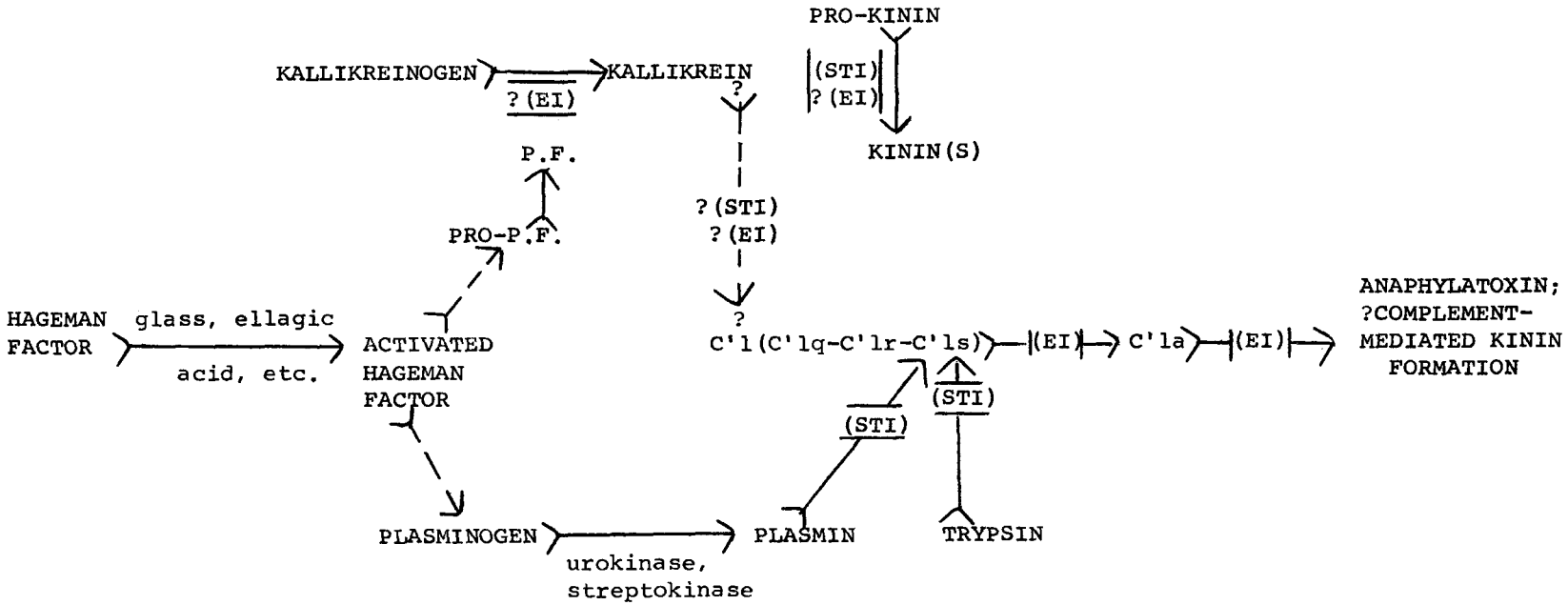

FIG. 5. A scheme of possible mechanisms through which activated Hageman factor might enhance the activation of C′1 esterase in hereditary angioneurotic edema plasma. Points of reported inhibition by serum inhibitor of C′1 esterase (EI) and by soy bean trypsin inhibitor (STI) are so designated. "P.F." refers to a globulin permeability factor, and "PRO-P.F." to its inactive precursor. C′1a is synonymous with C′1 esterase. C′1 esterase can generate an anaphylatoxin from purified preparations of later acting components of human complement (50), and can increase vascular permeability upon intradermal injection into humans, possibly by releasing kinin (51).

Hageman factor with other plasma enzymes leading eventually to kinin release (Fig. 5). In this regard, Landerman and his associates (41) and Burdon et al. (42) have reported that a kallikrein system may participate in the formation of plasma kinin in hereditary angioneurotic edema serum. Landerman et al. reported a deficiency of the normal serum inhibition directed against a preparation of plasma kallikrein and/or globulin permeability factor in hereditary angioneurotic edema plasma (41). Subsequently, Kagan has found that a partially purified preparation of inhibitor of C'1 esterase can inhibit preparations of plasma permeability globulin and of kallikrein (43). Perhaps, then, activated Hageman factor induces the formation of an active globulin permeability factor (34), which then directs events releasing kinin and culminating in the activation of C'1 esterase in hereditary angioneurotic edema plasma. We have found that a kinin-like substance forms readily in the blood of persons with hereditary angioneurotic edema (44) which can increase vascular permeability in the skins of experimental animals and of persons with hereditary angioneurotic edema (45). This effect is not mediated through the release of histamine but appears to be due to the action of a polypeptide (44). It is therefore logical to postulate that a kinin-forming system of plasma may be involved in the mechanism of activation of C'1 by activated Hageman factor (Fig. 5).

Trasylol can inhibit some kallikreins (46) and plasmin, a fibrinolytic enzyme of blood, but does not block the generation of clot-promoting activity in normal plasma in concentrations of 100 units/ml.[2] This substance was less effective in blocking the generation of C'1 esterase than either heparin or soy bean trypsin inhibitor. The explanation of the increased amount of esterolysis which eventually developed in incubation mixtures containing Trasylol is not apparent.

Plasma enzymes which participate in fibrinolysis might also direct a series of reactions initiated by activated Hageman factor and ultimately leading to the activation of C'1 esterase in plasma from persons with hereditary angioneurotic edema (Fig. 5).[1] Niewiarowski and Wartelle (47), and Iatridis and Ferguson (48) have provided some experimental support for the concept that the initiation of clot-promoting activity by the activation of the Hageman factor in plasma can accelerate the rate of formation of fibrinolytic activity. It has been repeatedly observed that plasmin, a fibrinolytic enzyme of blood, can activate the first component of complement (26, 49, and footnote 1). Therefore, activated Hageman factor might enhance the fibrinolytic activity of hereditary angioneurotic edema plasma, and this might in turn influence the rate of generation of C'1 esterase (Fig. 5).

These experiments in no way demonstrate a role for activated Hageman factor in the genesis of the sporadic bouts of edema to which persons with hereditary angioneurotic edema are subject. We know of no individual with an inherited deficiency of Hageman factor who also lacks serum inhibition of C'1 esterase. It would be interesting to know if such a doubly blighted person would

be subject to bouts of hereditary angioneurotic edema, as the absence of serum inhibition of C'1 esterase would lead one to predict. The fact that crude C'1 esterase, prepared from serum of a person markedly deficient in Hageman factor, hydrolyzed ALTEE and inactivated C'4, just as C'1 esterase from normal serum did, indicates that there is no absolute requirement for activated Hageman factor in the in vitro activation of crude C'1 esterase.

SUMMARY

The generation of C'1 esterase activity in siliconed plasma obtained from individuals with hereditary angioneurotic edema in remission tends to occur spontaneously, but can be hastened during its incubation with preparations of activated Hageman factor. This effect of activated Hageman factor could not be shown during its incubation with normal siliconed plasma, nor could consumption of normal serum inhibition of C'1 esterase be clearly shown.

Soy bean trypsin inhibitor and heparin could impair this enhanced generation of C'1 esterase but neither inhibits the esterolytic function of C'1 esterase once formed. Trasylol was less effective in blocking this effect of activated Hageman factor.

While the mechanism of the effect of activated Hageman factor upon C'1 activation remains obscure, it is apparent that some intermediate steps, possibly involving a kinin-forming system of plasma, may play a role.

Dr. Fred S. Rosen of The Children's Hospital Medical Center, Boston, Mass., provided some of the plasma samples from persons with hereditary angioneurotic edema, and Dr. Rudolf Goesswald of FBA Medical Research Division of Metachem, Inc., New York, provided the samples of Trasylol (Preparation A 128). Technical assistance was provided by P. Kent Taylor, and the secretarial assistance of Joyce Davidson permitted completion of the manuscript.

BIBLIOGRAPHY

1. Donaldson, V. H., and R. R. Evans. 1963. A biochemical abnormality in hereditary angioneurotic edema: Absence of serum inhibitor of C'1-esterase. *Am. J. Med.* **35**:37.
2. Rosen, F. S., P. Charache, J. Pensky, and V. H. Donaldson. 1965. Hereditary angioneurotic edema: Two genetic variants. Science. **148**:957.
3. Donaldson, V. H., and F. S. Rosen. 1964. The action of complement in hereditary angioneurotic edema: The role of C'1-esterase. *J. Clin. Invest.* **43**:2204.
4. Austen, K. F., and A. L. Sheffer. 1965. Detection of hereditary angioneurotic edema by demonstration of a reduction in the second component of human complement. *New Engl. J. Med.* **272**:649.
5. Lister, J. 1863. On the coagulation of the blood. *Proc. Roy. Soc. London.* **12**:580.
6. Bordet, J., and O. Gengou. 1901. Recherches sur la coagulation du sang et les serums anticoagulants. *Ann. Inst. Pasteur.* **15**:129.
7. Ratnoff, O. D., and C. L. Conley. 1951. The role of surface and of calcium in the coagulation of a globulin fraction of platelet-deficient plasma. *Bull. Johns Hopkins Hosp.* **89**:245.

8. Ratnoff, O. D., and J. Colopy. 1955. A familial hemorrhagic trait associated with a deficiency of clot-promoting fraction of plasma. *J. Clin. Invest.* **34**:602.
9. Ratnoff, O. D., and J. Rosenblum. 1958. Role of Hageman factor in the initiation of clotting by glass. *Am. J. Med.* **25**:160.
10. Ratnoff, O. D., E. W. Davie, and D. L. Mallett. 1961. Studies on the activation of Hageman factor: Evidence that activated Hageman factor in turn activates P.T.A. *J. Clin. Invest.* **40**:803.
11. Ratnoff, O. D., and E. W. Davie. 1962. The purification of activated Hageman factor. *Biochemistry.* **1**:967.
12. Bell, W. N., and H. G. Alton. 1954. A brain extract as a substitute for platelet suspension in the thromboplastin generation test. *Nature.* **174**:880.
13. Donaldson, V. H., and O. D. Ratnoff. 1965. Hageman factor: Alterations in physical properties during activation. *Science.* **150**:754.
14. Ratnoff, O. D., and J. Crum. 1964. Activation of Hageman factor by solutions of ellagic acid. *J. Lab. Clin. Med.* **63**:359.
15. Mayer, M. M. 1961. Complement and complement fixation. *In* Experimental Immunochemistry. E. A. Kabat and M. M. Mayer, editors. Charles C Thomas, Springfield. 2nd edition. 133.
16. Wedgwood, R. J. P. 1959. Measurement of the components of complement by reagent titration technique. *Z. Immunitaetsforsch.* **118**:358.
17. Kent, J. F., A. G. Otero, and R. E. Harrigan. 1957. Relative specificity of serologic tests for syphilis in Mycobacterium leprae infection. *Am. J. Clin. Pathol.* **27**:539.
18. Haines, A. L., and I. H. Lepow. 1964. Studies on human C'1-esterase. II. Function of purified C'1-esterase in the human complement system. *J. Immunol.* **92**:468.
19. MacFarlane, R. G. 1964. An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. *Nature.* **202**:498.
20. Davie, E. W., and O. D. Ratnoff. 1964. Waterfall sequence for intrinsic blood clotting. *Science.* **145**:1310.
21. Mirsky, I. A. 1944. Inhibition of β-hemolytic streptococcal fibrinolysin by trypsin inhibitor (antiprotease). *Science.* **100**:198.
22. Tagnon, H. J., and J-P. Soulier. 1946. Anticoagulant activity of the trypsin inhibitor from soya bean flour. *Proc. Soc. Exptl. Biol. Med.* **61**:440.
23. Webster, M. E., and J. V. Pierce. 1961. Action of the kallikreins on synthetic ester substrates. *Proc. Soc. Exptl. Biol. Med.* **107**:186.
24. Breckenridge, R. T., and O. D. Ratnoff. 1965. The role of proaccelerin in human blood coagulation. Evidence that proaccelerin is converted to a prothrombin converting principle by activated Stuart factor: with notes on the anticoagulant action of soybean trypsin inhibitor, protamine sulfate and hexadimethrine bromide. *J. C.in. Invest.* **44**:302.
25. Prentice, C. R. M., R. T. Breckenridge, and O. D. Ratnoff. 1967. Studies on the conversion of prothrombin to thrombin: with notes on the cation requirement for this reaction. *J. Lab. Clin. Med.* **69**:229.
26. Ratnoff, O. D., and I. H. Lepow. 1957. Some properties of an esterase derived from preparations of the first component of complement. *J. Exptl. Med.* **106**: 327.

27. Alkjaersig, N., A. P. Fletcher, and S. Sherry. 1959. Epsilon amino-caproic acid: an inhibitor of plasminogen activation. *J. Biol. Chem.* **234**:832.
28. Taylor, F. B., and H. Fudenberg. 1964. Inhibition of the C'1 component of complement by amino acids. *Immunology.* **7**:319.
29. Lepow, I. H., O. D. Ratnoff, F. S. Rosen, and L. Pillemer. 1956. Observations on a pro-esterase associated with partially purified first component of human complement (C'1). *Proc. Soc. Exptl. Biol. Med.* **92**:32.
30. Pensky, J., L. R. Levy, and I. H. Lepow. 1961. Partial purification of a serum inhibitor of C'1-esterase. *J. Biol. Chem.* **236**:1674.
31. McLean, J. 1916. The thromboplastic action of cephalin. *Am. J. Physiol.* **41**:250.
32. Ratnoff, O. D., and E. W. Davie. 1962. The activation of Christmas factor by activated plasma thromboplastin antecedent. *Biochemistry.* **1**:677.
33. Margolis, J. 1959. Hageman factor and capillary permeability. *Australian J. Exptl. Biol. Med. Sci.* **37**:239.
34. Ratnoff, O. D., and A. A. Miles. 1964. The induction of permeability-increasing activity in human plasma by activated Hageman factor. *Brit. J. Exptl. Pathol.* **45**:29.
35. Mason, B., and A. A. Miles. 1962. Globulin permeability factor without kininogenase activity. *Nature.* **196**:587.
36. Becker, E. L., and L. Kagan. 1964. The permeability globulins of human serum and the biochemical mechanism of hereditary angioneurotic edema. *Ann. N.Y. Acad. Sci.* **116**:866.
37. Margolis, J. 1958. Activation of plasma by contact with glass: Evidence for a common reaction which releases plasma kinin and initiates coagulation. *J. Physiol.* **144**:1.
38. Armstrong, D., J. B. Jepson, C. A. Keele, and J. W. Stewart. 1957. Pain producing substance in human inflammatory exudates and plasma. *J. Physiol.* **135**:250.
39. Spector, W. G. 1957. Activation of a globulin system controlling capillary permeability in inflammation. *J. Pathol. Bacteriol.* **74**:67.
40. Webster, M. E., and O. D. Ratnoff. 1961. Role of Hageman factor in the activation of vasodilator activity in human plasma. *Nature.* **192**:180.
41. Landerman, N. S., M. E. Webster, E. L. Becker, and H. E. Ratcliffe. 1962. Hereditary angioneurotic edema. II. Deficiency of inhibitor for serum globulin permeability factor and/or plasma kallikrein. *J. Allergy.* **33**:330.
42. Burdon, K. L., J. T. Queng, O. C. Thomas, and J. P. McGovern. 1965. Observations on biochemical abnormalities in hereditary angioneurotic edema. *J. Allergy.* **36**:546.
43. Kagan, L. J. 1964. Some biochemical and physical properties of the human permeability globulins. *Brit. J. Exptl. Pathol.* **45**:604.
44. Donaldson, V. H., O. D. Ratnoff, and F. S. Rosen. 1965. Permeability properties of plasma in hereditary angioneurotic edema. *J. Lab. Clin. Med.* **66**:867. (Abstr.)
45. Donaldson, V. H., and F. S. Rosen. 1966. Hereditary angioneurotic edema: A clinical survey. *Pediatrics.* **37**:1017.
46. Kraut, H., and N. Bhargava. 1964. Versuche zur Isolierung des Kallikrein-Inaktivators. V. *Z. Physiol. Chem.* **338**:231.

47. Niewiarowski, S., and O. Prou-Wartelle. 1959. Rôle du facteur contact (Facteur Hageman) dans la fibrinolyse. *Thromb. Diath. Haemorrhag.* **3**:593.
48. Iatridis, S. G., and J. H. Ferguson. 1962. Active Hageman factor: a plasma lysokinase of the human fibrinolytic system. *J. Clin. Invest.* **41**:1277.
49. Ratnoff, O. D., and G. B. Naff. 1967. The conversion of C'1s to C'1 esterase by plasmin and trypsin. *J. Exptl. Med.* **125**:337.
50. Dias da Silva, W., and I. H. Lepow. 1967. Anaphylatoxin formation by purified human C'1-esterase. *J. Immunol.* **95**:1080.
51. Klemperer, M. R., V. H. Donaldson, and F. S. Rosen. 1968. Effect of C'1 esterase on vascular permeability in man: Studies in normal and complement deficient individuals and in patients with hereditary angioneurotic edema. *J. Clin. Invest.* In press.