

ACTIVATION OF HAGEMAN FACTOR IN SOLID AND FLUID PHASES*

A CRITICAL ROLE OF KALLIKREIN

BY C. G. COCHRANE, S. D. REVAK, AND K. D. WUEPPER‡

(From the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037)

(Received for publication 23 July 1973)

The activation of Hageman factor results from its exposure to various insoluble particles having negative charges on their surfaces, such as glass, kaolin, extracellular membranes, etc., and to enzymes such as trypsin in solution. We have currently found that activation of Hageman factor by kaolin is highly inefficient in the presence of plasma, only a small percentage of the Hageman factor being utilized in the reaction (1). This led to the speculation that important soluble activators may exist in plasma that could greatly amplify the activation. Such activators could be released from cells at the moment of injury of tissues while others could be activated in the plasma. From previous data enzymes would comprise likely candidates for activators in the fluid phase, and one might reasonably suspect the involvement of those enzymes activated under the same circumstances as Hageman factor. One such enzyme, plasmin, has been shown to activate Hageman factor (2), a finding that was subsequently confirmed (3). A similar capacity of two other enzymes, kallikrein and clotting Factor XI (plasma thromboplastin antecedent, PTA)¹, both of which are activated directly by Hageman factor, is described herein. A key role of kallikrein was observed in amplifying the activation of Hageman factor which is essential for an adequate rate of generation of the fibrinolytic and intrinsic clotting systems.

We will also report upon changes observed in the structure of Hageman factor incurred during activation in fluid phase by plasma enzymes and in solid phase by insoluble, negatively charged particles in the absence of detectable enzymes. The data form a base for understanding the mechanisms of Hageman factor activation in pathologic conditions.

* This work was supported by U. S. Public Health Service Grant AI-07007, the American Heart Association, and the Council for Tobacco Research.

‡ Recipient of U. S. Public Health Service Research Career Development Award 1 KO4 AM 40086-02.

¹ Abbreviations used in this paper: AGLME, acetyl-glycine-lysine methyl ester; BAEE, benzoyl-L-arginine ethyl ester; BSA, bovine serum albumin; DFP, diisopropyl fluorophosphate; EACA, ϵ -amino-*n*-caproic acid; OMTI, Ovomuroid trypsin inhibitor; PMSF, phenyl methyl sulfonyl fluoride; PTA, plasma thromboplastin antecedent; SDS, sodium dodecyl sulfate; TBS, 0.01 M tris(hydroxymethyl)aminomethane buffer pH 7.4 in 0.15 M NaCl.

Materials and Methods

Preparation of Proteins.—

Hageman factor: Rabbit and human Hageman factor were purified according to previously published methods (4). Protein eluting early in the Hageman factor peak (as determined by ability to activate prekallikrein upon treatment with trypsin) from the final carboxymethyl Sephadex C-50 column was chosen for radioisotope labeling with ^{125}I by the Chloramine T method (5). 80-90% of the protein-bound iodine thus prepared was bound to inactive Hageman factor (1). Varying amounts of this labeled Hageman factor were added to cold (unlabeled) aliquots of Hageman factor and the mixture used for activation studies. The labeled Hageman factor was stored in aliquots at -70°C and thawed just before use.

Prekallikrein: Highly purified rabbit and human prekallikrein were prepared according to published methods (6). The prekallikrein tested in acrylamide gel electrophoresis in the presence or absence of sodium dodecyl sulfate (SDS) gave a single protein band when stained with Coomassie blue.

Kallikrein: Kallikrein was prepared by trypsin activation of prekallikrein followed by inhibition of the trypsin by 50/1 (wt/wt) excess ovomucoid trypsin inhibitor (OMTI) (Worthington Biochemical Corp., Freehold, N. J.). Generally $0.5\ \mu\text{g}$ trypsin ($2\times$ crystallized, Worthington Biochemical) was employed for each microgram of prekallikrein at pH 7.4. Activity of the kallikrein was then assayed by addition of 3 ml of 1 mM benzoyl-L-arginine ethyl ester (BAEe) (Schwartz/Mann Div., Becton, Dickinson and Co., Orangeburg, N. Y.) as described previously (6).

Clotting Factor XI: Factor XI (PTA) was prepared from human plasma according to the published methods.² The Factor XI recovered from its final separatory procedure, Pevikon block electrophoresis (Pevikon, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), was activated by the addition of $1\ \mu\text{g}$ trypsin to 0.3 ml of Factor XI. The quantity of Factor XI was in the order of 10-20 $\mu\text{g}/\text{ml}$, but an exact assay of protein was not possible. After incubation at 37°C for 30 min the trypsin activity was inhibited by the addition of 50 μg OMTI. Activity of Factor XI was determined by its ability to hydrolyze BAEe or promote clotting in Factor XI-deficient plasma.

Plasminogen: Purified human plasminogen was obtained through the courtesy of Dr. E. C. DeRenzo and Dr. P. H. Bell of Lederle Laboratories, Pearl River, N. Y. Plasminogen was converted to plasmin by the addition of 180 U streptokinase (Lederle Laboratories) to 40 μg plasminogen. After 10 min incubation at 37°C , conversion to plasmin was complete, with the final concentration being 4 $\mu\text{g}/10\ \mu\text{l}$ reaction volume. Plasmin activity was assayed in fibrin plates. The area of lysed circles was compared quantitatively with those produced by standard quantities of plasmin kindly provided by Dr. Alan J. Johnson, American National Red Cross Laboratory (New York University Medical Center, New York). For each plate 16 mg human fibrinogen (Nutritional Biochemical Corp., Cleveland, Ohio) were added to 2 ml of tris(hydroxymethyl)aminomethane buffer 0.01 M pH 7.4 containing 0.15 NaCl (TBS) and heated to 49°C . ϵ -amino-*n*-caproic acid (EACA) (Sigma Chemical Company, St. Louis, Mo.) was added to a concentration of 0.0044 M to prevent the activation by streptokinase of a small amount of plasminogen present as a contaminant of the fibrinogen. By this method, as little as 0.2 μg (equivalent to 0.007 CTA units) could be detected. For the activation studies discussed below, streptokinase-activated plasminogen, or on occasion, standard human plasmin, was employed after testing for activity by the fibrin plate method.

Activation of Hageman factor: Activation of Hageman factor was generally accomplished by the addition of increasing amounts of activator to a constant amount of Hageman factor at a pH of 7.4 in a plastic- or silicone-coated glass tube. Reaction volumes were 0.15-0.2 ml and

² Wuepper, K. D. 1973. Purification and characterization of human precursor of plasma thromboplastin antecedent. Manuscript submitted for publication.

contained from 0.5 to 2.0 μg Hageman factor. For maximum enzymatic activation 0.5 μg trypsin (inhibited after the reaction by addition of 50 μg OMTI), 0.5 μg kallikrein, or 3 μg plasmin (incubated at 37° C for 30 min) were generally sufficient. As noted above, the concentration of Factor XI could not be determined accurately, and the highest dose used was not sufficient to cause complete activation of the Hageman factor. Washed kaolin was tested for the quantity yielding maximum activation of Hageman factor. Generally, the optimum was between 50–250 μg kaolin as determined by preliminary assay. The kaolin was incubated with the ^{125}I -labeled Hageman factor for 15 min at 22°C with gentle shaking. The reaction mixture was generally centrifuged (3,400 rpm for 3 min) and the supernate removed. More than 80% of the Hageman factor was bound to the kaolin as determined quantitatively by radiolabeling. Activated Hageman factor was assayed by either its ability to activate 1.25 μg (exceptions noted) rabbit prekallikrein or decrease the clotting time of Factor XII-deficient human plasma (4). In the former assay, prekallikrein was converted to kallikrein and the activity of this enzyme assayed by its capacity to hydrolyze 1 mM BAEe. Data are presented as changes in optical density at 253 nm at 37°C over a period of 30 min.

Physical Methods.—

Acrylamide gel electrophoresis: Acrylamide gels (7 or 10%, see below) containing 0.1% SDS (Bio-Rad Laboratories, Richmond, Calif.) were employed for observation of possible cleavage products of activated [^{125}I]Hageman factor according to described methods (7). Samples of 0.1 ml were applied to gels 8 cm \times 5 mm and electrophoresis carried out at 4 mA/tube for 45 min followed by 8 mA/tube for 4 h. At the termination of electrophoresis 1.2-mm segments were cut and analyzed for radioactive content. Location of the ^{125}I peaks were recorded relative to either [^{131}I]bovine serum albumin (BSA) run in the same gel or to the tracking dye used (bromphenol blue) and molecular weights determined by comparison with the migration (R_f) of protein markers (human IgG, transferrin, BSA, ovalbumin, chymotrypsinogen, cytochrome *c*) of known molecular weight observed in a separate gel. Generally 7% acrylamide was employed in gels in which native Hageman factor was analyzed.

Ultracentrifugation in density gradients of sucrose: Ultracentrifugation studies were carried out in a 5–20% linear gradient of sucrose (Schwartz/Mann) in TBS at 65,000 rpm for 8 h or 45,000 rpm for 16 h. Fractions containing 10 or 15 drops/tube were obtained. These were generally assayed for Hageman factor by radioisotope detection (^{125}I), for spontaneous activity (addition directly to 1.25 μg prekallikrein), and for precursor, (activatable Hageman factor; activation with 0.2 μg trypsin before addition of prekallikrein as noted above).

Reduction of disulfide bonds and alkylation: Proteins in TBS were subjected to reduction in 0.02 M dithiothreitol for 1 h at room temperature and alkylated by addition of 0.05 M iodoacetamide for 30 min at 4°C.

RESULTS

Activation of Purified Hageman Factor on Negatively Charged Particles.—

Addition of plasma to glass or to particles with negative charges leads to binding and activation of a portion of the Hageman factor. It has not been clear whether the activation resulted from binding to the surfaces alone or to binding followed by subsequent activation by enzymes in the plasma. To examine this question, 0.5 μg highly purified rabbit Hageman factor was added to increasing quantities of washed kaolin in TBS as shown in Fig. 1. As noted, activation of the Hageman factor resulted, reaching a maximum when approximately 10 μg kaolin were added. The decrease in measureable Hageman factor activity in an excess of insoluble activator is apparently caused by binding of

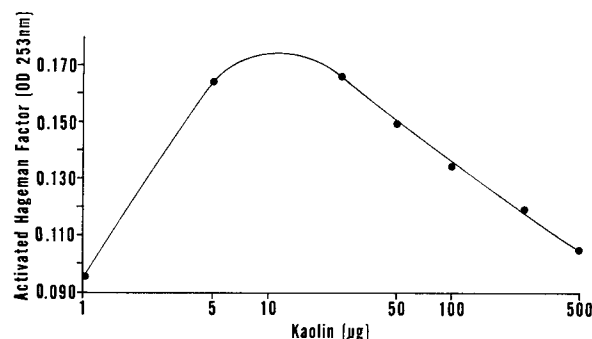


FIG. 1. Activation of 0.5 μg highly purified Hageman factor by kaolin. Final volume = 0.20 ml.

the prekallikrein used in the assay to the kaolin and thereby lost from the reaction. The diminution in activity does not appear when acetyl-glycine-lysine methylester (AGLME) is used in excess to assay for activity (8).

Despite the activation of highly purified Hageman factor on negatively charged particles, attempts were made to activate the molecule in the presence of an inhibitor of kallikrein, Factor XI, and plasmin. These enzymes, known to activate Hageman factor (see below), and possibly present in trace amounts, are susceptible to treatment with phenyl methyl sulfonyl fluoride (PMSF). Direct assay failed to reveal the presence of plasmin or kallikrein in the preparation of rabbit Hageman factor to the limit of sensitivity, i.e., about 0.1 μg . Nevertheless the preparation of purified Hageman factor was treated with PMSF in concentrations of 10^{-2} to 10^{-5} M for 10 min at 22°C. The preparations were then incubated with 50 μg kaolin followed by washing of the particles twice in TBS. Activation, as determined by prekallikrein activation, was not inhibited by treatment of the Hageman factor preparation with PMSF (Table I). In addition, 100 μg kaolin were added to 0.2 ml Hageman factor in the presence of EACA, 10^{-2} M final concentration. After incubation, the supernatant fluid was removed and the bound Hageman factor assayed. No diminution of activating capacity was noted despite the presence of EACA during activation.

Inhibition of binding of ^{125}I Hageman factor to kaolin by plasma: Previous studies have demonstrated that plasma inhibits the removal of Hageman factor from solution by kaolin as determined immunologically and by decreased prekallikrein-activating capacity of the treated kaolin (1). With the availability of ^{125}I -labeled Hageman factor, it was important to determine directly if the binding of Hageman factor to a negatively charged particle was inhibited by the presence of plasma or plasma proteins. Accordingly approximately 0.05 μg [^{125}I]human or [^{125}I] rabbit Hageman factor was mixed with increments of plasma deficient in Hageman factor or prekallikrein (Fletcher trait plasma), normal human plasma, or human albumin. 250 μg kaolin were then added and incubation allowed to proceed 15 min at 22°C. The kaolin was then

centrifuged and washed, and the [125 I]Hageman factor bound to the kaolin measured. As shown in Fig. 2, marked inhibition of binding of Hageman factor occurred with both Hageman factor-deficient and normal plasma. No consistent difference in inhibition between the various plasmas was noted. Human IgG and human albumin inhibited binding of Hageman factor also, each being

TABLE I
Activation of Hageman Factor Pretreated with PMSF on Negatively Charged Particles

Rabbit Hageman factor	Final conc. PMSF	Kaolin	Prekallikrein activation (OD 253 nm)
μg	M	μg	
0.5	10^{-2}	50	0.160
0.5	10^{-3}	50	0.163
0.5	10^{-4}	50	0.178
0.5	10^{-5}	50	0.140
0.5	—	50	0.152
0.5	—	50	0.160

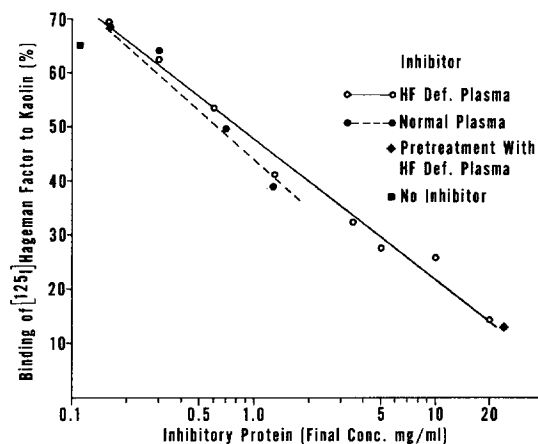


FIG. 2. Inhibition of binding of [125 I]rabbit Hageman factor to 250 μg kaolin by increments of normal or Hageman factor-deficient plasma. Final volume was 150 μl , yielding a dilution of the plasma of $\frac{1}{3}$ at the highest concentration employed.

about half as effective as whole plasma on a weight basis. Pretreatment of the kaolin with Hageman factor-deficient plasma, followed by washing, inhibited binding as well as when the kaolin was exposed to Hageman factor in the presence of plasma (Fig. 2). While rabbit Hageman factor was employed in the studies in Fig. 2, binding of human Hageman factor was similarly inhibited.

As presented previously (1), the inhibition of uptake of Hageman factor onto kaolin by plasma was accompanied by a decrease in the ability of the kaolin-bound Hageman factor to activate prekallikrein.

Activation of Hageman Factor in Fluid Phase with Enzymes.—

With the marked inhibition of binding and activation of Hageman factor to a negatively charged surface in the presence of plasma, the possibilities of fluid phase activation were explored. Enzymes were first studied that are converted to an active state during, or as a result of, the initial activation of Hageman factor.

Kallikrein: Human and rabbit Hageman factor, 0.6–1.0 μg , were exposed to increments of purified kallikrein of the corresponding and heterologous species at 37°C for 30 min. After treatment, the Hageman factor was assayed for activity of two of its known functions, the activation of prekallikrein and promotion of clotting. Examples of such experiments are shown in Figs. 3 and 4. A dose-response relationship was observed between the quantity of kalli-

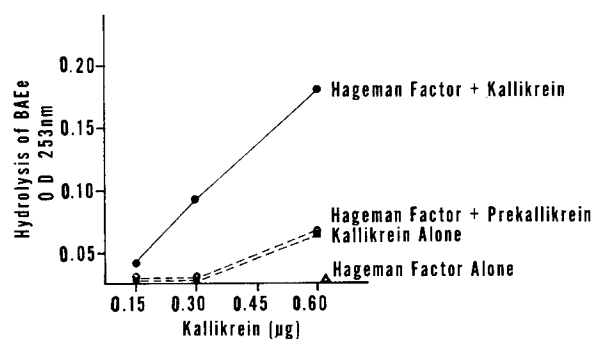


FIG. 3. Activation of rabbit Hageman factor by human kallikrein. Kallikrein or prekallikrein in the amounts shown was added to 0.6 μg Hageman factor in 0.05 ml. After incubation, 1.25 μg rabbit prekallikrein was added to indicate activation of Hageman factor as noted in Materials and Methods.

krein added and the activation of Hageman factor. In Fig. 4, kallikrein and trypsin are compared on a weight basis in their ability to activate Hageman factor. While it was found that by weight, trypsin was slightly more active, when compared on a molar basis, kallikrein possessed greater activity.

When prekallikrein was substituted for kallikrein, the Hageman factor was not activated to promote clotting. However, when doses as large as 0.60 μg human prekallikrein were incubated with Hageman factor, some Hageman factor activity did develop as noted in Fig. 3. This may be explained by a trace of activity present in either or both the precursor Hageman factor or prekalli-

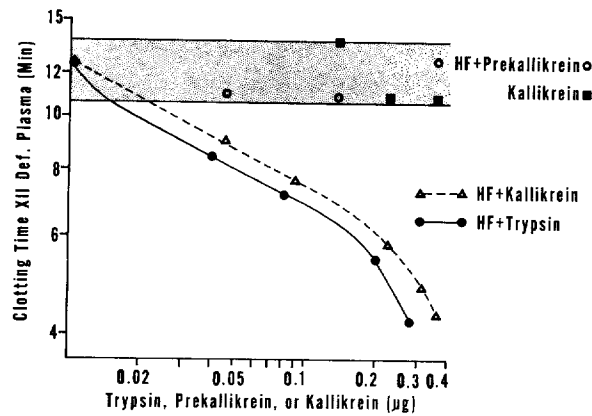


FIG. 4. Activation of human Hageman factor by human kallikrein and trypsin. Kallikrein, prekallikrein, and trypsin were added to Hageman factor as noted in Fig. 3. Activity of the Hageman factor was then assessed by its ability to promote clotting of Factor XII-deficient plasma. The shaded zone represents the range of normal clotting times.

krein. A small amount of activity of either reagent could initiate a cyclical process of activation.

In order to gain further evidence that kallikrein was responsible for the activation of Hageman factor, purified kallikrein was subjected to molecular exclusion chromatography on Sephadex G-200. The various fractions were then assayed for their capacity to activate Hageman factor. As shown in Fig. 5, activation of Hageman factor was observed in fractions containing kallikrein. Similarly, when kallikrein was sedimented in a gradient of sucrose and subjected to electrophoresis in Pevikon, the fractions found to activate Hageman factor corresponded to those containing kallikrein activity.

In additional control experiments, 12 µg rabbit kallikrein in 1.0 ml PBS were treated with diisopropyl fluorophosphate (DFP) (10^{-4} M final concentration) and after 10 min at room temperature dialyzed 18 h against three changes of PBS, 600 ml each. An identical quantity of untreated kallikrein was similarly dialyzed. 0.5 and 1.0 µg of the treated and untreated kallikrein were then added to Hageman factor as above. Marked inhibition of the Hageman factor activating capacity of kallikrein occurred following treatment with DFP in contrast to the control.

It should be noted that the two other plasma substrates of Hageman factor, Factor XI and plasminogen proactivator, were separated from human prekallikrein during elution from carboxymethyl Sephadex during purification. The effect of kallikrein on Hageman factor was thereby distinguished from any effect of plasminogen proactivator or Factor XI (PTA).

Clotting Factor XI, PTA: Purified Factor XI of human plasma was activated with trypsin (see Materials and Methods). Approximately 1–4 µg

were incubated with Hageman factor at 37°C for 30 min. Assays for activation of Hageman factor by addition of rabbit prekallikrein indicated that activation of the Hageman factor had occurred (Table II).

Plasmin activation of Hageman factor: Plasminogen was converted to plasmin by the action of streptokinase as noted in Materials and Methods. Varying

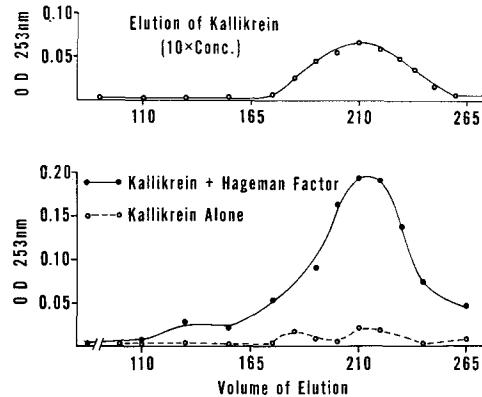


FIG. 5. Activation of human Hageman factor by human kallikrein. Purified human kallikrein was chromatographed by molecular exclusion on Sephadex G-200. The elution of kallikrein is shown in the upper panel. Aliquots of various fractions after dilution $\frac{1}{10}$ were activated to kallikrein with trypsin and tested for their capacity to activate Hageman factor. As shown, the fractions containing kallikrein were capable of activating Hageman factor. The diluted kallikrein, by itself, hydrolyzed BAEc only weakly (lower panel).

TABLE II
Activation of Human Hageman Factor (HHF) by Active Factor XI (PTA)

PTA	HHF	Prekallikrein activation (253 nm)
μl	μl	
50	100	0.143
50	100	0.136
50	—	0.064
50	—	0.065
—	100	0.018
—	100	0.025

quantities of plasmin were then exposed to 0.6–1.0 μg human Hageman factor. In confirmation of previous studies (2, 3) activation of Hageman factor ensued (Fig. 6). Streptokinase alone or plasminogen not treated with streptokinase failed to activate the Hageman factor.

Comparison of the capacities of kallikrein, Factor XI, and plasmin to activate Hageman factor: While accurate comparisons of kallikrein, plasmin, and Factor XI to activate Hageman factor are not yet possible (see Discussion),

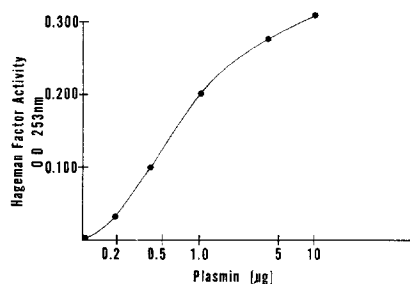


FIG. 6. Activation of human Hageman factor with plasmin. Plasmin was activated from plasminogen by streptokinase (see Materials and Methods). Activity of Hageman factor is expressed by its prekallikrein-activating capacity. The plasmin did not activate prekallikrein directly.

it was deemed important to gain some preliminary data on the relative capacities of these enzymes. Accordingly, varying amounts of human kallikrein, plasmin, and Factor XI were incubated with 0.5 µg of highly purified human Hageman factor. The activity of Hageman factor was tested by activation of prekallikrein. Assays of BAEe hydrolysis were performed at 20 min. The quantities of enzymes used to activate Hageman factor and the amount of prekallikrein used in the assay of the Hageman factor activity were selected to yield a linear increase in BAEe hydrolysis between 15 and 30 min incubation time. In the 20-min period the comparative results, expressed as the µM BAEe hydrolyzed per minute were as follows: kallikrein (0.13 µg)-36.8 µM BAEe; and plasmin (1.47 µg)-36.9 µM BAEe. Factor XI was less active than plasmin as calculated using the maximal possible quantity of Factor XI present. The figures are inaccurate in that the quantity of Factor XI was below that required for precise protein determinations. In each case, the background hydrolysis of BAEe by the enzyme used to activate Hageman factor was subtracted from the total. This amount was in each case quite small, as quantities of the activating enzymes were kept at a minimum.

Kallikrein was therefore found to be about 11 times more active than plasmin, and more than 10 times more active than Factor XI in its capacity to activate Hageman factor. As noted previously, kallikrein is slightly more active than trypsin on a molar basis. However it is clear that for truly quantitative values, an assay of primary Hageman factor is essential. One such assay is being developed (8).

Changes in Physical Characteristics of Hageman Factor Accompanying Activation.—

Molecular characteristics of Hageman factor activated with particles bearing negatively charged surfaces: [¹²⁵I]human and [¹²⁵I]rabbit Hageman factor, containing approximately 0.5–1 µg Hageman factor, were exposed to 250 µg kaolin. After incubation with shaking at 22°C for 15 min, 88–90% of the human

Hageman factor, and 93–95% of the rabbit protein became firmly bound to the kaolin and resisted removal by washing in TBS. To find if the bound Hageman factor was fully activated, the particles containing Hageman factor were exposed to the fluid phase activators, trypsin and kallikrein. Neither enzyme augmented the activity of the Hageman factor. In addition, the activity of the kaolin-bound Hageman factor was identical to that generated maximally when an equal quantity of Hageman factor was treated with kallikrein or trypsin in solution.

To elute the [125 I]Hageman factor from the kaolin, the particles were exposed to 0.3% SDS which resulted in elution of 67% of the bound Hageman factor. The eluted protein was then examined in SDS acrylamide gel electrophoresis. A typical pattern is shown in Fig. 7. All of the [125 I]Hageman factor

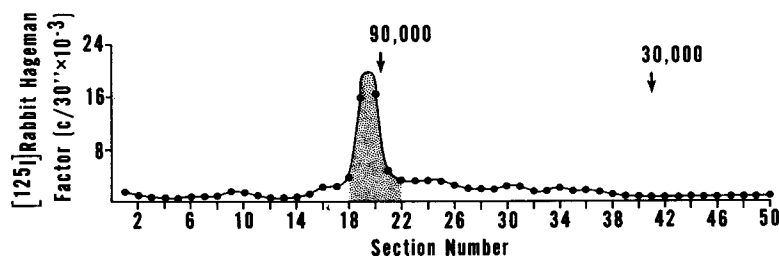


FIG. 7. Electrophoresis in SDS acrylamide gel of [125 I]rabbit Hageman factor eluted from kaolin by 0.3% SDS. 67% of the Hageman factor was eluted from the particles. As noted, the molecule eluted from kaolin in its native, 90,000 mol wt form, i.e., without evidence of cleavage. Similar results were obtained with [125 I]human Hageman factor.

was recovered in the mol wt range of 80,000 (human) to 90,000 (rabbit). Cleavage to smaller mol wt fragments was not observed. Milder methods of removing the protein from kaolin or other negatively charged particles, in which activity of the solubilized Hageman factor could be tested, were not successful.

Molecular characteristics of rabbit Hageman factor activated in fluid phase with enzymes: A comparison was made of the physical characteristics of the Hageman factor molecule accompanying activation by trypsin, kallikrein and plasmin, and during spontaneous activation accompanying aging. [125 I]rabbit Hageman factor (0.2 μ g in 0.1 ml) was activated with trypsin (0.2–0.5 μ g), kallikrein (0.2–1.25 μ g), and plasmin (2–30 μ g) for 1.5, 5, 15, and 30 min. In each case the degree of activation was assessed, and changes in physical properties studied by SDS acrylamide gel and ultracentrifugation in a gradient of sucrose. In the latter case, the degree of activity of the Hageman factor could be determined in each eluted fraction of the sedimentation tube. As noted in Fig. 8, when trypsin was used to activate the [125 I]rabbit Hageman factor, the sedimentation rate was lowered from 4.5 to 2.6S over a 30-min period. The fragmentation of the molecule was accompanied by activation. Active Hage-

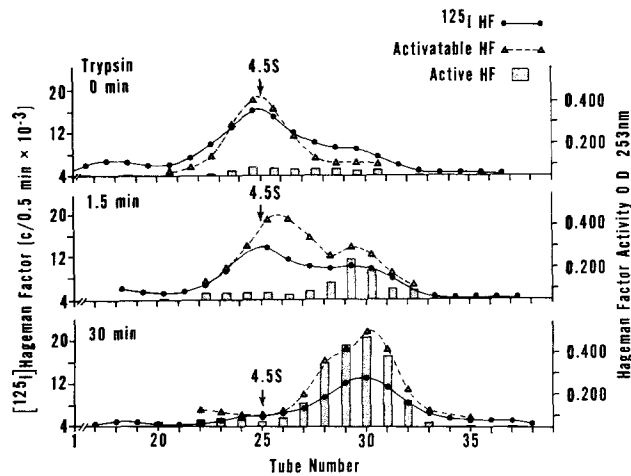


FIG. 8. Sedimentation of [^{125}I]rabbit Hageman factor in a gradient of sucrose before and after treatment with $2\ \mu\text{g}$ trypsin for the time periods shown. The 4.5S marker consisted of [^{131}I]BSA incorporated in each sample as an internal marker. Active Hageman factor (solid bars) was tested by addition of the fractions directly to prekallikrein. Activatable Hageman factor was assayed by treatment with trypsin and then OMTI preceding the addition of prekallikrein.

man factor was found only in fractions at 2.6S and not at 4.5S, with increasing quantities of active Hageman factor appearing at 2.6S as more of the native molecule was cleaved. Activatable (native) Hageman factor, measureable only at 4.5S, was lost over the 30-min period as active Hageman factor appeared at 2.6S. There was no evidence of molecules lying intermediate to those at 4.5 and 2.6S as demonstrated by radioactivity or biologic activity. The apparent cleavage of Hageman factor accompanying treatment and activation by trypsin was supported by assays of the reactions in acrylamide gel electrophoresis in SDS as shown in Fig. 9. Treatment with trypsin was associated with cleavage of the rabbit protein to 30,000 mol wt fragments. Recent experiments to be reported separately indicate a more complex pattern of cleavage of human Hageman factor by trypsin.

By contrast, when [^{125}I]rabbit Hageman factor was treated with the two other enzymes, plasmin and rabbit kallikrein, there was no apparent change in the sedimentation rate and/or position in electrophoresis of the molecule in SDS acrylamide gel. In Fig. 10 is shown the sedimentation profile of [^{125}I]rabbit Hageman factor treated with plasmin ($16\ \mu\text{g}$) and rabbit kallikrein ($4.2\ \mu\text{g}$). As noted, the peak of ^{125}I activity sedimented at 4.5S (determined by [^{131}I]BSA standard which was incorporated as an internal marker in each sample). There was no shift of the ^{125}I to 2.6S in contrast to the decrease in sedimentation rate of [^{125}I]Hageman factor treated with trypsin as noted in Fig. 8. The activated

Hageman factor was detected in a peak at 4.5S after treatment with both plasmin and kallikrein. These sedimentation patterns are identical to that of untreated Hageman factor (Fig. 10).

When the [125 I]rabbit Hageman factor after treatment with kallikrein and

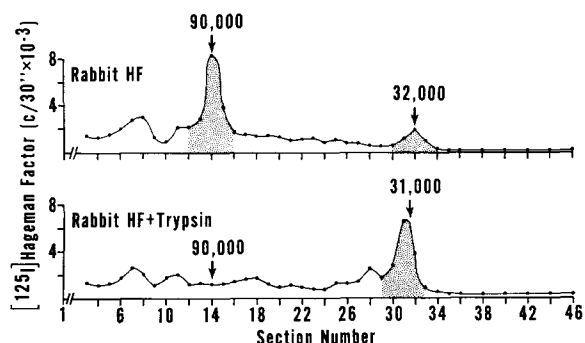


FIG. 9. Electrophoresis of [125 I]rabbit Hageman factor in SDS acrylamide gels before and after treatment with trypsin for 30 min (as in Fig. 8). The [125 I]Hageman factor is converted from a peak at 90,000 to 30,000 mol wt.

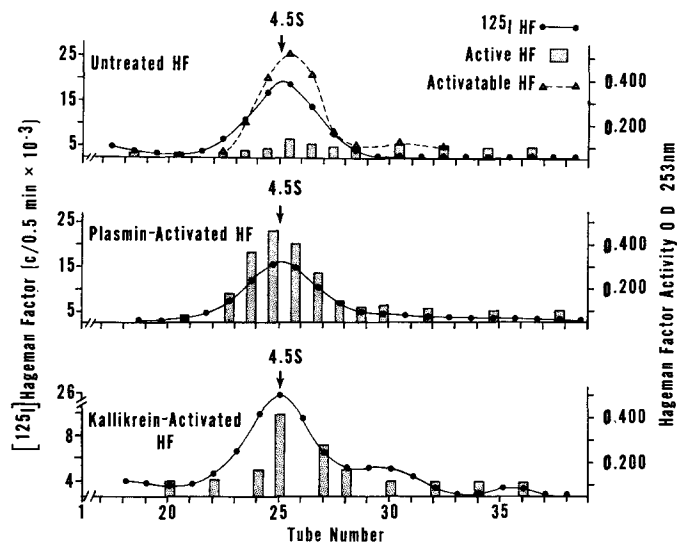


FIG. 10. Sedimentation of [125 I]rabbit Hageman factor in a gradient of sucrose before and after treatment with 16 μ g plasmin or 4.2 μ g rabbit kallikrein (see Materials and Methods). The various fractions were tested for Hageman factor activity as in Fig. 8. Activity was found in the peak at 4.5S in the enzyme-treated preparations (middle and lower panels) with necessity of further activation with trypsin as in the top panel. The Hageman factor used for tests with kallikrein was the same used in the studies of Fig. 8, yielding a slightly different pattern on sedimentation than that of the upper and middle panels.

plasmin was subjected to electrophoresis in SDS acrylamide gels (Fig. 11), the lack of cleavage of the Hageman factor into large fragments was confirmed. It should be noted that small fragments might not be detected, especially those lacking tyrosine. Virtually all of the radiolabeled protein remained at 90,000 mol wt. It was possible that the rabbit Hageman factor molecule was cleaved by plasmin or kallikrein but failed to dissociate by virtue of intrachain disulfide bridges. To test this, rabbit Hageman factor was treated with plasmin or kallikrein and then subjected to reduction in 0.02 M dithiothreitol followed by alkylation with iodoacetamide. The molecule upon reduction dissociated into fragments of approximately 30,000 mol wt as determined in SDS acrylamide electrophoresis. These fragments were identical in size to those obtained by reduction of the native molecule. Thus, enzymatic cleavage had probably not occurred between intrachain disulfide bridges.

Experiments were performed to find if factors in plasma could cleave rabbit Hageman factor activated by kallikrein or affect the fragments obtained after treatment with trypsin. Accordingly, [125 I]rabbit Hageman factor was activated by those enzymes as noted above. The active material was then incubated with fresh, nonchelated rabbit plasma obtained by rapidly sedimenting rabbit arterial blood in plastic without anticoagulants. Equal volumes of activated Hageman factor and plasma were employed. After incubation at 37°C for 20 min and removal of any formed fibrin by centrifugation, the possible fragmenta-

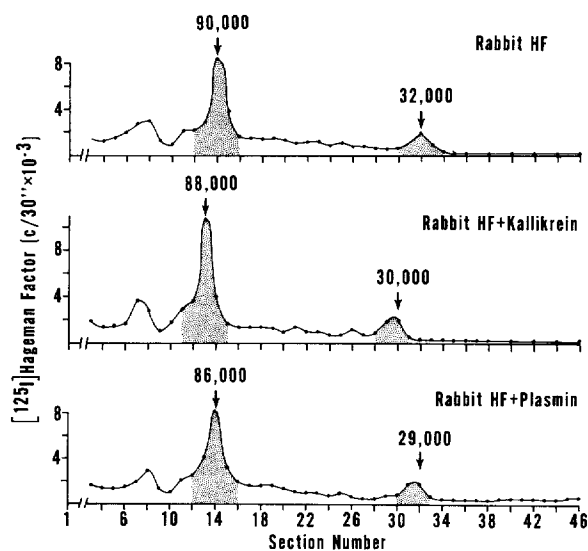


FIG. 11. Electrophoresis of [125 I]rabbit Hageman factor in SDS acrylamide gel before and after treatment with plasmin and kallikrein as in Fig. 10. The small amount of Hageman factor that had spontaneously converted to the 30,000 mol wt degradation product in the control test did not increase after treatment with plasmin or kallikrein.

tion of the ^{125}I -labeled protein was assayed by ultracentrifugation in a gradient of sucrose. The Hageman factor activated by kallikrein and exposed to plasma did not appear in the 2.6S zone, but instead sedimented with a velocity of 6–7S. Trypsin-cleaved fragments of 2.6S, upon exposure to plasma sedimented at approximately 4.5S. Presumably the activated molecule became bound to an inhibitor in plasma, or had aggregated, accounting for the greater rate of sedimentation.

In contrast to the observations with the rabbit protein, human Hageman factor, activated by plasmin and kallikrein, was found to be cleaved during activation. Upon treatment with human kallikrein or plasmin, human Hageman factor was activated and cleaved into fragments of approximately 52,000, 40,000, and 28,000 mol wt as determined by SDS electrophoresis. These results, together with a study correlating activity with the fragments, will be presented separately.

DISCUSSION

Two major considerations emerge from these studies on the activation of Hageman factor:

The Activation of Hageman Factor in Fluid-Phase and the Importance of this Mechanism in the Initiation of the Kinin-Forming, Intrinsic Clotting, and Fibrinolytic Systems.—

A comparison has been made between two general mechanisms of activating Hageman factor, i.e., that rendered by an insoluble negatively charged surface such as kaolin or glass which might be termed solid-phase activation, and that induced enzymatically in free solution which might be called fluid-phase activation.

Regarding solid-phase activation, the present and previous data from this laboratory (1) indicate that the binding and activation of Hageman factor by negatively charged surfaces is greatly inhibited by plasma. The combined data indicate that only a small percentage of the available Hageman factor in undiluted plasma is bound and activated by 250 μg of kaolin. While greater amounts of kaolin remove more Hageman factor from plasma, the effective activating surface provided by 250 μg of kaolin is greater than that provided by two known tissue activators of Hageman factor, vascular basement membranes and collagen (unpublished observation). Total inhibition in solid-phase activation results from a combination of inhibition of binding of Hageman factor to the surface by plasma proteins and inhibition of the activity of the bound Hageman factor by natural inhibitors in plasma.

These findings, together with data derived from studies on prekallikrein-deficient plasma noted below, stress the importance of the fluid-phase activation of Hageman factor. It was thought probable that important fluid-phase activators might be found among the enzymes set into motion by the same process inducing the activation of Hageman factor. The natural substrates of

Hageman factor were therefore examined initially and it was found that kallikrein and clotting Factor XI activate Hageman factor. Plasmin, which is activated secondarily, also induced activation of Hageman factor as observed previously (2, 3). The capacity of isolated plasminogen activator to activate Hageman factor was not tested in the present study.

But which of these three fluid-phase activators is most significant? The semi-quantitative comparisons in the present studies indicate that kallikrein is over 10-fold more active than plasmin and clotting Factor XI which suggests that kallikrein plays the most significant role. This suggestion is supported by the recent studies in this laboratory by Wuepper (9) employing plasma deficient in prekallikrein (Fletcher trait plasma) (10, 11). Prekallikrein is absent from the Fletcher trait plasma by both functional and immunochemical assays. This plasma exhibits a prolongation of clotting time, a failure to generate kinin, and globulins derived from Fletcher trait plasma fail to form plasmin upon exposure to glass or kaolin. These deficiencies were readily corrected by addition of purified human prekallikrein. The data indicate that prekallikrein is essential for generation of not only the kinin-forming system, but the intrinsic clotting and fibrinolytic systems as well. Support for this concept stems from the finding that purified kallikrein promotes clotting of normal plasma (12).

It would appear that insufficient Hageman factor is activated in solid phase when prekallikrein-deficient plasma contacts glass tubes for the generation of adequate amounts of active Factor XI to initiate clotting. Possibly, for the same reason, the rate of plasmin formation is also low. However, in the presence of prekallikrein, the small amount of bound, activated Hageman factor converts prekallikrein to its active form and the kallikrein activates additional Hageman factor in fluid phase. The Hageman factor activated in solution then generates sufficient active Factor XI for a normal rate of clotting to occur, and accelerates the formation of plasminogen activator, plasmin, and additional kallikrein.

A scheme of activation of Hageman factor in solid and fluid phase is shown in Fig. 12. The heavy arrow denotes the greater degree of activation exerted by activation in fluid phases. The significance of kallikrein is underscored. Of importance in this hypothesis is the finding that kallikrein does not generate directly Factor XI or plasmin activity from the respective precursor molecules (unpublished observations). Whether kallikrein will activate plasminogen proactivator has not been tested.

Despite the apparent importance of reciprocal activation of Hageman factor in the stimulation of the intrinsic clotting, kinin-and plasmin-forming systems, an accurate appraisal of the role of kallikrein vs. plasmin and Factor XI will require information of the precise quantity of each in plasma, and their rates of activation by Hageman factor and inhibition by natural inhibitors in plasma.

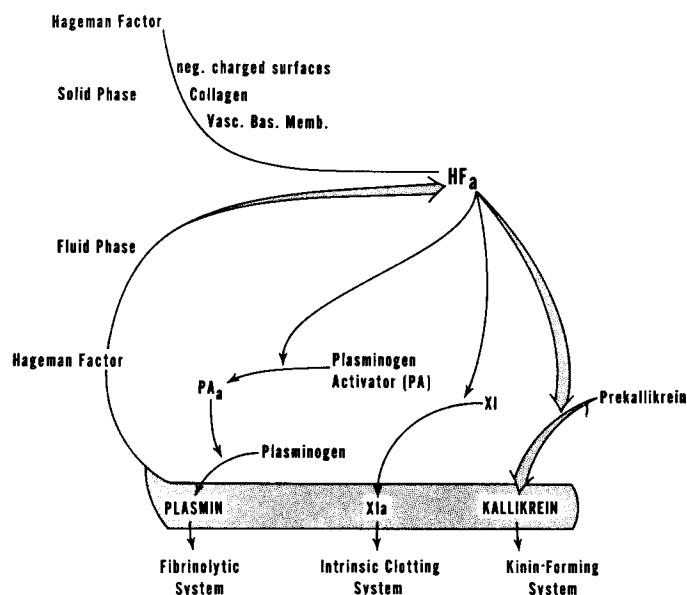


FIG. 12. Scheme of the activation of Hageman factor in solid and fluid phases. The importance of kallikrein in fluid-phase activation of Hageman factor is noted by the accentuated arrows.

Structural Changes Accompanying Activation of the Hageman Factor Molecule.—

Molecular changes after solid-phase activation of Hageman factor: The activation of Hageman factor on insoluble, negatively charged surfaces such as provided by glass, kaolin, celite, and diatomaceous earth has been recognized for many years from the data of Margolis (13). The observations led to the hypothesis that Hageman factor unfolds partially on the negatively charged surface to induce its activation. Pretreatment of the surfaces with positively charged molecules prevents activation of the Hageman factor (14-16), underscoring the importance of the negative surface charge. However it has not been clear whether activation resulted from unfolding of the molecule, or from enzymatic cleavage by plasma constituents after it became bound. The possibility that enzymatic action could account for the activation must be considered in view of the data presented herein and since in previous studies activation of native Hageman factor on insoluble surfaces has been performed with whole plasma or with partially purified fractions containing Hageman factor. The present experiments support the view that Hageman factor can be activated on a negatively charged surface independently of external enzymatic activity. The conclusion is supported by the findings that Hageman factor, prepared in highly purified form, free of other clotting agents (4) plasmin and kallikrein, was readily bound to and activated by kaolin; that activation was noted even after the Hageman

factor was pretreated with DFP and PMSF, treatment designed to inactivate enzymes of the plasma known to activate the Hageman factor, and that activation also occurred in the presence of EACA. In addition, Hageman factor added to and activated by kaolin in purified form, was recovered in its native size, i.e., 80,000–90,000 mol wt in eluates of the kaolin. In this regard, it must be recalled that Hageman factor, exposed to kaolin in the presence of whole plasma and eluted with NaCl is cleaved into smaller mol wt fragments some of which possess biologic activity (17). The molecule bound to the negatively charged surface is apparently particularly vulnerable to proteolytic cleavage since rabbit as well as human Hageman factor is cleaved by plasma constituents when bound to kaolin.

These data therefore support the earlier premise (13) that upon binding of Hageman factor to a negatively charged surface, conformational changes occur that allow combination with its natural substrates, prekallikrein, clotting Factor XI, and plasminogen activator. As opposed to previous theories (18) data from this laboratory have indicated that the action of activated Hageman factor on prekallikrein and clotting Factor XI is one of an enzyme-substrate reaction. Hageman factor activates prekallikrein and clotting Factor XI by partial proteolytic cleavage (6, 19); it hydrolyzes synthetic substituted lysine methyl esters; its action is blocked by certain inhibitors of trypsin (20); and, as has recently been found in this laboratory, Hageman factor activated on a negatively charged surface (kaolin) cleaves the synthetic ester AGLME, a reaction that is blocked by pretreatment of the active Hageman factor with DFP (8). Thus, Hageman factor appears to acquire esterolytic activity under these conditions while retaining full molecular size.

Changes in the molecular structure of Hageman factor after fluid-phase activation: Hageman factor, activated in fluid phase by kallikrein or plasmin, was cleaved at three positions in the case of the human protein while evidence of cleavage of rabbit Hageman factor during activation was not obtained.³ In studies to be published separately, clotting and prekallikrein activating capacities were associated with the fragments. It is not clear, however, that cleavage is essential to activation of the molecule in fluid phase. Donaldson and Ratnoff (21) observed a marked increase in sedimentation rate of human Hageman factor that was activated with ellagic acid. An increase in size of the molecule, presumably by aggregation, was not observed in the present studies. In addition we have been unable to maintain ellagic acid in a soluble form at 10^{-4} – 10^{-5} M at 4°C, the temperature at which the ultracentrifugation is conducted.

Repeated measurements of the activated rabbit molecule by ultracentrifuga-

³ Revak, S. D., C. G. Cochrane, and A. Johnston. 1973. Structural changes accompanying activation of human Hageman factor. Submitted for publication.

tion and by electrophoresis in SDS acrylamide gel indicated that rabbit Hageman factor retained its native size of 90,000 mol wt. In addition, cleavage of peptide bonds between an intrachain disulfide bridge had apparently not taken place since reduction and alkylation of the plasmin or kallikrein-activated Hageman factor did not result in fragmentation of the molecule. These data contrasted with active rabbit Hageman factor resulting from treatment with trypsin in which the fragments possessing biologic activity were recovered in the region of 2.6S (Fig. 8) with a mol wt of 30,000. It is apparent that proteolytic activity by kallikrein is essential, nevertheless, since prekallikrein did not induce activation of the Hageman factor, and since pretreatment of kallikrein with diisopropylfluorophosphate prevented its action on Hageman factor. The results suggest that cleavage of a small, as yet undetected, fragment takes place when kallikrein or plasmin activate rabbit Hageman factor. The loss of a small fragment may also accompany activation with trypsin. Such a cleavage fragment would probably be smaller than 2,500 mol wt since little difference in size is observed in the parent molecule after activation with kallikrein or plasmin.

With such restricted cleavage apparently essential for activation with kallikrein and plasmin, and with no fragmentation observable after activation with kaolin, the question must be raised whether cleavage into 30,000–40,000 mol wt fragments is essential for activation even with trypsin, or whether such fragmentation is a secondary phenomenon.

SUMMARY

The activation of Hageman factor in solid and fluid phase has been analyzed. Activation of highly purified Hageman factor occurred after it interacted with and became bound to a negatively charged surface. Activation was observed in the absence of enzymes that are inhibitable with diisopropylfluorophosphate, phenyl methyl sulfonyl fluoride and ϵ -amino-*n*-caproic acid. The binding of [¹²⁵I]Hageman factor to the negatively charged surface was markedly inhibited by plasma or purified plasma proteins.

Activation of Hageman factor in solution (fluid phase) was obtained with kallikrein, plasmin, and Factor XI (plasma thromboplastin antecedent). Kallikrein was greater than 10 times more active in its ability to activate Hageman factor than plasmin and Factor XI. The data offer a plausible explanation for the finding that highly purified kallikrein promotes clotting of normal plasma. In addition, the combined results of this and previously reported data from this laboratory indicate that the reciprocal activation of Hageman factor by kallikrein in fluid phase is essential for normal rate of activation of the intrinsic-clotting, kinin-forming, and fibrinolytic systems.

Activation of Hageman factor was associated with three different structural changes in the molecule: (a) Purified Hageman factor, activated on negatively charged surfaces retained its native mol wt of 80–90,000. Presumably a con-

formational change accompanied activation. (b) In fluid phase, activation with kallikrein and plasmin did not result in cleavage of large fragments of rabbit Hageman factor, although the activation required hydrolytic capacity of the enzymes. (c) Activation of human Hageman factor with kallikrein or plasmin was associated with cleavage of the molecule to 52,000, 40,000, and 28,000 mol wt fragments. Activation of rabbit Hageman factor with trypsin resulted in cleavage of the molecule into three fragments, each of 30,000 mol wt as noted previously. This major cleavage occurred simultaneously with activation.

The authors wish to acknowledge the capable assistance of Mr. Alan Johnston and the generous supplies of Hageman deficient blood from Mrs. Gunda Hiatt.

REFERENCES

1. Cochrane, C. G., K. D. Wuepper, B. S. Aikin, S. D. Revak, and H. L. Spiegelberg. 1972. The interaction of Hageman factor and immune complexes. *J. Clin. Invest.* **51**:2736.
2. Kaplan, A. P., and K. F. Austen. 1971. A prealbumin activator of prekallikrein. II. Derivation of activators of prekallikrein from active Hageman factor by digestion with plasmin. *J. Exp. Med.* **133**:696.
3. Burrowes, C. E., H. Z. Movat, and M. J. Soltay. 1971. The kinin system in human plasma. VI. The action of plasmin. *Proc. Soc. Exp. Biol. Med.* **138**:959.
4. Cochrane, C. G., and K. D. Wuepper. 1971. The first component of the kinin forming system in human and rabbit plasma. Its relationship to clotting factor XII (Hageman factor). *J. Exp. Med.* **134**:986.
5. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* **29**:185.
6. Wuepper, K. D., and C. G. Cochrane. 1972. Plasma prekallikrein: isolation, characterization, and mechanism of activation. *J. Exp. Med.* **135**:1.
7. Weber, K., and Osborn, M. 1969. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**:4406.
8. Ulevitch, R. J., and D. J. Letchford. 1973. A direct enzymatic assay of Hageman factor. *Fed. Proc.* **32**:845.
9. Wuepper, K. D. 1972. Biochemistry and biology of components of the plasma kinin-forming system. *In* Inflammation. Mechanisms and Control. I. Lepow and P. A. Ward, editors. Academic Press, Inc., N. Y. 93.
10. Hathaway, W. E., L. P. Belhasen, and H. S. Hathaway. 1965. Evidence for a new plasma thromboplastin factor: I. Case report, coagulation studies and physicochemical properties. *Blood J. Hematol.* **26**:521.
11. Hattersby, P. G., and D. Hayse. 1970. Fletcher deficiency: a report of three unrelated cases. *Br. J. Haematol.* **18**:411.
12. Wuepper, K. D., and C. G. Cochrane. 1972. Effect of plasma kallikrein on coagulation *in vitro*. *Proc. Soc. Exp. Biol. Med.* **141**:271.
13. Margolis, J. 1963. The relationship of coagulation of plasma and release of peptides. *Ann. N. Y. Acad. Sci.* **104**:133.
14. Haanen, C., F. Hommes, and G. Morselt. 1961. Some observations on the role of Hageman factor in blood coagulation. *Thromb. Diath. Haemorrh.* **6**:261.

15. Eisen, V. 1964. Effect of hexadimethrine bromide on plasma kinin formation, hydrolysis of *p*-tosyl-*L*-arginine methyl ester and fibrinolysis. *Brit. J. Pharmacol.* **22**:87.
16. Nossel, H. L., H. Rubin, M. Drillings, and R. Hsieh. 1968. Inhibition of Hageman factor activation. *J. Clin. Invest.* **47**:1172.
17. Wuepper, K. D., E. S. Tucker, and C. G. Cochrane. 1970. Plasma kinin system: proenzyme components. *J. Immunol.* **105**:1307.
18. Vroman, L. 1964. Effects of hydrophobic surfaces upon blood coagulation. *Thromb. Diath. Haemorrh.* **10**:455.
19. Wuepper, K. D. 1972. Precursor plasma thromboplastin antecedent (PTA, clotting factor XI). *Fed. Proc.* **31**:624.
20. Wuepper, K. D., and C. G. Cochrane. 1971. Plasma prokininogenase (prekallikrein): isolation and activation. *J. Clin. Invest.* **50**:100a. (Abstr.)
21. Donaldson, V. H., and O. D. Ratnoff. 1965. Hageman factor: alterations in physical properties during activation. *Science (Wash. D. C.)*. **150**:754.