

Activation and Function of Human Hageman Factor

THE ROLE OF HIGH MOLECULAR WEIGHT KININOGEN AND PREKALLIKREIN

HENRY L. MEIER, JACK V. PIERCE, ROBERT W. COLMAN, and ALLEN P. KAPLAN

From the Allergic Diseases Section, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases; the Section on Physiological Chemistry, Laboratory of Chemistry, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014; and the Coagulation Unit of the Hematology-Oncology Section, Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

ABSTRACT The activation and function of surface-bound Hageman factor in human plasma are dependent upon both high molecular weight (HMW) kininogen and prekallikrein. HMW kininogen does not affect the binding of Hageman factor to surfaces, but it enhances the function of surface-bound Hageman factor as assessed by its ability to activate prekallikrein and Factor XI. The initial conversion of prekallikrein to kallikrein by the surface-bound Hageman factor in the presence of HMW kininogen is followed by a rapid enzymatic activation of Hageman factor by kallikrein. The latter interaction is also facilitated by HMW kininogen. Kallikrein therefore functions as an activator of Hageman factor by a positive feedback mechanism and generates most of the activated Hageman factor during brief exposure of plasma to activating surfaces. HMW kininogen is a cofactor in the enzymatic activation of Hageman factor by kallikrein and it also augments the function of the activated Hageman factor generated. The stoichiometry of the Hageman factor interaction with HMW kininogen suggests that it enhances the activity of the active site of Hageman factor. Since HMW kininogen and prekallikrein circulate as a complex, HMW kininogen may also place the prekallikrein in an optimal position for its reciprocal interaction with Hageman factor to proceed. The surface appears to play a passive role upon which bound Hageman factor and the prekallikrein-HMW kininogen complex can interact.

H. L. Meier is a Predoctoral fellow, Department of Biochemistry, Georgetown University, Wash., D. C.

Received for publication 12 July 1976 and in revised form 3 March 1977.

INTRODUCTION

Hageman factor (1, 2), prekallikrein (3-5), and high molecular weight kininogen (6-11) have been shown to be critical constituents of the initiation phase of the intrinsic coagulation and fibrinolytic pathways as well as the generation of the vasoactive peptide, bradykinin. Two of these proteins, namely, prekallikrein and high molecular weight (HMW)¹ kininogen appear to circulate in plasma as a complex (12). Upon binding to certain negatively charged surfaces, this complex presumably interacts with surface-bound Hageman factor. In this manuscript we have sought to further delineate the functions of the surface, prekallikrein, and HMW kininogen in the activation of Hageman factor and in the expression of Hageman factor activity.

An additional factor has been shown to be needed when surface-bound Hageman factor is used to activate either prekallikrein (13) or Factor XI (14) even though it is clear that activated Hageman factor is capable of activating prekallikrein (15) and Factor XI (16) in the fluid phase. Similarly, the feedback activation of Hageman factor by kallikrein that is demonstrable in the fluid phase (17, 18) does not appear to be evident in plasma that are deficient in HMW kininogen (6-11). Thus HMW kininogen might directly activate

¹Abbreviations used in this paper: BPVAN, α -benzoyl-phenylalanyl-valyl-arginine *p*-nitroanilide; DFP, diisopropyl fluorophosphate; HMW, high molecular weight; LMW, low molecular weight; PTT, partial thromboplastin time; QAE, quaternary aminoethyl; SDS, sodium dodecyl sulfate; SP, sulfopropyl.

surface-bound Hageman factor or act to facilitate the activation of Hageman factor by kallikrein. HMW kininogen might also augment the function of Hageman factor subsequent to its activation or interact with the Hageman factor substrates to facilitate their cleavage. The studies to follow examine each of these possibilities.

METHODS

Bradykinin triacetate (Sandoz Ltd., Basel, Switzerland) was used as the standard for native bradykinin. Hexadimethrine bromide and diisopropyl fluorophosphate (DFP) (Aldrich Chemical Co., Inc., Milwaukee, Wis.); enzodiffusion fibrin plates and streptokinase (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.); quaternary aminoethyl (QAE)-Sephadex A-25, sulfopropyl (SP)-Sephadex, Sephadex G-50, G-100, G-150, and G-200 and Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.); DEAE-52 and CM-cellulose (Whatman Chemicals, Div., W. & R. Balston, Maidstone, Kent, England); Bio-Gel A 0.5 m (Bio-Rad Laboratories, Richmond, Calif.); hemostatic phosphatide (cephalin) (Nutritional Biochemicals Div., International Chemical and Nuclear Corp., Cleveland, Ohio); guanidine hydrochloride and Tris base (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.); kaolin, potassium thiocyanate, and disodium EDTA (Fisher Scientific Co., Pittsburgh, Pa.); [³H]DFP (3 Ci/mM) (Amersham/Searle Corp., Arlington Heights, Ill.); chloramine-T (Matheson Coleman & Bell, East Rutherford, N. J.); Aquasol and ¹²⁵I-sodium iodide (New England Nuclear, Boston, Mass.) cyanogen bromide (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.); and L-lysine hydrochloride (Sigma Chemical Co., St. Louis, Mo.) were obtained as indicated.

Hageman factor-deficient plasma and Factor XI-deficient plasma containing 0.38% citrate were fresh-frozen, platelet-poor plasma of severely deficient patients collected by ourselves or by Sera-Tec Biologicals, New Brunswick, N. J. Fletcher factor (prekallikrein)-deficient plasma was a gift from Dr. C. Abildgaard (University of California, Davis, Calif.). Supercel was supplied by Johns-Manville, Co., Baltimore, Md. α -benzoyl-phenylalanyl-valyl-arginine *p*-nitroanilide (BPVAN) was synthesized by Bachem Chemical Co., Marina Del Ray, Calif.

Preparation of plasma proteins. To obtain deficient human plasma for use in assays, 9 vol of blood from the donor was drawn directly into plastic tubes containing 1 vol of 3.8% sodium citrate. The plasma was centrifuged at 900 *g* for 20 min at 4°C to remove the cells. 1-ml aliquots of plasma were stored at -70°C and used immediately after thawing. Any residual plasma was discarded. Normal human plasma used for assays of kinin generation was collected in EDTA (9 mg/10 ml blood), centrifuged at 900 *g* for 20 min at 4°C, and the plasma removed.

Plasma utilized for the isolation of unactivated Hageman factor, Hageman factor fragments, prekallikrein, Factor XI, plasminogen, and kininogen was collected in 3.8% sodium citrate (final concentration 0.38%). 3.6 mg of hexadimethrine bromide in 0.1 ml of 0.15 M saline was added for each 10 ml of blood drawn. The tubes were centrifuged at 900 *g* for 20 min at 4°C and the plasma was separated with plastic pipettes. Plastic columns and test tubes were utilized throughout all chromatographic procedures to minimize contact activation of Hageman factor. Samples were routinely concentrated by ultrafiltration

through a UM-10 membrane (Amicon Corp., Lexington, Mass.). Gel filtration on Sephadex G-100 (15) or G-150 (19) alkaline disc gel electrophoresis (15), and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (20) were performed as previously described. Protein was estimated by absorbance at 280 nm, with $A_{1\text{cm}}^{1\%}$ assumed to equal 10, or was determined by the Lowry technique (21).

Radioiodination of proteins was performed by the chloramine-T method (22) using ¹²⁵I-sodium iodide. The iodinated protein was immediately fractionated on a 2 × 100-cm column of Sephadex G-50 in 0.003 M phosphate buffer 0.15 M NaCl, pH 8.0. The initial peak of radioactivity was completely separated from the peak of free iodine and, when concentrated, contained over 99% of the trichloroacetic acid-precipitable counts. Radioactivity was determined in a sodium iodide well scintillation counter (Searle Analytic Inc., Des Plaines, Ill., model 1185) with automatic subtraction of background and an efficiency of 82%.

[³H]DFP was counted in a Tri-Carb model 3375 liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) calibrated with a [³H]toluene standard with a counting efficiency of 45%.

Unactivated Hageman factor. Unactivated Hageman factor was isolated utilizing chromatography on QAE-Sephadex, elution from an anti-Hageman factor immunoadsorbent, and fractionation on SP-Sephadex as previously reported (23, 24). 2 liters of fresh human plasma collected as described above was made 1 mM in DFP, dialyzed at 4°C for 12 h against four 30-liter changes of 0.003 M PO₄ buffer pH 8.3, and again made 1 mM in DFP. The plasma was centrifuged at 900 *g* for 15 min to remove any precipitate that had formed and was applied to a 18.5 × 90-cm column of QAE-Sephadex equilibrated in the same buffer. All subsequent buffers were made 0.1 mM in DFP to inhibit enzymatic activation of the Hageman factor. The column was washed with 30 liters of equilibrating buffer and the effluent was used subsequently to obtain prekallikrein and Factor XI. Unactivated Hageman factor was then eluted from the column with 30 liters of 0.003 M PO₄ buffer containing 0.06 M NaCl pH 8.3. The unactivated Hageman factor peak was concentrated to 1 liter by ultrafiltration using a 150-mm UM-10 membrane and the concentrate was then passed over a 9 × 20-cm immunoadsorbent prepared with sheep anti-human Hageman factor coupled to Sepharose 4B. The column was washed with 10 liters of 0.003 M PO₄ buffer 0.35 M NaCl, pH 7.4, and the Hageman factor was eluted with 500 ml 1 M potassium thiocyanate in 0.003 M PO₄ buffer pH 8.0. The unactivated Hageman factor peak was pooled, concentrated to 200 ml, dialyzed overnight against 10 liters of 0.003 M PO₄ buffer pH 6.0 and fractionated on a 9 × 76-cm column of SP-Sephadex equilibrated with the same buffer. The column was washed with 4 liters equilibrating buffer and then with 2 liters equilibrating buffer containing 0.09 M NaCl. A linear gradient consisting of 4 liters 0.003 M PO₄ buffer with 0.09 M NaCl pH 6.0 and 4 liters 0.003 M PO₄ with 0.35 M NaCl pH 6.0 was then applied. The unactivated Hageman factor peak eluting between 0.15 and 0.20 M NaCl was pooled and concentrated to 35 ml. When the Hageman factor preparation was assessed by alkaline disc gel electrophoresis, one major and two minor bands were observed. Elution of a sliced, unstained, alkaline disc gel run simultaneously revealed that these bands coincided with the Hageman factor activity. When the preparation was subjected to SDS gel electrophoresis, a single band at mol wt 82,000 was observed. The final preparation at a concentration of 20 μg/ml corrected the activated partial thromboplastin time (PTT) of Hageman factor-deficient plasma from a control time of 31 min-1 min, 20 s. This represents 0.6 U/ml when

the Hageman factor activity of a pool of 10 normal plasmas was arbitrarily set at 1.0 U/ml. Samples were dialyzed against phosphate-buffered saline before assay to remove the DFP. The Hageman factor contained less than 1% activated Hageman factor and was free of detectable kininogen, Factor XI, prekallikrein, plasminogen, or ability to cleave BPVAN.

The anti-Hageman factor immunoadsorbent (24) was prepared as follows: Hageman factor isolated from 200 ml of plasma by chromatography on QAE-Sephadex and SP-Sephadex was concentrated and emulsified in complete Freund's adjuvant. A sheep was immunized intramuscularly and then boosted intradermally three times at 2-wk intervals using the Hageman factor preparation emulsified in incomplete Freund's adjuvant. The animal was bled and the serum absorbed with 20% (by volume) Hageman factor-deficient plasma. 500 ml of absorbed sheep serum was then made 45% in ammonium sulfate, the precipitate was dissolved in 200 ml distilled water, dialyzed against 0.003 M PO_4 buffer 0.15 M NaCl pH 8.0, and coupled to Sepharose 4B by the cyanogen bromide method (25).

Activated Hageman factor. Activated Hageman factor was prepared in a similar fashion as the precursor; however, DFP was eliminated from the buffers and CM-cellulose was substituted for SP-Sephadex. The 0.06 M NaCl eluate from QAE-Sephadex was concentrated, stirred in a glass vessel overnight, and fractionated on CM-cellulose. The Hageman factor was then concentrated to 10 ml and fractionated on a 5×100 -cm column of Sephadex G-100 to remove Hageman factor fragments. The preparation was active upon the Hageman factor substrates and, when utilized at a concentration of 20 $\mu\text{g/ml}$, corrected the coagulation defect of Hageman factor-deficient plasma from a control time of 30 min–1 min, 35 s in the presence or absence of kaolin, indicating that the preparation was completely activated. It had no effect upon Factor XI-deficient plasma. Its mol wt was 80,000 in unreduced SDS gels.

Hageman factor fragments. Hageman factor prealbumin fragments were purified by chromatography of plasma on QAE-Sephadex twice, Sephadex G-100 gel filtration, SP-Sephadex, and elution from alkaline disc gels after electrophoresis, as previously reported (15, 23). 20- μg Hageman factor fragments revealed two prealbumin bands upon disc gel electrophoresis and a single band at mol wt 28,000 upon SDS gel electrophoresis. After dialysis, the fragments were concentrated and routinely used at 25 $\mu\text{g/ml}$. When assessed functionally, 5 μl of Hageman factor fragment generated 100 ng of bradykinin after incubation with 0.2 ml fresh plasma for 2 min at 37°C as assessed by bioassay. There was no detectable contamination with any of the Hageman factor substrates, plasminogen, plasmin, or kininogen.

Hageman factor substrates. Factor XI and prekallikrein were isolated from the QAE-Sephadex effluent obtained above during the isolation of unactivated Hageman factor. The proenzyme mixture was concentrated to 500 ml by ultrafiltration, the pH brought to pH 6.0 with 1 N HCl and each substrate was isolated by sequential chromatography on SP-Sephadex, twice on Sephadex G-150 (26), and passage over an anti-IgG immunoadsorbent as described previously (26, 27). All buffers were made 1 mM in DFP. When 30 μg of prekallikrein (2 mg/ml) was subjected to alkaline disc gel electrophoresis or SDS gel electrophoresis, a single band was observed and it contained no detectable Factor XI. 15 μl of this preparation (10 $\mu\text{g/ml}$) incubated with 15- μl Hageman factor fragments (2.5 $\mu\text{g/ml}$) generated 150 ng bradykinin from 0.2 ml of heat-inactivated plasma (15). The Factor XI preparation had a single band upon disc gel or SDS gel electrophoresis (mol wt 160,000),

contained no detectable prekallikrein or kallikrein, and completely corrected Factor XI-deficient plasma at a concentration of 7 $\mu\text{g/ml}$. We therefore utilized this concentration in our experiments.

For studies in which the active enzyme kallikrein was used, the prekallikrein was prepared through the QAE-Sephadex, SP-Sephadex, and the first Sephadex G-150 step and concentrated to 2.5 ml. It was then activated by addition of 50 μl Hageman factor fragment (25 $\mu\text{g/ml}$) for 24 h at 4°C. The preparation was then found to be fully activated since further addition of equal volumes of Hageman factor fragments and incubation for 10 min at 37°C yielded no additional activity. Kallikrein was then fractionated on Sephadex G-150 which separated kallikrein from the Hageman factor fragments. The kallikrein preparation was then passed over the anti-IgG immunoadsorbent. 15 μl of a 10- $\mu\text{g/ml}$ preparation generated 130 ng bradykinin after a 2-min incubation with heat-inactivated plasma.

Kininogen. Highly purified kininogens were prepared by the method of Pierce et al.² described briefly in earlier reports (9, 28, 29). 10 liters of fresh citrated human plasma, diluted to 50 liters with distilled water and made 0.01% in hexadimethrine bromide, was stirred with 500 g of DEAE-cellulose (DEAE-23) at pH 6.0 and 25°C. The filtered and washed adsorbent was eluted with 0.4 M Tris-HCl, pH 6.0. The eluate, adjusted to pH 7.1, was passed over an anti-low molecular weight kininogen immunoadsorbent, followed by several bed volumes of phosphate-buffered saline, pH 7.0. The kininogen was eluted with 8 M guanidine, dialyzed, and chromatographed on DEAE-cellulose at 4°C. Four main peaks of kininogen activity, coincident with the A_{280} peaks and named B1, B2, B3, and B4, were eluted from the column by a gradient of 0.05–0.30 M PO_4 buffer, pH 6.0. Gel filtration of the B4 peak on Bio-Gel A 0.5 m at 24°C yielded kininogens with an approximate mol wt of 80,000, 160,000, and 225,000, called B4 α , B4 β , and B4 γ , respectively, each of which corrected the abnormalities in Williams trait plasma. B4 β and B4 γ are designated HMW kininogen and were functionally indistinguishable in all the experiments to be presented (29). The results with B4 γ kininogen are reported in these studies and yielded a single major band upon alkaline disc gel electrophoresis. Treatment of this kininogen with kallikrein yielded 11 μg of bradykinin/mg protein.

The antikininogen immunoadsorbent was prepared as follows: sheep anti-low molecular weight (LMW) kininogen (30) was passed over a column of LMW kininogen coupled to agarose by the cyanogen bromide procedure (25). The antikininogen was eluted with 8 M guanidine, the eluate was dialyzed against phosphate-buffered saline, and the purified antikininogen was coupled to agarose by the cyanogen bromide procedure (25). In this fashion a high-capacity column that specifically binds kininogen was prepared. Since antibody to LMW kininogen cross-reacts with HMW kininogen (29), this column was used to isolate both forms. A column of 450 ml bed volume removes all the kininogen from 1.8 liters of plasma.

Plasminogen. Plasminogen was prepared by affinity chromatography using a modification of the procedure of Deutsch and Mertz (26, 31). The preparation contained less than 1% plasmin and gave a single band of mol wt 105,000 upon SDS gel electrophoresis after reduction and alkylation. Plasminogen was routinely utilized at 200 $\mu\text{g/ml}$ and had 26.5 azocaseinolytic U/ml.

² Manuscript in preparation.

Assays

Coagulation assays. The PTT was measured by a modification of the method of Proctor and Rapaport (32); after recalcification, the end point was determined at 24°C. Factor XI, Hageman factor, prekallikrein, and HMW kininogen were determined by a modification of the PTT using congenitally deficient plasma (9). Activated Hageman factor and Factor XI_A were assayed in an identical fashion except that kaolin was omitted from the incubation mixture. All coagulation assays were performed in 10 × 75-mm polypropylene test tubes.

Fibrinolytic assays. Plasmin was assayed with Hyland fibrin plates (Hyland Div., Travenol Laboratories, Inc.) containing plasminogen-free human fibrin as previously described (26). A standard curve relating ring diameter to plasmin concentration was obtained by activating 0.2 ml of a reference preparation of purified plasminogen containing 250 μg/ml with 140 U of streptokinase for 30 min at 37°C; a linear plot relating log plasmin concentration to ring diameter was obtained between 3 and 250 μg plasminogen/ml.

Plasminogen was assayed by incubating 0.2 ml of plasminogen source with 140 U streptokinase for 30 min at 37°C and determining the plasmin generated by the fibrin plate assay.

Plasminogen activator activity was assayed by incubating 20 μl of a plasminogen activator source with 20 μl of plasminogen (200 μg/ml) for 1 h at 37°C, and determining the plasmin generated in a fibrin plate.

Plasminogen proactivator activity was assayed by incubation of 10 μl plasminogen proactivator source with 10-μl Hageman factor fragments (25 μg/ml) for 10 min at 37°C. 20 μl of plasminogen (200 μg/ml) was added, the mixture was incubated for 1 h at 37°C, and the plasmin generated was determined by the fibrin plate assay.

The rate of generation of kaolin-activated fibrinolytic activity of plasma was assessed by a modification of the procedure described by Ogston et al. (33). To 0.2 ml of plasma or 0.1 ml of plasma and 0.1 ml test sample were added 0.1 ml kaolin suspension (4 mg/ml in saline) and 3.7 ml 0.01 M sodium acetate buffer, pH 4.8. After incubation at 37°C, the samples were centrifuged at 900 g for 10 min at 4°C, the supernate was aspirated, and the precipitate was washed with 2.0 ml 0.01 M sodium acetate buffer pH 4.8 and again centrifuged at 900 g for 10 min at 4°C. The precipitate obtained was dissolved in 0.1 ml 0.003 M PO₄ buffer, pH 7.5, containing 0.15 M NaCl, and centrifuged at 900 g for 5 min to sediment the kaolin. The supernate was then assayed for plasmin by the fibrin plate assay.

Assays of kinin-forming enzymes and substrates. Kallikrein activity was assayed by its ability to cleave *p*-nitroaniline from the synthetic substrate BPVAN (34). 10–100 μl of kallikrein source was incubated with 100 μl of substrate (0.7 mg/ml) for 60 min at room temperature, the sample was diluted to 1.0 ml, and the release of *p*-nitroaniline was determined by its absorbance at 405 nm. Initial experiments showed that the liberation of *p*-nitroaniline was proportional to the kallikrein concentration between 0.05 and 30.0 μg kallikrein/ml and was linear for a given concentration of kallikrein up to 60 min. The proteolytic activity of kallikrein was measured by its ability to release bradykinin from heat-inactivated plasma (15). Routinely, 25 μl of kallikrein source was incubated with 0.2 ml substrate for 2 min at 37°C and the bradykinin liberated was quantitated by bioassay.

Prekallikrein was determined by incubation of 25 μl of proenzyme source with 25 μl of Hageman factor fragments

(25 μg/ml) for 5 min at 37°C and the kallikrein generated was determined.

Kininogen was assayed functionally from column fractions by incubation of 0.5 ml of each fraction with 1 μg trypsin for 30 min at 37°C. The trypsin was inactivated by heating for 15 min in a boiling water bath, and the bradykinin generated was determined by bioassay. The ability of highly purified B4γ kininogen (1 mg/ml) to generate bradykinin upon incubation with plasma kallikrein was determined by incubating 100 μg kininogen with 100 μl of kallikrein (50 μg/ml) for 10 min at 37°C.

Determination of the effect of HMW kininogen and/or kallikrein upon surface-bound Hageman factor. Assessment of the requirements for surface activation of Hageman factor was performed by a modification of the method of Webster and Pierce (13). 5 mg of supercel was washed once with 0.04 M Tris-HCl-0.15 M NaCl pH 8.0, centrifuged at 1,000 g for 5 min at 4°C and the supernate was discarded. Buffer was then added to the pellet followed by the reactants (Hageman factor, HMW kininogen, kallikrein) such that the final volume was 1.0 ml. The suspension was then mixed by vortexing for 10 s, and incubated at either 4°, 24°, or 37°C for varying time intervals. The mixture was centrifuged at 1,000 g for 5 min at 4°C and the pellet washed three times with the Tris-saline buffer. This activation mixture was then assayed for activated Hageman factor by incubation with the Hageman factor substrates thus separating the reaction into two steps. The pellet was resuspended in 0.5 ml of a 10-μg/ml solution containing prekallikrein or Factor XI, and incubated at 24°C for 5 min to 1 h. The suspension was then centrifuged at 1,000 g for 5 min at 4°C, the supernate was removed and an aliquot assayed for either kallikrein, Factor XI_A, or plasminogen activating activity, respectively. In these experiments Factor XI_A was assayed by mixing 50 μl of each of the reactants (sample, cephalin reagent, calcium, and Factor XI-deficient plasma) and determining the time for formation of a clot at 37°C in the absence of any preincubation. Kallikrein and plasminogen activator were assayed as described above. In this two-stage reaction the conditions utilized allowed little binding of the Hageman factor substrates to the supercel pellet during the final incubation. In separate experiments we determined that the quantity of prekallikrein and Factor XI bound to the supercel under these conditions was less than 10% of that added. Of the fraction of prekallikrein and Factor XI activated to kallikrein and Factor XI_A, <2% of the kallikrein was bound to the surface, while 5–10% of the Factor XI_A was bound.

The quantity of Hageman factor bound to the supercel surface was determined by adding a trace quantity of ¹²⁵I-Hageman factor (1.2 μCi/μg) to purified unactivated Hageman factor. The fraction of bound ¹²⁵I was determined and multiplied by the initial Hageman factor added to determine the quantity bound.

RESULTS

To determine the conditions for optimal activation of Hageman factor, a time-course of interaction of Hageman factor and HMW kininogen with supercel was determined. 1 μg of Hageman factor, 3 μg of HMW kininogen or a mixture of 1 μg of Hageman factor and 3 μg of HMW kininogen were incubated with the supercel for varying time intervals before centrifugation. An aliquot of the resuspended pellet was then incubated with prekallikrein for 20 min at 37°C, the

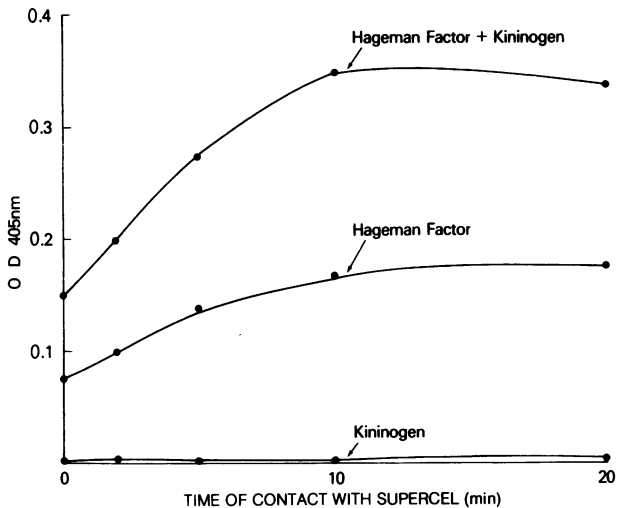


FIGURE 1 A time-course of interaction of Hageman factor and HMW kininogen with supercel. After each time interval the washed pellet was incubated with prekallikrein for 30 min at 37°C and the kallikrein generated was determined.

mixture centrifuged, and the release of *p*-nitroaniline from BPVAN was determined. As shown in Fig. 1, as the time of incubation of Hageman factor with supercel was increased from 0 to 10 min, the activity of the surface-bound Hageman factor increased. Significant activity was already evident when the reactants were mixed and then centrifuged without any incubation. The kininogen alone yielded no activation of the prekallikrein whereas the addition of HMW kininogen to the Hageman factor resulted in enhancement of the ability of the surface-bound Hageman factor to activate prekallikrein at each time interval. There was little difference, however, between the value at 10 and 20 min for the bound Hageman factor alone or the mixture of Hageman factor and HMW kininogen. When Factor XI was used as the Hageman factor substrate, the same result was obtained. Subsequent experiments were therefore performed using a 10-min incubation of the reactants with the surface.

This effect of HMW kininogen upon the Hageman factor activity might reflect an increase in the quantity of Hageman factor bound to the surface, or an increase in the rate of Hageman factor activation, or an enhancement of the function of the bound Hageman factor. To distinguish these possibilities the ability of HMW kininogen to affect binding of Hageman factor to the supercel surface was next investigated. ^{125}I -Hageman factor was incubated with supercel and buffer or supercel and 5 μg HMW kininogen for periods of 1–10 min. The suspension was centrifuged at 1,000 *g* for 5 min at 4°C, and washed with Tris-saline buffer until the supernate was free of radioactivity. The bound ^{125}I -Hageman factor was then determined. At each time interval, the presence of HMW kininogen had no effect

upon the quantity of Hageman factor bound. Thus it appeared that HMW kininogen affected either the activation of surface-bound Hageman factor and/or enhanced the ability of surface-bound Hageman factor to activate its substrates.

We next investigated the conditions for maximal interaction of surface-bound Hageman factor with its substrates. Fig. 2 presents the effect of time and temperature upon the ability of surface-bound Hageman factor to activate Factor XI in the presence or absence of HMW kininogen. Aliquots of supercel were incubated with buffer, 1 μg of Hageman factor, 3 μg of HMW kininogen, or a mixture of 1 μg of Hageman factor and 3 μg of HMW kininogen for 10 min at 24°C. An aliquot of the resuspended pellet was then incubated with Factor XI for varying time intervals from 0 to 60 min at 24°C. Addition of either buffer or HMW kininogen to the Factor XI did not yield conversion to Factor XI_A during the entire time-course. A gradual increase in Factor XI_A was observed upon incubation with the Hageman factor alone; however the addition of HMW

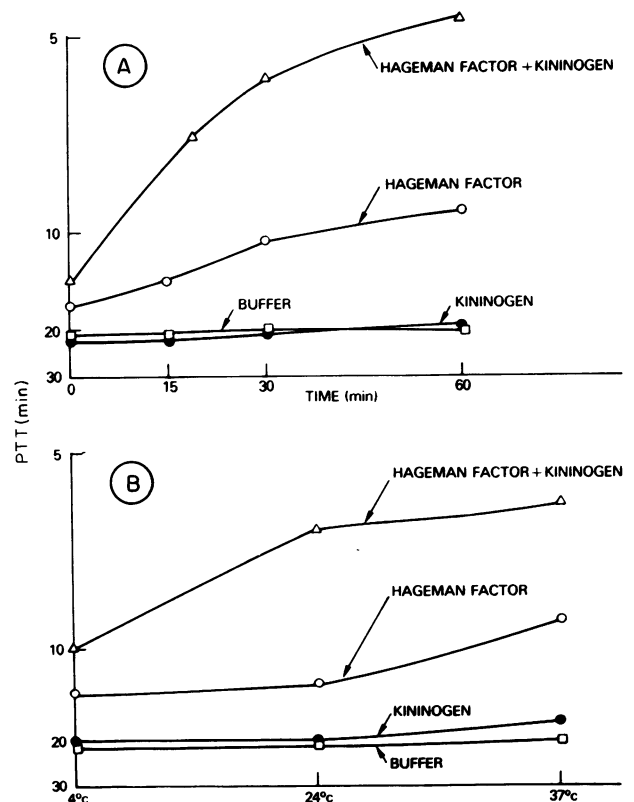


FIGURE 2 (A) A time-course of activation of Factor XI after interaction with surface-bound Hageman factor, surface-bound HMW kininogen, or surface-bound Hageman factor plus HMW kininogen. (B) The temperature dependence of Factor XI activation after a 30-min incubation with surface-bound Hageman factor, surface-bound HMW kininogen, and surface-bound Hageman factor plus HMW kininogen.

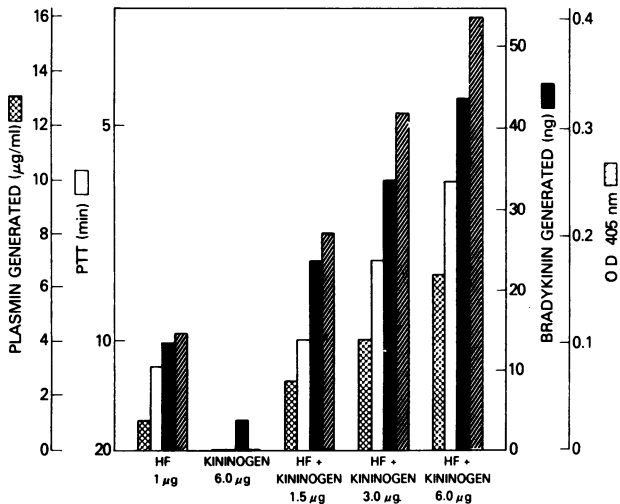


FIGURE 3 The effect of increasing quantities of HMW kininogen upon the ability of Hageman factor (HF) to activate prekallikrein, Factor XI, and generate plasminogen activating activity.

kininogen to the Hageman factor resulted in an increase in Factor XI_A at each time interval. Fig. 2B demonstrates the difference in Factor XI activation by surface-bound Hageman factor in the presence or absence of HMW kininogen at 4°, 24°, and 37°C after a 30-min incubation with the washed supercel. At each temperature an enhancement of the ability of Hageman factor to activate Factor XI was observed in the presence of HMW kininogen, however activation of Factor XI was diminished at 4°C compared to either 24° or 37°C. Although the rate of activation of Factor XI by Hageman factor alone or by Hageman factor plus HMW kininogen increased with increasing temperature, the optimal effect of the Hageman factor-HMW kininogen mixture compared to the Hageman factor alone was observed at 24°C. The time-course and temperature dependence of prekallikrein activation by Hageman factor in the presence or absence of HMW kininogen paralleled that observed for Factor XI activation. Thus experiments were routinely carried out at 24°C utilizing a 30-min incubation time of the bound Hageman factor with its substrates.

In the above experiments HMW kininogen enhanced the ability of bound Hageman factor to activate either prekallikrein or Factor XI. In the next series of experiments 1.0 µg of Hageman factor was incubated with supercel and the quantity of HMW kininogen added was varied to determine the effect upon the ability of Hageman factor to subsequently activate Factor XI or prekallikrein, and generate plasminogen activating activity. Kallikrein was assayed by its ability to generate bradykinin from kininogen as well as by its ability to cleave BPVAN. As shown in Fig. 3, the bound Hageman factor alone was

capable of activating each substrate. When 6 µg of HMW kininogen alone was incubated with supercel and the washed pellet then incubated with the Hageman factor substrates, no significant activation was observed although a small quantity of free bradykinin was detectable in the kininogen preparation. When HMW kininogen was added to the supercel together with the Hageman factor, the ability of Hageman factor to activate each substrate was augmented and the increase in observed activity in each case appeared proportional to the concentration of HMW kininogen added. When equal concentrations of B2α LMW kininogen was similarly assessed, there was no augmentation of the ability of surface-bound Hageman factor to activate its substrates. HMW kininogen had no effect upon the ability of kallikrein to cleave BPVAN nor did it affect the coagulant activity of activated Factor XI.

To determine the optimal quantity of HMW kininogen required to augment the activity of a fixed quantity of Hageman factor, 1.5 µg Hageman factor was bound to supercel and its ability to convert prekallikrein to kallikrein was determined after addition of either buffer or increasing concentrations of HMW kininogen. The enhancement obtained with each dose of HMW kininogen was then expressed as a function of the micrograms of HMW kininogen added. As shown in Fig. 4, Hageman factor activity increased linearly with the quantity of HMW kininogen added. The optimal enhancement was observed when 5.0 µg HMW kininogen was added and further addition of HMW kininogen did not yield further enhancement. An excess of HMW kininogen was inhibitory.

To distinguish an effect upon the generation of the

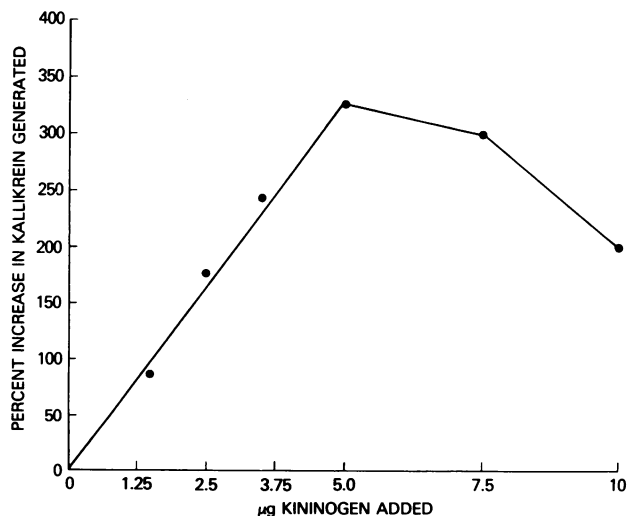


FIGURE 4 Enhancement of the activity of surface-bound Hageman factor upon prekallikrein as a function of the HMW kininogen added.

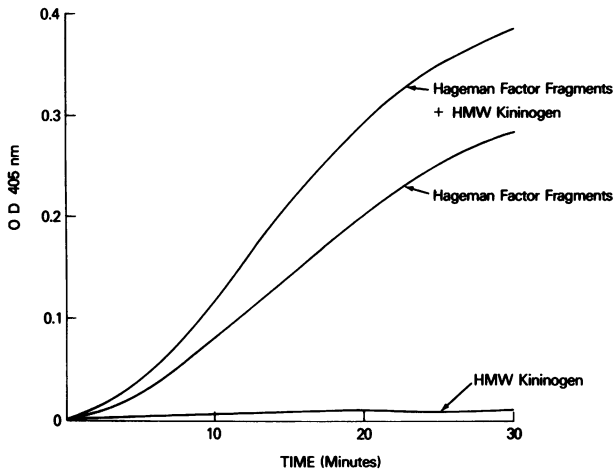


FIGURE 5 Enhancement of the activity of Hageman factor fragments upon the conversion of prekallikrein to kallikrein in the presence and absence of HMW kininogen. Kallikrein was assessed by the release of *p*-nitroaniline from BPVAN using a continuous recording spectrophotometer. The first 30 min of the tracing was then replotted and the points connected with a smooth curve.

active site in Hageman factor from augmentation of the function of an activated form of Hageman factor, we examined the effect of HMW kininogen upon a fluid-phase form of activated Hageman factor. 10- μ g samples of Hageman factor fragments were incubated together with 50 μ g prekallikrein and BPVAN in the presence or absence of 25 μ g of HMW kininogen and the rate of evolution of *p*-nitroaniline was continuously monitored with a recording spectrophotometer. The rate of conversion of prekallikrein to kallikrein as indicated by the liberation of *p*-nitroaniline from BPVAN (Fig. 5) was enhanced by HMW kininogen. This enhancement of the activity of Hageman factor fragments by HMW kininogen supports the conclusion that HMW kininogen is capable of augmenting the activity of activated Hageman factor.

Activation of Hageman factor by kallikrein and its dependence upon the presence of a surface and HMW kininogen. Fig. 6 illustrates the comparison of the rate of generation of kaolin-activable coagulation and fibrinolysis in normal plasma compared to plasma deficient in Hageman factor, prekallikrein, and HMW kininogen. The Hageman factor-deficient plasma had a markedly prolonged PTT (Fig. 6A) and generated no detectable plasmin (Fig. 6B). The HMW kininogen-deficient plasma had a similarly prolonged PTT (Fig. 6A) and generated a minimal quantity of plasmin (Fig. 6B). The prekallikrein-deficient plasma had an abnormal PTT and generated little plasmin at 2 min; however when the time of incubation with kaolin was increased, the PTT diminished and approached normal (Fig. 6A) and plasmin generation gradually increased

(Fig. 6B). In contrast the normal plasma had a short PTT and rapidly generated plasmin within 3 min of incubation with kaolin. Since the prekallikrein-deficient plasma initially generated little plasmin and had a prolonged PTT, the major activation of Hageman factor occurring within the first few minutes appeared to be a function of the prekallikrein content. Yet plasma that is deficient in HMW kininogen but contains prekallikrein did not express this effect of prekallikrein upon Hageman factor activation.

We next assessed the surface and enzymatic conditions required to correct the coagulation abnormality of prekallikrein-deficient plasma. As shown in Table I, the PTT of the prekallikrein-deficient plasma was 32 min in the absence of kaolin and 6 min, 15 s in the presence of kaolin. The PTT could be corrected to 1 min, 30 s by addition of 10 μ g/ml of intact activated Hageman factor in the absence of kaolin indicating that the abnormality lies in the rate of formation of activated Hageman factor. Addition of 3 μ g of kallikrein in the absence of kaolin did not yield a significant shortening of the PTT, while the addition of the same amount of kallikrein in the presence of kaolin corrected the PTT to 1 min, 20 s. 3 μ g of DFP-treated kallikrein (which was inactive as a kinin-forming enzyme) did not shorten the PTT even in the presence of kaolin indicating that the active site of kallikrein is necessary. In the presence of kaolin, 3 μ g of DFP-treated prekallikrein corrected the PTT as well as 3 μ g of untreated prekallikrein. Since the active-site serine residue in prekallikrein is unavailable to DFP (19) subsequent conversion to kallikrein would then yield a functional enzyme. Thus, correction of prekallikrein deficiency appeared to require conversion of prekallikrein to kallikrein, thus generating an active site and the kallikrein interacted with Hageman factor only in the presence of a surface. DFP treatment of HMW kininogen had no effect upon its ability to correct the coagulation abnormality in HWM kininogen-deficient plasma.

The ability of kallikrein to activate Hageman factor was next studied using purified components bound to the supercel surface. 1 μ g of unactivated Hageman factor was incubated with supercel in the presence of either buffer, 0.5 μ g of kallikrein, 3 μ g of HMW kininogen, or a mixture of 0.5 μ g of kallikrein and 3 μ g of HMW kininogen. After incubation the suspensions were centrifuged, the pellets were washed three times with Tris-saline buffer, and the resuspended pellets were assayed for their ability to activate prekallikrein. Activation of prekallikrein was observed with the bound Hageman factor alone, and addition of 0.5 μ g of kallikrein to the Hageman factor yielded a small increment in the subsequent conversion of prekallikrein to kallikrein (Fig. 7). The combinations of kallikrein and buffer, HMW kininogen and buffer, and

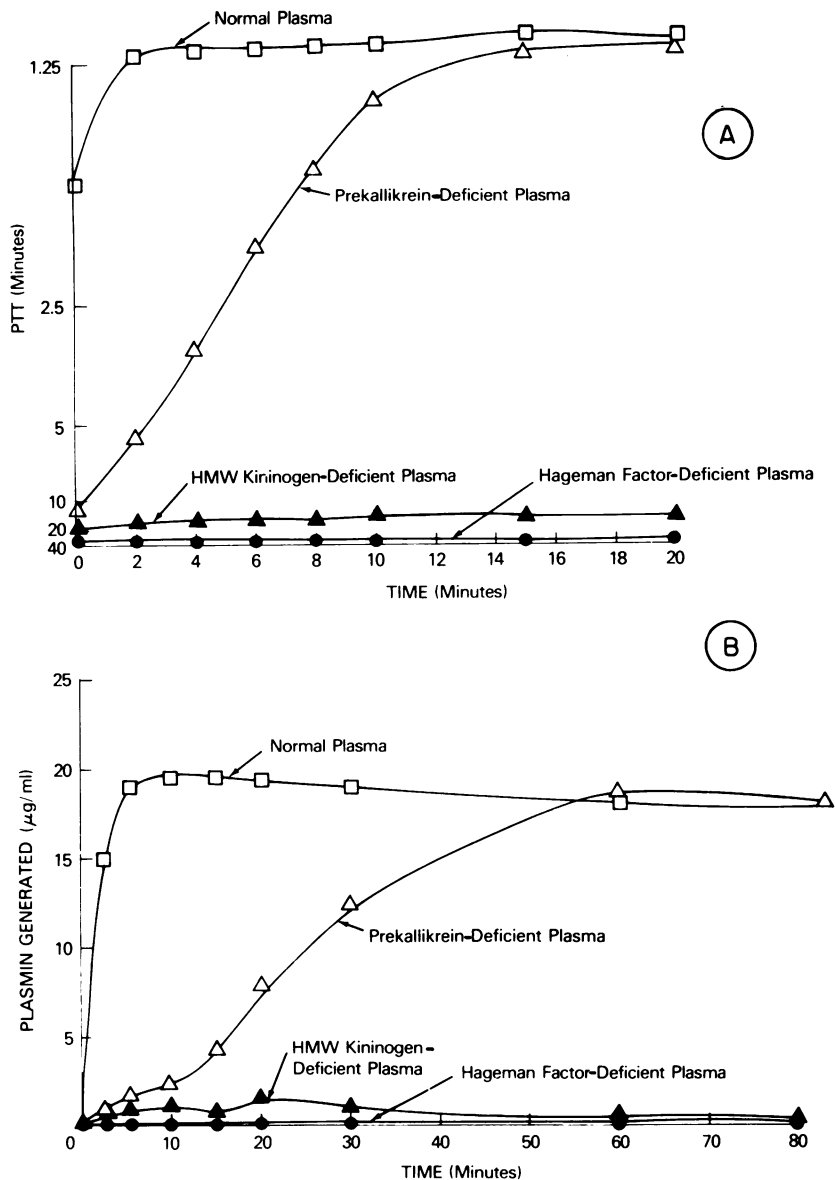


FIGURE 6 (A) The partial thromboplastin time (PTT) of normal plasma, prekallikrein-deficient plasma, HMW kininogen-deficient plasma, and Hageman factor-deficient plasma as a function of time of incubation with kaolin. (B) A time-course of generation of kaolin-activatable fibrinolytic activity in normal plasma, prekallikrein-deficient plasma, HMW kininogen-deficient plasma, and Hageman factor-deficient plasma.

kallikrein plus HMW kininogen in the absence of Hageman factor had no effect upon the subsequent conversion of prekallikrein to kallikrein. Addition of 3 μg of HMW kininogen to the Hageman factor yielded a threefold increase in the subsequent conversion of prekallikrein to kallikrein. However, addition of 0.5 μg of kallikrein to the Hageman factor-HMW kininogen mixture doubled the prekallikrein-activating ability, suggesting that the presence of HMW kininogen either facilitated the ability of kallikrein to activate

the Hageman factor and/or augmented the function of the activated Hageman factor generated.

When the same mixture of supercel-bound proteins were assayed for their ability to activate Factor XI (Fig. 8), a similar result was obtained. The combination of HMW kininogen and kallikrein in the absence of Hageman factor did not convert Factor XI to Factor XI_A beyond that observed in the buffer control. The activation observed with bound Hageman factor alone was augmented by HMW kininogen but not by kalli-

TABLE I
The Requirement for a Surface and the Active Site of
Kallikrein in the Normalization of the PTT of
Prekallikrein-Deficient Plasma*

	Cephalin	Cephalin and kaolin	PTT
Buffer	+	-	32 min
Buffer	-	+	6 min, 15 s
Activated Hageman factor	+	-	1 min, 30 s
Kallikrein	+	-	27 min, 30 s
Kallikrein	-	+	1 min, 20 s
DFP-kallikrein†	-	+	6 min, 10 s
Prekallikrein	-	+	1 min, 25 s
DFP-prekallikrein†	-	+	1 min, 30 s

* The PTT was performed utilizing 50 μ l of the cephalin reagent (6 mg/ml cephalin in normal saline) with or without kaolin (10 mg/ml) added to 50 μ l prekallikrein-deficient plasma plus 50 μ l of either buffer or sample. The mixture was incubated for 2 min at 37°C before recalcification and the clotting time determined.

† Samples were made 0.1 mM in DFP, incubated for 90 min at 37°C, and dialyzed against three changes of 4 liters 0.003 M PO₄ buffer 0.15 M NaCl pH 8.0 for 12 h at 4°C.

krein alone. However addition of kallikrein to the mixture of Hageman factor and HMW kininogen yielded a significant augmentation in the activation of Factor XI.

As evident in Fig. 6, most of the activated Hageman factor generated in normal plasma occurred within the first 2 or 3 min of incubation with the surface. This activation was dependent upon the presence of prekallikrein. Therefore the ability of kallikrein to activate Hageman factor in the presence or absence of HMW kininogen was evaluated under conditions such that the resuspended pellet containing HMW kininogen and Hageman factor possessed minimal ability to convert prekallikrein to kallikrein. All reagents (except kallikrein) were made 10 mM in DFP and incubated for 90 min at 37°C to inhibit any trace of activated enzyme. Each protein was dialyzed extensively to remove the excess DFP. 1 μ g Hageman factor in the presence or absence of 4.5 μ g HMW kininogen was incubated with varying quantities of kallikrein. The washed pellet was then assayed for its ability to convert prekallikrein to kallikrein, however the incubation time with prekallikrein was decreased to 10 min at 24°C. As shown in Fig. 9, the addition of kallikrein to the bound Hageman factor in the absence of HMW kininogen did not yield a significant conversion of prekallikrein to kallikrein. The Hageman factor-HMW kininogen mixture (with no kallikrein) yielded little activity above that of the Hageman factor alone. However addition of increasing quantities of kallikrein to the Hageman factor-HMW kininogen mix-

ture yielded a rapid increase in amidolysis indicating an augmentation of the activated Hageman factor detected. In contrast to the 3-fold enhancement observed with HMW kininogen-alone in previous experiments, the enhancement observed upon addition of kallikrein to the Hageman factor-HMW kininogen mixture was approximately 40-fold.

Since kallikrein appeared to activate Hageman factor in the presence of HMW kininogen, we next compared the incorporation of [³H]DFP into surface-bound Hageman factor upon addition of HMW kininogen, kallikrein, and a mixture of HMW kininogen and kallikrein. Triplicate samples containing 1.0 μ g Hageman factor were incubated with supercel in the presence or absence of 1.5 μ g HMW kininogen and/or 0.05 μ g kallikrein for 20 min at 24°C in a final volume of 0.1 ml. The pellets were washed three times with Tris-saline buffer, 50 μ Ci [³H]DFP was added, and the mixtures were incubated for 90 min at 37°C. The samples were then diluted with equal volumes of 24% trichloroacetic acid to precipitate any Hageman factor that might have eluted from the surface during the 90-

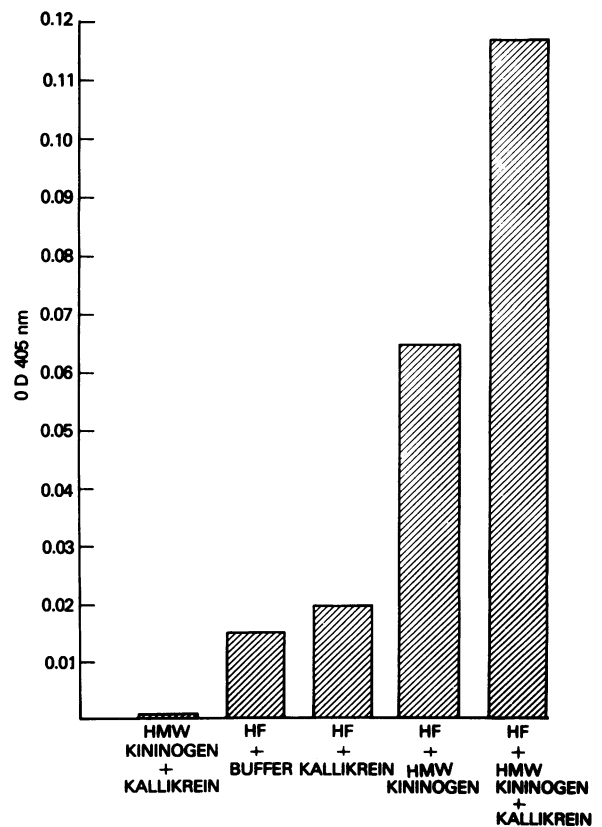


FIGURE 7 The effect of kallikrein (0.5 μ g) and HMW kininogen (3 μ g) upon the activation and function of bound Hageman factor (1.0 μ g) as assessed by the subsequent conversion of prekallikrein to kallikrein.

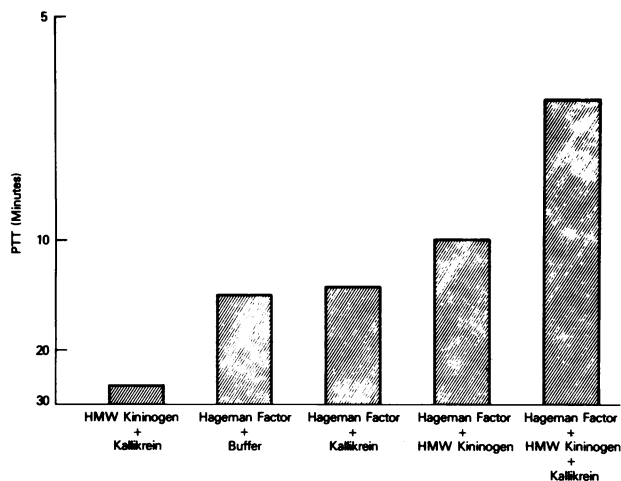


FIGURE 8 The effect of kallikrein (0.5 μ g) and HMW kininogen (3 μ g) upon the activation and function of bound Hageman factor (1.0 μ g) as assessed by the subsequent activation of Factor XI.

min incubation. The pellets were then centrifuged at 1,000 g for 10 min at 4°C and the pellets were washed with 12% trichloroacetic acid until the supernate was free of counts. The pellets were then suspended in 10 ml Aquasol and counted. As shown in Table II, Hageman factor alone, HMW kininogen alone, or Hageman factor + HMW kininogen incorporated few counts beyond that of the supercell alone. The addition of HMW kininogen to the Hageman factor yielded no significant increase in the incorporation of [3 H]DFP. Kallikrein alone contributed approximately 1,200 cpm above background and kallikrein + HMW kininogen did not differ significantly from this value. However the mixture of kallikrein + Hageman factor yielded a 6,000-cpm increase in the incorporation of [3 H]DFP while kallikrein + Hageman factor + HMW kininogen yielded a 26,000-cpm increase. Kallikrein was next adsorbed to the surface and inactivated with 10 mM nonradiolabeled DFP for 1 h at 37°C. The pellet was washed three times in 1.0 ml Tris-saline buffer, and the reaction sequence continued by incubation with Hageman factor and HMW kininogen followed by [3 H]DFP as described above. As seen at the bottom line of Table II, incorporation of [3 H]DFP was prevented. In separate experiments such two-stage adsorption to the surface either in the absence of nonradiolabeled DFP or subsequent to exposing the surface to 10 mM DFP was evaluated. Each control incorporated over 30,000 cpm indicating that this procedure is as effective as incubating the three reactants together and that non-specific adsorption of nonradiolabeled DFP did not interfere with the reaction. The data indicate that the incorporation of [3 H]DFP into surface-bound Hageman factor is a result of activation by kallikrein which was clearly augmented in the presence of HMW kinino-

TABLE II
Incorporation of [3 H]DFP by Surface-Bound Hageman Factor, HMW Kininogen, and Kallikrein

Proteins added to surface	Counts [3 H]DFP
	cpm
—	7,240 \pm 442
Hageman factor	7,924 \pm 438
HMW kininogen	7,635 \pm 480
Hageman factor + HMW kininogen	8,081 \pm 505
Kallikrein	8,426 \pm 470
Kallikrein + HMW kininogen	8,269 \pm 498
Kallikrein + Hageman factor	14,175 \pm 770
Kallikrein + Hageman factor + HMW kininogen	34,395 \pm 1,123
DFP-Kallikrein + Hageman factor + HMW kininogen	9,230 \pm 521

gen and that such activation was dependent upon the active site of kallikrein.

DISCUSSION

Recent studies have demonstrated that HMW kininogen is a critical constituent of the Hageman factor-dependent pathways. Wuepper et al. reported a patient with abnormalities of coagulation, fibrinolysis,

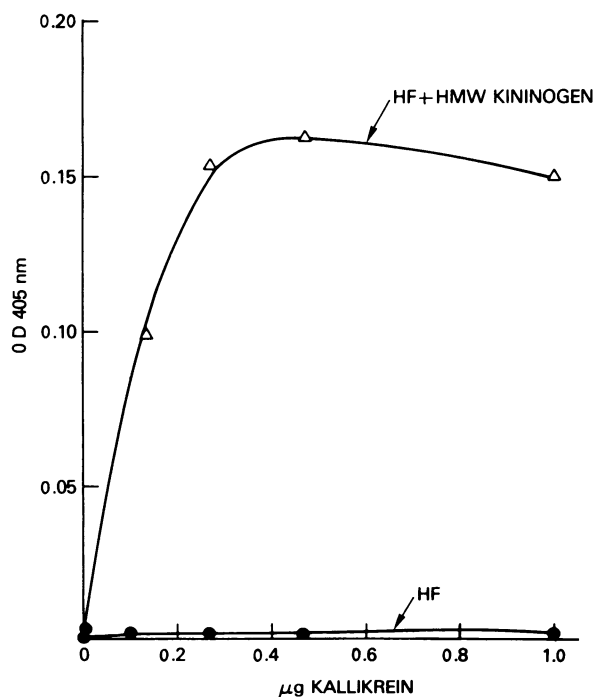


FIGURE 9 Dose response of the activation and function of bound Hageman factor by kallikrein in the presence and absence of HMW kininogen.

and kinin-generation attributable to HMW kininogen deficiency (6, 7), and, Colman et al. reported a patient who had no detectable kininogen and whose functional abnormalities were attributed to a new factor (8) which was independently identified to be HMW kininogen (9). Confirmation of these observations appeared shortly thereafter when Fitzgerald trait, an abnormal plasma described earlier by Saito et al. (10) was also shown to be deficient in HMW kininogen (11).

In an attempt to more precisely localize the role of HMW kininogen in coagulation, Saito et al. observed that activated Factor XI functions normally in HMW kininogen-deficient plasma (10) indicating that the contribution of HMW kininogen must precede the formation of Factor XI. We have shown that HMW kininogen enhanced the action of surface-bound Hageman factor upon each of its substrates (Fig. 3), as reported in preliminary form (35), but did not increase the incorporation of [³H]DFP into surface-bound Hageman factor (Table II). Thus HMW kininogen did not activate surface-bound Hageman factor but appeared to augment the function of the activated molecule. Liu et al. (36) reported a similar functional enhancement when the activity of Hageman factor fragments was assessed in the presence and absence of HMW kininogen (Fig. 5). In this case, a surface is not necessary since the fragments are already active, and the enhancement observed with HMW kininogen is clearly an effect upon the function of Hageman factor. The interaction of activated Hageman factor and HMW kininogen appears to be stoichiometric since the percentage enhancement of Hageman factor activity is directly proportional to the quantity of HMW kininogen added and further addition of HMW kininogen yields a gradual inhibition of Hageman factor activation. Schiffman and Lee have reported such an incremental increase in the quantity of Factor XI_A formed when increasing amounts of "contact activation cofactor" were added to surface-bound Hageman factor preparations (37). Griffin and Cochrane (38, 39) have also reported that the reaction of HMW kininogen and Hageman factor was stoichiometric and presented similar evidence that HMW kininogen augments the activity of Hageman factor upon its substrates. A physical interaction between activated Hageman factor and HMW kininogen has not, however, been demonstrated.

Prekallikrein-deficient plasma possesses normal HMW kininogen and incubation of this plasma with appropriate surfaces yields a gradual activation of Hageman factor. However the rate is abnormal when compared to normal plasma. Weiss et al. (4) have shown that reconstitution of prekallikrein-deficient plasma with activated Hageman factor can correct the abnormal rate of coagulation and fibrinolysis indicating that the defect reflects a diminished rate of formation of activated Hageman factor. When activated

Hageman factor is then added to this plasma, its function can be augmented by the HMW kininogen present. The gradual activation of Hageman factor observed upon incubation of prekallikrein-deficient plasma with kaolin (Fig. 6) (3-5) may reflect the gradual formation of the active site of Hageman factor upon the surface as well as augmentation of the function of that site by HMW kininogen. Enzymatic activation of the Hageman factor by enzymes such as plasmin (40) or Factor XI_A (17) may function instead of kallikrein and contribute to this autocorrection with time. It has been shown that kallikrein can activate and fragment Hageman factor in the fluid phase; yet addition of kallikrein to prekallikrein-deficient plasma in the absence of a surface does not generate activated Hageman factor (Table I) (5, 27). Thus the critical activation of Hageman factor by kallikrein takes place upon a surface. Since the active site of kallikrein is required for this interaction (Table I), enzymatic activation of Hageman factor is likely.

Incubation of plasma deficient in HMW kininogen with kaolin does not yield detectable conversion of prekallikrein to kallikrein (9, 10), indicating that the HMW kininogen enhancement of the function of Hageman factor is needed to convert sufficient prekallikrein to kallikrein so that the kallikrein can activate the bound Hageman factor. However, as shown in Figs. 7 and 8, augmentation of the formation and/or function of activated Hageman factor is small if bound Hageman factor is interacted with kallikrein in the absence of HMW kininogen. HMW kininogen may be enhancing the function of the Hageman factor activated by the kallikrein (which is likely), however it also appears to facilitate the interaction of kallikrein with the Hageman factor; thus a 40-fold augmentation was observed when HMW kininogen and kallikrein were incubated together while a 2- to 3-fold enhancement was observed with HMW kininogen alone and little enhancement with kallikrein. Incorporation of [³H]DFP into surface-bound Hageman factor was observed with kallikrein alone, however in the absence of HMW kininogen, little activation of the Hageman factor substrates was observed. HMW kininogen, which did not increase the incorporation of [³H]DFP into surface-bound Hageman factor, clearly augmented the kallikrein-dependent incorporation of [³H]DFP into surface-bound Hageman factor and activated Hageman factor was rapidly generated.

Ratnoff and Saito (41) have presented evidence that kaolin masks the enzymatic activity of kallikrein when it is bound to a surface and that HMW kininogen reverses this inhibition. However IgG as well as cytochrome *c* had the same effect as the HMW kininogen suggesting that such inhibition may not be a specific function of HMW kininogen. HMW kininogen has also been shown to accelerate the cleavage of Hage-

factor. Kallikrein digests the HMW kininogen to yield the vasoactive peptide bradykinin and in the bovine system, digestion of HMW kininogen liberates a histidine-rich peptide which can inhibit surface-dependent activation of Hageman factor (45). This might relate to the inhibition observed with excess kininogen (Fig. 4) although similar inhibition was observed when testing the function of Hageman factor fragments in the absence of a surface.

REFERENCES

- Ratnoff, O. D., and J. E. Colopy. 1955. A familial hemorrhagic trait associated with a deficiency of clot-promoting fraction of plasma. *J. Clin. Invest.* **34**: 602-613.
- Ratnoff, O. D., and E. W. Davie. 1962. The purification of activated Hageman factor (activated Factor XII). *Biochemistry*. **1**: 967-975.
- Wuepper, K. D. 1973. Prekallikrein deficiency in man. *J. Exp. Med.* **138**: 1345-1355.
- Weiss, A. S., J. I. Gallin, and A. P. Kaplan. 1974. Fletcher factor deficiency. A diminished rate of Hageman factor activation caused by absence of prekallikrein with abnormalities of coagulation, fibrinolysis, chemotactic activity, and kinin generation. *J. Clin. Invest.* **53**: 622-633.
- Saito, H., O. D. Ratnoff, and V. H. Donaldson. 1974. Defective activation of clotting, fibrinolytic, and permeability-enhancing systems in human Fletcher trait plasma. *Circ. Res.* **34**: 641-651.
- Wuepper, K. D., D. R. Miller, and M. J. Lacombe. 1975. Flaujeac trait: deficiency of kininogen in man. *Fed. Proc.* **34**: 859. (Abstr.)
- Wuepper, K. D., D. R. Miller, and M. J. Lacombe. 1975. Flaujeac trait. Deficiency of human plasma kininogen. *J. Clin. Invest.* **56**: 1663-1672.
- Colman, R. W., A. Bagdasarian, R. C. Talamo, M. Seavey, C. F. Scott, and A. P. Kaplan. 1975. Williams trait: combined deficiency of plasma plasminogen proactivator, kininogen, and a new procoagulant factor. *Fed. Proc.* **34**: 859. (Abstr.)
- Colman, R. W., A. Bagdasarian, R. C. Talamo, C. F. Scott, M. Seavey, J. A. Guimaraes, J. V. Pierce, and A. P. Kaplan. 1975. Williams trait. Human kininogen deficiency with diminished levels of plasminogen proactivator and prekallikrein associated with abnormalities of the Hageman factor-dependent pathways. *J. Clin. Invest.* **56**: 1650-1662.
- Saito, H., O. D. Ratnoff, R. Waldmann, and J. P. Abraham. 1975. Fitzgerald trait. Deficiency of a hitherto unrecognized agent, Fitzgerald factor, participating in surface-mediated reactions of clotting, fibrinolysis, generation of kinins, and the property of diluted plasma enhancing vascular permeability (PF-dil). *J. Clin. Invest.* **55**: 1082-1089.
- Donaldson, V. H., H. I. Glueck, M. A. Miller, H. Z. Movat, and F. Habal. 1976. Kininogen deficiency in Fitzgerald trait: Role of high molecular weight kininogen in clotting and fibrinolysis. *J. Lab. Clin. Med.* **87**: 327-337.
- Mandle, Jr., R., R. W. Colman, and A. P. Kaplan. 1976. Identification of prekallikrein and HMW kininogen as a circulating complex in human plasma. *Proc. Natl. Acad. Sci. U. S. A.* **73**: 4179-4183.
- Webster, M. E., and J. V. Pierce. 1973. Activators of Hageman factor (Factor XII): Identification and relationship to kallikrein-kinin system. *Fed. Proc.* **32**: 845. (Abstr.)
- Schiffman, S., and P. Lee. 1974. Preparation, characterization, and activation of a highly purified Factor XI: Evidence that a hitherto unrecognized plasma activity participates in the interaction of Factors XI and XII. *Br. J. Haematol.* **27**: 101-114.
- Kaplan, A. P., and K. F. Austen. 1970. A pre-albumin activator of prekallikrein. *J. Immunol.* **105**: 802-811.
- Heck, L. W., and A. P. Kaplan. 1974. Substrates of Hageman factor. I. Isolation and characterization of human factor XI (PTA) and inhibition of the activated enzyme by α_1 -antitrypsin. *J. Exp. Med.* **140**: 1615-1630.
- 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.
- Bagdasarian, A., B. Lahiri, and R. W. Colman. 1973. Origin of the high molecular weight activator of prekallikrein. *J. Biol. Chem.* **248**: 7742-7747.
- Kaplan, A. P., A. B. Kay, and K. F. Austen. 1972. A prealbumin activator of prekallikrein. III. Appearance of chemotactic activity for human neutrophils by the conversion of human prekallikrein to kallikrein. *J. Exp. Med.* **135**: 81-97.
- Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**: 4406-4412.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.
- Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of ^{125}I -labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89**: 114-123.
- Kaplan, A. P., J. Spragg, and K. F. Austen. 1971. The bradykinin-forming system of man. In *Biochemistry of the Acute Allergic Reactions*. K. F. Austen and E. L. Becker, editors. Blackwell Scientific Publications Ltd., Oxford. 279-298.
- Meier, H. L., and A. P. Kaplan. 1975. A new method for the rapid purification of human unactivated Hageman factor. *Fed. Proc.* **34**: 860. (Abstr.)
- Axén, R., J. Porath, and S. Ernback. 1967. Chemical coupling of peptides and proteins to polysaccharides by cyanogen halides. *Nature (Lond.)* **214**: 1302-1304.
- Kaplan, A. P., and K. F. Austen. 1972. The fibrinolytic pathway of human plasma. Isolation and characterization of the plasminogen proactivator. *J. Exp. Med.* **136**: 1378-1393.
- Kaplan, A. P., H. L. Meier, L. D. Yecies, and L. W. Heck. 1976. Hageman factor and its substrates: The role of Factor XI (PTA), prekallikrein, and plasminogen proactivator in coagulation, fibrinolysis, and kinin-generation. In *The Kallikrein System in Health and Disease*. Fogarty International Center Proceedings, no. 27. J. J. Pisano and K. F. Austen, editors. U. S. Government Printing Office, Washington, D. C. 237-254.
- Guimaraes, J. A., R. C-Lu, M. E. Webster, and J. V. Pierce. 1974. Multiple forms of human plasma kininogen. *Fed. Proc.* **33**: 641. (Abstr.)
- Pierce, J. V., and J. A. Guimaraes. 1976. Further characterization of highly purified human plasma kininogens. In *The Kallikrein System in Health and Disease*. Fogarty International Center Proceedings, no. 27. J. J. Pisano and K. F. Austen, editors. U. S. Government Printing Office, Washington, D. C. 121-126.
- Pierce, J. V., and M. E. Webster. 1966. The purification and some properties of two different kallidinogens from human plasma. In *Hypotensive Peptides*. E. G. Erdös, N.

- Back, and F. Sicuteri, editors. Springer-Verlag New York, Inc., New York. 130–138.
31. Deutsch, D. G., and E. T. Mertz. 1970. Plasminogen: purification from human plasma by affinity chromatography. *Science (Wash. D. C.)*. **170**: 1095–1096.
 32. Proctor, R. R., and S. I. Rapaport. 1961. The partial thromboplastin time with kaolin: A simple screening test for first stage plasma clotting deficiencies. *Am. J. Clin. Pathol.* **35**: 212–219.
 33. Ogston, D., N. B. Bennett, C. M. Ogston, and O. D. Ratnoff. 1971. The assay of a plasma component necessary for the generation of a plasminogen activator in the presence of Hageman factor (Hageman factor cofactor). *Br. J. Haematol.* **20**: 209–216.
 34. Svendsen, L., B. Blombäck, M. Blombäck, and P. I. Olsson. 1972. Synthetic chromogenic substrates for determination of trypsin, thrombin, and thrombin-like enzymes. *Thromb. Res.* **1**: 267–278.
 35. Meier, H. L., M. Webster, C. Y. Liu, R. W. Colman, and A. P. Kaplan. 1976. Enhancement of surface dependent Hageman factor activation by high molecular weight kininogen. *Fed. Proc.* **35**: 692. (Abstr.)
 36. Liu, C. Y., A. Bagdasarian, H. Meier, C. F. Scott, J. Pierce, A. P. Kaplan, and R. W. Colman. 1976. Potentiation of the action of Hageman factor fragments (HF_i) by high molecular weight kininogen (HMW Kng). *Fed. Proc.* **35**: 692. (Abstr.)
 37. Schiffman, S., and P. Lee. 1975. Partial purification and characterization of contact activation cofactor. *J. Clin. Invest.* **56**: 1082–1092.
 38. Griffin, J. H., and C. G. Cochrane. 1976. Involvement of high M.W. kininogen (HK) in surface dependent reactions of Hageman factor (HF). *Fed. Proc.* **35**: 692 (Abstr.)
 39. Griffin, J., and C. G. Cochrane. 1976. Mechanism for the involvement of high molecular weight kininogen in surface-dependent reactions of Hageman factor. *Proc. Natl. Acad. Sci. U. S. A.* **73**: 2559–2563.
 40. 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**: 696–712.
 41. Ratnoff, O. D., and H. Saito. 1976. Evidence that Fitzgerald factor counteracts inhibition by kaolin or ellagic acid of the amidolytic properties of a plasma kallikrein. *Blood.* **47**: 243–251.
 42. Revak, S. D., C. G. Cochrane, and J. H. Griffin. 1976. Plasma proteins necessary for the cleavage of Hageman factor during surface activation. *Fed. Proc.* **35**: 692. (Abstr.)
 43. McMillin, C. 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.
 44. Stead, N., A. P. Kaplan, and R. D. Rosenberg. 1976. The inhibition of activated Factor XII by antithrombin-heparin cofactor. *J. Biol. Chem.* **251**: 6481–6488.
 45. Katori, M., S. Iwanaga, A. M. Komiya, Y. N. Han, T. Suzuki, and S. Oh-Ishi. 1975. Structure and a possible physiological function of a fragment (“histidine-rich peptide”) released from bovine plasma high molecular weight kininogen by plasma kallikrein. In Kininogenases. G. L. Haberland, J. W. Rohen, G. Blumel, and P. Huber, editors. F. K. Schattauer-Verlag, Stuttgart. **3**: 11–18.