

The relationship of structure and function in human Hageman factor. The association of enzymatic and binding activities with separate regions of the molecule.

S D Revak, C G Cochrane

J Clin Invest. 1976;57(4):852-860. <https://doi.org/10.1172/JCI108361>.

Research Article

Three regions of the human Hageman factor molecule termed the c, d, and e regions have been defined. Division of the molecule into these three regions is based on the analysis of fragments obtained by enzymatic cleavage during fluid-phase activation. The three regions have the following properties: (a) the c region has a mol wt of 40,000, has the capacity to bind to negatively charged surfaces, and does not have detectable enzymatic activity; (b) the e region possess a mol wt of 28,000 has enzymatic activity, and does not bind to negatively charged surfaces; (c) the d region has a mol wt of 12,000, is located between the c and e fragments but has not been detected as a freely existing polypeptide, and can bind firmly to negatively charged surfaces. The preparation of antibodies specific for the c and e regions is described as well as their use in defining the electrophoretic characteristics of the cde, cd, de, c, and e polypeptide fragments of Hageman factor. Evidence is given showing that the e region, but not the c or d, is released from a negatively charged surface when bound Hageman factor is exposed to proteolytic enzymes or whole plasma and that when this occurs in the presence of normal plasma, the e fragment becomes bound to C1 esterase inhibitor.

Find the latest version:

<https://jci.me/108361/pdf>



The Relationship of Structure and Function in Human Hageman Factor

THE ASSOCIATION OF ENZYMATIC AND BINDING ACTIVITIES WITH SEPARATE REGIONS OF THE MOLECULE

SUSAN D. REVAK and CHARLES G. COCHRANE

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

ABSTRACT Three regions of the human Hageman factor molecule termed the c, d, and e regions have been defined. Division of the molecule into these three regions is based on the analysis of fragments obtained by enzymatic cleavage during fluid-phase activation. The three regions have the following properties: (a) the c region has a mol wt of 40,000, has the capacity to bind to negatively charged surfaces, and does not have detectable enzymatic activity; (b) the e region possesses a mol wt of 28,000, has enzymatic activity, and does not bind to negatively charged surfaces; (c) the d region has a mol wt of 12,000, is located between the c and e fragments but has not been detected as a freely existing polypeptide, and can bind firmly to negatively charged surfaces. The preparation of antibodies specific for the c and e regions is described as well as their use in defining the electrophoretic characteristics of the cde, cd, de, c, and e polypeptide fragments of Hageman factor. Evidence is given showing that the e region, but not the c or d, is released from a negatively charged surface when bound Hageman factor is exposed to proteolytic enzymes or whole plasma and that when this occurs in the presence of normal plasma, the e fragment becomes bound to C1 esterase inhibitor.

INTRODUCTION

Since the identification of Hageman factor in 1958 (1) a great deal has been learned concerning its role in the coagulation, kinin-forming, and fibrinolytic systems of

plasma. Until recently, however, information concerning the molecular structure of the protein has been sparse and sometimes conflicting (2-6). The confusion has been undoubtedly due, in part, to the multiple forms of the enzymatically active Hageman factor moiety that result from differing purification and manipulatory techniques used by various investigators. In addition, there is little understanding of the portions of the molecule that do not have enzymatic activity, although such regions may have other important functions.

In the studies reported here, we have combined the use of radiolabeled Hageman factor, functional assays of activity, and immunologic techniques to probe the structure of the native Hageman factor molecule. In a previous report (5) the cleavage of the native molecule into three fragments by various enzymes was documented. This report further details the participation of these regions in the two known biologic activities of Hageman factor, namely enzymatic activity on various substrates and the ability to bind to negative surfaces. For the former, we studied the action of the various regions on prekallikrein (presumably the active site is the same for the activity on the three known substrates, although other portions of the molecule may influence the enzyme-substrate interaction) and as a model of surface binding we chose to study the interaction of the regions with kaolin.

METHODS

Plasma proteins. Human Hageman factor was purified and labeled with ¹²⁵I as described previously (5). The preparation used contained 1.05 mg Hageman factor (determined by quantitative radial immunodiffusion assays) per mg protein (measured by Kjeldahl nitrogen determination). Prekallikrein was prepared from human plasma by techniques previously published (5) with the following modifications: elution from the DEAE-Sephadex column was

This is publication no. 1025 from the Scripps Clinic and Research Foundation.

Received for publication 2 September 1975 and in revised form 9 December 1975.

effected in a single step with 0.01 M phosphate buffer + 0.06 M NaCl pH 7.7. After dialysis against 0.01 M phosphate + 0.1 M NaCl pH 6.0 the prekallikrein was applied to a column of carboxymethyl-Sephadex C-50 and washed with this buffer until no further protein eluted. A linear salt gradient of 0.1–0.25 M NaCl was used to remove the prekallikrein. The prekallikrein-containing fractions were then passed over Sepharose 4B beads (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) to which soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo.) was covalently coupled to remove any traces of active kallikrein. Contaminating proteins present at this point were identified as β_2 -glycoprotein I and γ -globulin. The presence of plasminogen activator was not assayed. The prekallikrein was concentrated by negative pressure before use. Activation to kallikrein was accomplished by incubation of 50 μ l prekallikrein with 50 μ l suspended Enzite agarose trypsin beads (Miles Laboratories, Elkhart, Ind.) for 60 min at 22°C. After removal of the beads by centrifugation, the kallikrein activity was assayed by incubation of 10 μ l enzyme (75 μ g total protein) with 1.5 ml of 1 mM benzoyl-L-arginine ethyl ester (BAEe)¹ at 37°C. Hydrolysis was found to occur at a rate of 0.33 μ M BAEe/min per mg protein. Partially purified rabbit prekallikrein, used for assays of Hageman factor, was prepared as previously described (7) being used after the second DEAE-Sephadex column at a concentration of 12.5 μ g/ml (1,000 mU²). The 28,000 mol wt fragment was isolated by preparative polyacrylamide-gel electrophoresis from a highly concentrated pool of Hageman factor (approximately 600 μ g/ml Hageman factor, 3.3 mg/ml total protein) which had undergone partial spontaneous fragmentation during 2 mo of storage at 4°C. 2 ml of the sample was applied to a rectangular gel of 20 ml of 7% acrylamide, and electrophoresis was carried out in the cold at 200 V for 5 h using a Tris-glycine pH 8.6 buffer system. The gel was sectioned into 38 slices, and each slice was eluted into 2 ml Tris-buffered saline. Assays of the eluates for their prekallikrein-activating ability yielded a strong, sharp peak in the anodal region. Further analysis showed this to be the 28,000 mol wt prekallikrein activator derived from Hageman factor.

Antibody to Hageman factor. Goat antibody to human Hageman factor was prepared and absorbed as previously described (5). For use in immunoelectrophoresis, the absorbed antiserum was diluted 1:10.

Assay of Hageman factor activity. Enzymatic activity of Hageman factor or its fragments was determined by its ability to convert prekallikrein to kallikrein. Rabbit prekallikrein, known to be activated by human as well as rabbit Hageman factor (4), was employed at a concentration of 100 mU (1.25 μ g)/0.1 ml. In general, the sample to be tested was added at neutral pH in a volume not exceeding 0.2 ml–100 mU/0.1 ml rabbit prekallikrein in plastic tubes. After incubation at 37°C for 20 min, 1.5 or 3.0 ml of 1 mM BAEe was added to each tube. Absorbance at 253 nm was measured at various times after the addition of BAEe, an increase with time indicating the presence of kallikrein.

Sodium dodecyl sulfate (SDS)-acrylamide gel electrophoresis. Electrophoresis in 7% polyacrylamide gels containing 0.1% SDS was performed according to the method

¹ Abbreviations used in this paper: BAEe, benzoyl-L-arginine ethyl ester; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

² 1 U of kallikrein activity is that amount of enzyme which when added to 3 ml of 1 mM BAEe causes hydrolysis of 1 μ mol/min at 37°C.

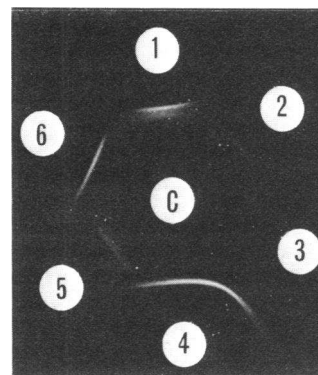


FIGURE 1 Double immunodiffusion in agar showing the separation of anti-Hageman factor antibody into two non-cross-reacting fractions. Trypsin-treated Hageman factor was incubated with kaolin (see text for details) and separated into two pools based on ability to bind to kaolin. Separate aliquots of anti-Hageman factor were absorbed with either the kaolin-bound Hageman factor fragments or fragments not bound to kaolin which remain in the supernate. 10 μ l of the following were placed in the wells as numbered: C, trypsin-treated Hageman factor diluted 1:10; 1, anti-Hageman factor; 2 and 5—anti-Hageman factor absorbed with Hageman factor fragments not binding to kaolin (anti-c); 3, Hageman factor fragments not binding to kaolin diluted 1:10; 4 and 6, anti-Hageman factor absorbed with kaolin-bound Hageman factor fragments (anti-e). See Fig. 7 for explanation of c and e.

of Weber and Osborn (8) with modifications as described previously (5).

Immuno-electrophoresis. Immuno-electrophoresis based on the method of Grabar and Williams (9) as modified by Scheidegger (10) was performed in 1% agarose at a current of 40 V/plate for 75 min. Immunoprecipitation was allowed to occur for 18 h followed by 24–48 h of washing in phosphate-buffered saline (PBS), drying, and staining of the slides with Amido-Schwartz. Photographic reproductions of faint precipitin bands reported in this paper are augmented by line drawings where necessary.

Preparation of antibodies to specific portions of the Hageman factor molecule. Antibodies specific to portions of the Hageman factor molecule were prepared by absorption of whole antiserum with isolated portions of the enzymatically treated Hageman factor molecule as follows: partially purified human Hageman factor was concentrated by lyophilization to a concentration of approximately 720 μ g/1.2 ml to which 50 μ l containing 2.5 μ g of ¹²⁵I-labeled Hageman factor (0.94 μ Ci/ μ g) was added. 1 ml was incubated with 1 μ g trypsin (an amount calculated to give complete cleavage) for 30 min at 37°C followed by the addition of 250 μ g/25 μ l ovomucoid trypsin inhibitor. 0.5 ml of the trypsin-treated material was incubated with 5 mg kaolin for 60 min at 22°C with shaking. After removal of the supernate following a 3-min centrifugation at 3,400 rpm, the kaolin was washed twice with 0.5 ml PBS. The radioactivity content of the initial supernate and the washed kaolin was determined. The supernate was sequentially absorbed with fresh 5-mg aliquots of kaolin until no more counts were removed. This was found to require four absorptions. 0.2 ml of monospecific antihuman Hageman factor was absorbed with either 50 μ l of the fourth supernate or 2.5 mg of the kaolin used for the first absorption. The two resulting antibodies

TABLE I
Effect of Kaolin Absorption on the Ability of Hageman Factor Fragments to Activate Prekallikrein

	¹²⁵ I cpm	OD 253 nm
A*	2,581	0.047
B	2,261	0.345
C	782	0.231
D	2,721	0.025
Buffer	—	0.014

* A, 5 μ l of untreated ¹²⁵I-human Hageman factor; B, 5 μ l trypsin-treated Hageman factor; C, 5 μ l supernate of trypsin-treated Hageman factor after absorption with kaolin; D, 200 μ g/20 μ l resuspended washed kaolin used for absorption in C. A, B, C, D, and 5 μ l buffer were incubated with 100 mU/100 μ l rabbit prekallikrein for 20 min at 37°C. 1.5 ml BAEe was then added to each tube and absorption at 253 nm read after 10 min incubation at 37°C. (See text for complete experimental details.)

show no cross-reactivity as noted in Fig. 1, where there is no coalescence of the bands formed between trypsin-treated Hageman factor (center well) and the two antibodies (wells 5 and 6). Immunoelectrophoretic studies employing radio-labeled Hageman factor fragments (such as those described under "binding of Hageman factor fragments to kaolin") showed the antibody absorbed with the kaolin-bound fragments (hereafter called "anti-e," see Fig. 7) to react immunologically with solutions containing the Hageman factor fragment having a mol wt of 28,000 while the antibody absorbed with the unbound fragment (hereafter called "anti-c," see Fig. 7) did not react with fragments of 28,000 mol wt. Both of the antibodies reacted with untreated native Hageman factor.

RESULTS

Binding of Hageman factor fragments to kaolin. Partially purified human Hageman factor was concentrated to approximately 75 μ g/ml by ultrafiltration. 125 μ l was mixed with an equal volume of ¹²⁵I-labeled Hageman factor. This mixture was shown to have minimal prekallikrein-activating capacity (Table I). To activate and cleave the Hageman factor, 175 μ l was then incubated with 0.5 μ g trypsin for 20 min at 37°C followed by a blocking of the trypsin with 50 μ g ovomucoid trypsin inhibitor. That activation of the Hageman factor had occurred was confirmed by BAEe hydrolysis after incubation of 5 μ l of the trypsin-treated material with 100 mU rabbit prekallikrein for 20 min at 37°C (Table I). This activation was accompanied by a cleavage of the 80,000 mol wt molecule into fragments of 52,000, 40,000, and 28,000 mol wt as determined by an examination of the radioactivity pattern obtained when 20 μ l was subjected to electrophoresis in acrylamide gels containing 0.1% SDS (Fig. 2, bottom panel). To determine which fragment(s) were capable of binding to a negatively charged surface, 90 μ l of the trypsin-treated Hageman factor was incubated with 1.5 mg kaolin for 20 min at 22°C. The

supernate (containing 31% of the radioactive counts added) was removed after a brief centrifugation and the kaolin was washed several times with buffer. SDS was added to a final concentration of 0.1% to 50 μ l of the supernate and a suspension containing 500 μ g of the washed kaolin. The samples were then applied to SDS acrylamide gels. (Greater than 90% of the radioactivity bound to the kaolin is removed during the electrophoresis.) The results, shown in the middle and top panels of Fig. 2, show only the 28,000 mol wt fragment remaining in the supernate while the 52,000, 40,000, and residual 80,000 moieties were bound to the kaolin. Prekallikrein-activating activity was found only in the supernate (Table I).

Further evidence that the prekallikrein-activating capacity found in the supernate described above was indeed associated with the 28,000 mol wt fragment was obtained by subjecting duplicate 8- μ l samples of the supernate material to electrophoresis in agarose on a microscope slide for 75 min. One section of the plate was then developed with anti-Hageman factor antibody placed in a trough alongside the electrophoresis path, and the other portion of the plate was sectioned into 2-mm segments and allowed to elute overnight into 0.1 ml PBS. The eluates were counted for radioactivity and assayed for their capacity to activate prekallikrein. As shown in Fig. 3, a region of activity was found at the anodal end of the

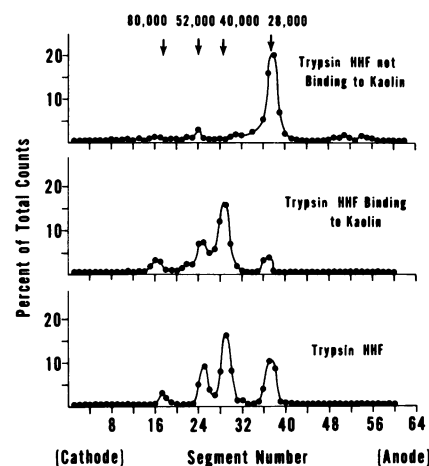


FIGURE 2 Binding of trypsin-treated human Hageman factor (HNF) to kaolin. Bottom panel shows the pattern of peaks observed on SDS-polyacrylamide gel electrophoresis of ¹²⁵I-labeled Hageman factor after incubation with trypsin. Such material was then incubated with kaolin and after centrifugation, the supernate, containing unbound fragments, was removed and subjected to SDS-polyacrylamide gel electrophoresis with the results shown in the top panel. Removal of the bound fragments was accomplished by electrophoresis into an SDS-polyacrylamide gel which revealed that maximal binding of the 52,000 and 40,000 fragments to the kaolin had occurred with a trace of 28,000 also present, as shown in the center panel.

electrophoretic pattern, coincident with the peak of radiolabel. The portion of the plate allowed to develop with anti-Hageman factor antibody revealed a single precipitin band in the corresponding area.

Release of a Hageman factor fragment from a surface after enzymatic treatment. 10 μ l of 125 I-labeled Hageman factor was added to triplicate aliquots of 500 μ g kaolin in 50 μ l PBS. Binding of the radiolabeled Hageman factor was allowed to occur for 15 min at room temperature. The kaolin was then pelleted by a 3-min centrifugation at 3,400 rpm and washed twice with 0.2 ml PBS to remove any unbound material. To the kaolin pellets was then added 50 μ l of buffer, human kallikrein, or human prekallikrein. After 60 min incubation at 22°C with shaking, the tubes were centrifuged, the supernates were removed, and the kaolin was washed. Essentially no counts were removed by the buffer incubation, while 18 and 16% of the 125 I counts initially bound to the kaolin were released into the supernate after incubation with the kallikrein or prekallikrein, respectively. The supernates and precipitates were then analyzed by SDS-acrylamide gel electrophoresis. As shown in Fig. 4, bottom panel, the

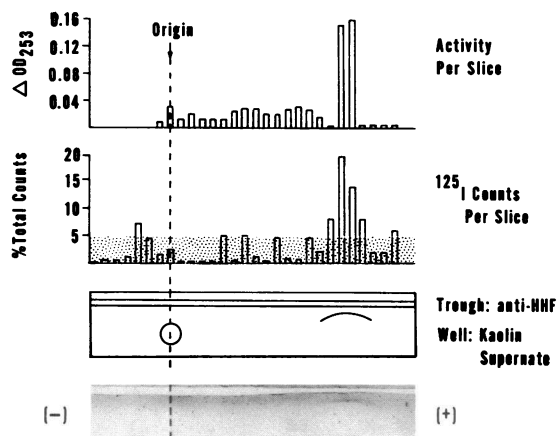


FIGURE 3 Correspondence of activity, radiolabel, and immunoprecipitate band of the fragment incapable of binding to kaolin derived from trypsin treatment of 125 I-human Hageman factor (HHF). 125 I-Hageman factor was activated and cleaved with trypsin as described in the text. After incubation with kaolin, 8 μ l of the supernate containing the material not binding was placed in each of the two wells and subjected to electrophoresis in agarose at 40 V/plate for 75 min. One portion of the plate was sliced into segments approximately 2 mm wide, and after elution, was assayed for radioactivity and ability to activate prekallikrein with the results shown in the top two panels. (It should be noted that the total radioactivity recovered was 615 counts/2 min which gives rise to a graph showing a definite region of increased counts but also a highly variable background as indicated by the gray zone). The remainder of the plate was allowed to incubate overnight with anti-Hageman factor in the trough. A faint band, shown here with a line drawing for clarification, could be seen after staining of the plate. Anode is to the right.

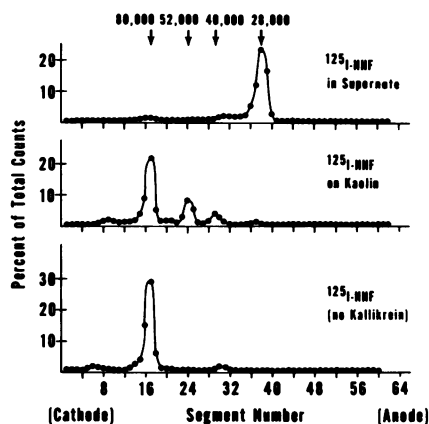


FIGURE 4 Effect of kallikrein on surface-bound Hageman factor. 500 μ g kaolin containing bound 125 I-labeled Hageman factor (125 I-HHF) was incubated with 50 μ l human kallikrein for 60 min at 22°C with shaking. After centrifugation, the supernate and washed precipitate were subjected to electrophoresis on SDS-polyacrylamide gels as shown in the top two panels. For comparison, a gel of 125 I-HHF bound to kaolin, not treated with kallikrein, is shown in the bottom panel.

untreated bound 125 I-labeled Hageman factor retained a mol wt of 80,000 while that incubated with kallikrein showed the appearance of fragments of 52,000 and 40,000 on the kaolin (center panel) and 28,000 mol wt fragments in the supernate (top panel). The results from prekallikrein were essentially identical to the kallikrein, presumably due to rapid conversion of the zymogen by the bound Hageman factor which was activated on the surface. (The prekallikrein employed was shown in preliminary experiments to contain no detectable enzymatic activity and did not activate Hageman factor in fluid phase.)

Cleavage of surface-bound 125 I-labeled Hageman factor by normal human plasma. 10 μ l of 125 I-labeled Hageman factor was incubated with 500 μ g kaolin in 50 μ l PBS for 15 min at 22°C. Unbound material was removed by centrifugation and washing. 100 μ l normal human plasma was then added and allowed to incubate 60 min at 22°C with shaking. Separation of the supernate by centrifugation revealed 29% of the radiolabel had been released from the kaolin. 10 μ l of this supernate and the 500 μ g kaolin were applied to separate SDS-acrylamide gels and electrophoresis was performed with the results shown in Fig. 5. The majority of the Hageman factor had been cleaved from its native 80,000 mol wt with the appearance of fragments of 52,000 and 40,000 mol wt which remained bound to the kaolin. The electrophoretic pattern of the supernate showed a peak of radioactivity at approximately 140,000 mol wt.

Alteration of the electrophoretic migration of the 28,000 mol wt fragment of Hageman factor when mixed

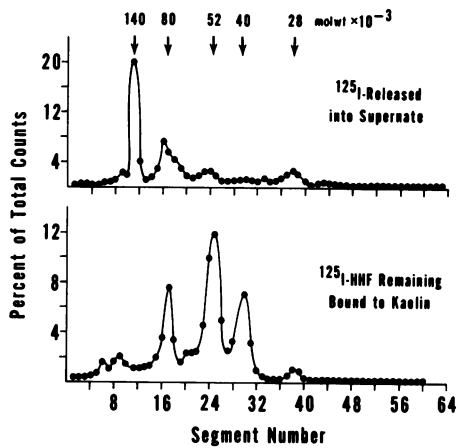


FIGURE 5 Cleavage of surface-bound ^{125}I -labeled Hageman factor (^{125}I -HHF) by normal human plasma. 500 μg of kaolin containing bound ^{125}I -HHF was incubated with 100 μl normal human plasma for 60 min at 22°C with shaking. After separation of the supernate by centrifugation, 10 μl of it was subjected to SDS-polyacrylamide gel electrophoresis with the resultant profile shown in the top panel. After washing, the kaolin precipitate was layered on top of an SDS-polyacrylamide gel and the pattern shown in the bottom panel was obtained.

with plasma. Mixtures of enzymatically active 28,000 mol wt Hageman factor fragments and normal human plasma or plasma from a patient with hereditary angioneurotic edema were mixed in a ratio of 10:1 (vol:vol) and incubated at 37°C for 10 min. 10 μl of each mixture plus a control of 28,000 fragment alone were placed in separate wells cut in a layer of agarose on a microscope slide. Electrophoresis was carried out for 75 min after which troughs were cut and filled with a 1:10 dilution of a stock monospecific anti-human Hageman factor antibody. After 24 h the plate was washed, dried, and stained with the results shown in Fig. 6. A new precipitin band showing immunologic identity with the 28,000 fragment

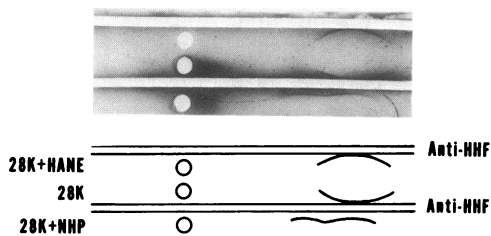


FIGURE 6 Immunoelectrophoresis of 28,000 mol wt Hageman factor fragment (28K) showing a change in pattern of migration when added to normal human plasma (NHP) but not to hereditary angioneurotic edema plasma (HANE). Diagrammatic representation is shown below for clarification. The ratio of fragment to plasma placed in the wells was 10:1 (vol:vol) at which dilution of human plasma no precipitin band can be seen for the native Hageman factor present in the plasmas. Anode is to the right.

appeared with the incubation with normal plasma but not with the plasma of the hereditary angioneurotic edema patient. The same shift was seen when purified C1 inhibitor (kindly supplied by Dr. Peter Harpel) was pre-incubated with the 28,000 fragment. Development of similar plates containing normal human plasma with anti-C1 inhibitor revealed migration of this protein occurred coincidentally with the new, cathodally migrating 28,000 fragment after it was mixed with normal plasma.

Characterization of various regions of the Hageman factor molecule by their immunologic and electrophoretic properties. The single polypeptide chain of human Hageman factor, previously shown to be enzymatically cleaved at two primary sites (5), was divided into three regions based on these two cleavage points. As diagrammed in Fig. 7, the region resulting in a 40,000 mol wt fragment

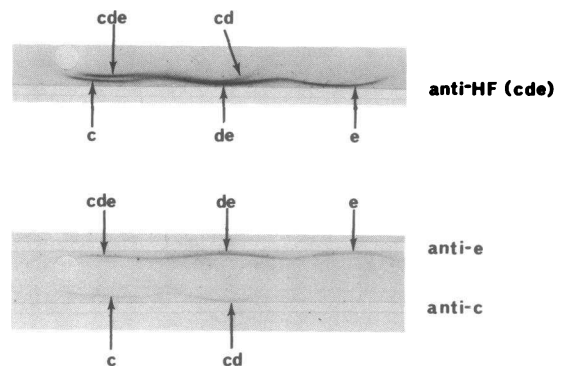
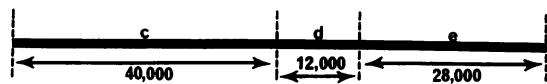


FIGURE 7 Immunoelectrophoretic patterns of the Hageman factor polypeptide fragments resulting from enzymatic cleavage. The 80,000 mol wt Hageman factor molecule was divided into three regions based on the cleavage pattern one obtains with enzymatic activators. These regions, termed the c, d, and e regions, and their corresponding molecular weights are shown in the diagram at the top. Immunoelectrophoresis was carried out at 40 V for 75 min on 10- μl samples of a highly concentrated Hageman factor pool known to contain the cleavage products, using anti-Hageman factor [anti-HF (cde)], anti-e, and anti-c antibodies. Five distinct bands appeared with the anti-HF (cde) antibody (although some heterogeneity can be seen upon close examination of the e band) as shown in the upper photograph. In the lower photograph, antibody to the e region (anti-e) forms three precipitin bands while anti-c yields two (the precipitin band one would expect between cde and anti-c does not form due to precipitation of the antibody by the more rapidly diffusing c fragment). Identification of the bands as indicated by the labeled arrows is described in the text. Anode is to the right.



FIGURE 8 Immunoelectrophoretic pattern of normal human plasma (NHP) demonstrating the position of Hageman factor [HF (cde)]. Electrophoresis was for 75 min at 40 V in 1% agarose. Anode is to the right.

after cleavage may be designated the c region,³ the 28,000 mol wt fragment-producing region may be designated as e, and the 12,000 mol wt region between the two as d. The other polypeptides appearing upon cleavage will be called cd or de with the 80,000 mol wt "whole chain" to be considered cde.⁴ Immunoelectrophoresis in agarose of normal human plasma yields a single precipitin band in the β -globulin region with antiserum to Hageman factor (anti-cde) as shown in Fig. 8. As described under Methods, anti-Hageman factor antibody was fractionated into two subclasses, one of which reacted immunologically with the 28,000 mol wt fragment appearing after complete enzymatic cleavage and therefore designated anti-e and a second which reacted with the 40,000 mol wt fragment, therefore being designated anti-c. For immunologic studies, a highly concentrated pool of partially purified Hageman factor (approximately 600 μ g Hageman factor/ml) which had undergone partial cleavage was used. This cleavage is presumably due to trace amounts of contaminating proteases and has been shown in other studies to be indistinguishable from the pattern that is obtained by controlled enzymatic cleavage with trypsin, kallikrein, or plasmin. By immunoelectrophoresis this material was found to contain five immunologically and/or electrophoretically distinct Hageman factor-derived molecular species. These are evident in Fig. 7 where development with anti-Hageman factor (cde) shows two bands in the β -globulin region, two in the α -globulin area, and one in the region of albumin migration. (It should be noted, however, that some heterogeneity of this last band can be seen. A possible explanation of this is included in Discussion.) The bottom portion of Fig. 7 shows three bands, one in each of the three migratory positions developing with antibody to the e region and two migrating in the β - and α -globulin regions, appearing with anti-c antibody. The band between cde and anti-c is prevented from forming by the faster diffusing

³The choice of the letters c, d, and e to designate the various regions of the human Hageman factor molecule was arbitrary. The use of the letter a to designate one region was eliminated due to the potential confusion with the symbol HFa which is commonly used to designate an active form of Hageman factor.

⁴The designation cde will be used here although it is equally possible that analysis of the terminal amino acids of the fragments will show the actual order to be edc.

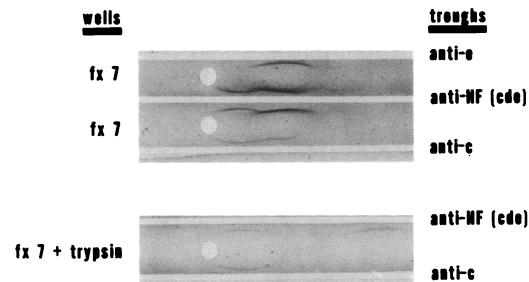


FIGURE 9 Immunoelectrophoretic pattern of an eluate from a preparative polyacrylamide gel electrophoresis of fragmented Hageman factor before and after trypsin treatment. A preparative polyacrylamide gel electrophoresis block of highly concentrated, partially cleaved Hageman factor was run as described in the text. The eluate of the seventh fraction from the cathodal end (fx 7) was subjected to electrophoresis in 1% agarose for 75 min at 40 V. Results of precipitation with anti-Hageman factor [HF (cde)], anti-e, and anti-c antibodies are shown in the top panel. Incubation of the eluate with trypsin before electrophoresis is shown at the bottom. Anode is to the right.

fragment c, which is first to precipitate the anti-c antibody in this region.

To determine which visible precipitin band corresponded with which of the five definable Hageman factor species known to result from interaction with enzymes, the Hageman factor was subjected to preparative polyacrylamide gel electrophoresis as described in Methods in an attempt to isolate the various fragments. After 5 h of electrophoresis, the block was sliced into 38 segments and each slice was eluted into 2 ml Tris-buffered saline. Immunoelectrophoresis was then performed on various eluates using the anti-c and anti-e antibodies in addition to the anti-Hageman factor (cde). Only the e fragment, found in the anodal eluates, was fully separated from the other fragments. It appeared in immunoelectrophoresis in the albumin-migrating position with either anti-Hageman factor (cde) or anti-e, giving no band with anti-c antibody (see Fig. 6). The other fragments were all found in overlapping peaks in the top (cathodal) half of the block. The eluate of the seventh slice from

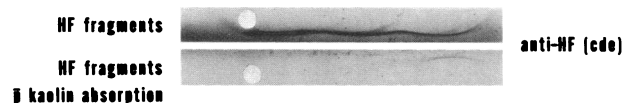


FIGURE 10 Immunoelectrophoretic pattern of a solution of Hageman factor (HF) fragments before and after being absorbed with kaolin. 100 μ l of a solution of highly concentrated, partially cleaved Hageman factor (HF fragments) was incubated with 1 mg of kaolin for 60 min at 22°C with shaking, followed by centrifugation and removal of the supernate. 10 μ l of the supernate material and 10 μ l of the mixture before absorption were subjected to electrophoresis for 75 min at 40 V in 1% agarose. The slide was developed with antibody to Hageman factor [anti-HF (cde)]. The anode is to the right.

the cathodal end (fx 7) is shown in Fig. 9 as an example of the type of pattern obtained and the identification of the fragment positions which could be determined. The eluate gave one band in the β -position and two bands in the α -position with anti-Hageman factor (cde). The β -migrating molecule did not react with anti-e, thereby eliminating the possibility of it representing the cde (whole Hageman factor molecule), de, or e fragment, and implying that it was either the c or cd fragment. Of the two bands in the α -region, one reacted with anti-c and not with anti-e, and the other gave a precipitin arc with anti-e and not anti-c. Both of these bands disappeared if the fraction was treated with trypsin before immunoelectrophoresis (bottom panel, Fig. 9), and only two bands, one in the β -region and the other in the albumin location, appeared. Since it is known (5) that complete trypsin degradation yields the c (40,000 mol wt) and e (28,000 mol wt) fragments (an intact d fragment of 12,000 mol wt has never been observed after enzymatic cleavage) and the e has been shown to be the albumin-migrating protein, it can be concluded that the c fragment migrates in the β -region. Immunoelectrophoresis of whole plasma in agarose shows the intact Hageman factor molecule (cde) also migrates in the slow β -region. Since only two bands are seen in the β -region when the electrophoresed fragments are precipitated with anti-Hageman factor (cde), the cd and de fragments must account for the two α -bands. Fraction 7, therefore, must contain fragments c, cd, and de. Based on such data from the individual fractions, we were able to identify the five precipitin bands as designated in Fig. 7.

The ability of the fraction eluates to activate prekallikrein was tested, and in each case such a capacity was noted wherever the e region of the molecule was present (i.e., if the fraction contained cde, de, or e). The capacity of various fragments to bind to negative surfaces was determined by immunoelectrophoresis before and after incubation with kaolin. The results of such an incubation are shown in Fig. 10. The c, cd, and de fragments, and the whole molecule cde, are removed from the solution by the kaolin; only the e fragment remains unbound.

DISCUSSION

The 80,000 mol wt Hageman factor molecule can be divided into three regions based on the fragments obtained after enzymatic treatment. Enzymatic cleavage of the molecule occurs during activation of Hageman factor in fluid phase by kallikrein, plasmin, clotting Factor XI, and perhaps other enzymes. The fragments of Hageman factor were shown previously (5) to be of 40,000, 12,000, and 28,000 mol wt. The Hageman factor molecule with its cleavage fragments is shown in the upper panel of Fig. 7. The ability of the regions to bind to surfaces was first determined by incubating radiolabeled frag-

ments with kaolin and examining by SDS-acrylamide gel electrophoresis which ones became bound. By this means it was determined that the 52,000 and 40,000 mol wt fragments, as well as the 80,000 mol wt native molecule, were able to bind, while the 28,000 mol wt fragment remained in the supernate. Conversely, when the whole molecule was first bound to the kaolin and then a proteolytic enzyme was added, cleavage occurred with the 28,000 mol wt fragment being released from the surface. From these data one can conclude that the 28,000 mol wt fragment of the molecule, designated region e, does not contain sites capable of binding to a negatively charged surface. The observation that this region is released into the supernate when kaolin-bound Hageman factor is incubated with kallikrein or prekallikrein bears interesting implications in the interpretation of studies of surface activation performed in the presence of such enzymes (i.e., in whole plasma). It will be important to ascertain the amount and rate of release of the enzymatically active 28,000 mol wt fragment from a surface in the presence of whole plasma and the rate of inhibition of activated Hageman factor both bound to the surface and free in the supernate after release.

The cleavage of surface-bound Hageman factor observed with plasma could be caused by several enzymes: kallikrein (5, 11), plasmin (5, 12), and Factor XI (5) have all been shown to cleave Hageman factor. It was of great interest to note that in the presence of normal plasma, the 28,000 fragment appeared to be bound to a protein of 110,000 mol wt, yielding a peak of radiolabel at approximately 140,000 mol wt. Immunoelectrophoresis of the fragment with normal or hereditary angioneurotic edema (C1 inhibitor-deficient) plasma suggests that the fragment is binding to C1 esterase inhibitor. A complex of C1 inhibitor and the e fragment would correlate with the 140,000 mol wt peak of radiolabel observed in the SDS studies. Inhibition of Hageman factor activity by C1 esterase inhibitor was first reported by Forbes et al., in 1970 (13). Schreiber et al. (14) has also reported inhibition of prealbumin Hageman factor fragments by C1 esterase inhibitor (14), although he was unable to show complex formation between the two.

By knowing the binding characteristics of the c, d, and e regions of the molecule and being able to separate these regions by selective proteolytic cleavage of the native molecule, we were able to prepare isolated e region in free solution and c region bound to kaolin which were then used to absorb anti-Hageman factor antibody. The 12,000 mol wt d region could not be detected as a freely existing polypeptide with either radiolabel or antibody although its presence when attached to either the c or e region changed the properties (mol wt and electrophoretic mobility) of both. It could represent a region of the native molecule which is rapidly broken down into small

polypeptide pieces when initially separated from the larger c and e regions, rendering it nonantigenic in immunoelectrophoresis and undetectable on SDS-acrylamide gels. The facts that one never detects a de fragment (having a mol wt of 40,000) in the supernate after enzymatic cleavage of radiolabeled fragments on kaolin, and the removal of the de fragment (as determined either by radiolabel or immunoelectrophoresis) from the supernate after kaolin absorption of the fragments leads one to the conclusion, however, that there is a "kaolin-binding" site located in the d region when it is attached to the e, since the e region itself is clearly not able to bind to kaolin. The characteristics of the native Hageman factor molecule and the five fragments one can isolate after selective proteolytic cleavage are given in Table II.

When highly concentrated partially cleaved Hageman factor solutions were subjected to immunoelectrophoresis, heterogeneity in the albumin-migrating band could often be seen (see Figs. 7 and 10). Whether this represents various configurations of the e fragment, genetic heterogeneity, or actual multiple forms of the polypeptide is unclear. It should be noted that multiple prealbumin bands have been observed on polyacrylamide gel electrophoresis after enzymatic cleavage of Hageman factor (3, 4, 12).

Other investigators have previously reported evidence of different forms of Hageman factor-derived molecules in clotted serum (3, 15) or glass-activated plasma fractions (12), with the capacity to activate prekallikrein although the exact nature of these forms was not determined. It is clear from the studies reported here that cleavage of the native Hageman factor molecule by kallikrein or plasmin, both of which might be present in serum, could give rise to two smaller molecular weight molecules (de and e) containing the enzymatically active region. Kaplan and Austen (3, 12) showed the conversion of one intermediate form of Hageman factor to a smaller, prealbumin molecule as well as an additional intermediate which cannot be directly accounted for by the model presented here. Presumably, action by other serum proteases on the molecule may give rise to other, as yet unknown, cleavage patterns. Bagdasarian et al. (16) have also reported molecules of differing molecular weights capable of activating prekallikrein and suggested that these may be derived from Factor XII by limited proteolysis.

The localization of the sites on the Hageman factor molecule responsible for the binding to surfaces and the enzymatic activity on substrates to unique, separable regions will undoubtedly aid in future studies of the exact nature of "contact activation." Studies to determine the amino acid sequence around the active site are being undertaken. Further investigation into the primary, secondary, and tertiary structure necessary to cause the molecule to bind and become active will be of great im-

TABLE II
Characteristics of the Hageman Factor Regions

Region	Mol wt	Electrophoretic mobility	Ability to bind to surface	Enzymatic ability
cde	80,000	β	+	+
c	40,000	β	+	-
cd	52,000	α	+	-
de	40,000	α	+	+
e	28,000	Albumin	-	+
d*	$\leq 12,000$?	+	-

* Has not been detected as a freely existing polypeptide.

portance. The identification of the various regions of the whole molecule and recognition of their biologic properties will facilitate such studies. Also, an understanding of the behavior of the regions under various conditions (e.g. the binding of the free e region to C1 esterase inhibitor in the presence of plasma) is essential for proper interpretation of data obtained from experiments in vivo.

ACKNOWLEDGMENTS

We wish to thank Mr. Alan Johnston, Dr. Peter Henson, and Dr. Richard Ulevitch for the numerous discussions held during the course of these experiments.

This work was supported by U. S. Public Health Service grant HL-16411 and the Council for Tobacco Research.

REFERENCES

- Margolis, J. 1958. Activation of plasma by contact with glass: evidence of a common reaction which releases plasma kinin and initiates coagulation. *J. Physiol. (Lond.)* **144**: 1-22.
- Speer, R. J., H. Ridgway, and J. M. Hill. 1965. Activated human Hageman factor (XII). *Thromb. Diath. Haemorrh.* **14**: 1-11.
- Kaplan, A. P., and K. F. Austen. 1970. A pre-albumin activator of prekallikrein. *J. Immunol.* **105**: 802-811.
- 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**: 986-1004.
- Revak, S. D., C. G. Cochrane, A. R. Johnston, and T. E. Hugli. 1974. Structural changes accompanying enzymatic activation of human Hageman factor. *J. Clin. Invest.* **54**: 619-627.
- McMillan, R., H. Saito, O. D. Ratnoff, and A. G. Walton. 1974. The secondary structure of human Hageman factor (Factor XII) and its alteration by activating agents. *J. Clin. Invest.* **54**: 1312-1322.
- Wuepper, K. D., and C. G. Cochrane. 1972. Plasma prekallikrein: isolation, characterization and mechanism of activation. *J. Exp. Med.* **135**: 1-20.
- Weber, K., and M. Osborn. 1969. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**: 4406-4412.
- Grabar, P., and C. A. Williams. 1953. Méthodes permettant l'étude conjuguée des propriétés électrophorétiques et immunochimiques d'un mélange de protéines Applica-

- tion au sérum sanguin. *Biochim. Biophys. Acta.* **10**: 193-194.
10. Scheidegger, J. J. 1955. Une micromethode de l'immuno-electrophorese. *Int. Arch. Allergy Appl. Immunol.* **7**: 103-110.
 11. Cochrane, C. G., S. D. Revak, and K. D. Wuepper. 1973. Activation of Hageman factor in solid and fluid phases. A critical role of kallikrein. *J. Exp. Med.* **138**: 1564-1583.
 12. Kaplan, A. P., and K. F. Austen. 1971. A pre-albumin activator of prekallikrein. II. Derivation of activators of prekallikrein from active Hageman factor by digestion with plasmin. *J. Exp. Med.* **133**: 696-712.
 13. Forbes, C. D., J. Pensky, and O. D. Ratnoff. 1970. Inhibition of activated Hageman factor and activated plasma thromboplastin antecedent by purified serum C1 inactivator. *J. Lab. Clin. Med.* **76**: 809-815.
 14. Schreiber, A. D., A. P. Kaplan, and K. F. Austen. 1973. Inhibition by C1 inhibitor of Hageman factor fragment activation of coagulation, fibrinolysis, and kinin generation. *J. Clin. Invest.* **52**: 1402-1409.
 15. Webster, Marian E. 1968. Human plasma kallikrein, its activation and pathological role. *Fed. Proc.* **27**: 84-89.
 16. Bagdasarian, A., R. C. Talamo, and R. W. Colman. 1973. Isolation of high molecular weight activators of human plasma prekallikrein. *J. Biol. Chem.* **248**: 3456-3463.