

THE FIRST COMPONENT OF THE KININ-FORMING SYSTEM
IN HUMAN AND RABBIT PLASMA .

ITS RELATIONSHIP TO CLOTTING FACTOR XII (HAGEMAN FACTOR)*

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The role of the kinin-forming system in inflammatory injury is not well understood. Its role in pathologic states has been assessed generally in plasma or plasma-rich fluids by association of free kinin with inflammatory conditions, by a decrease in kininogen levels (1), or by measurement of esterase activities (2). The interpretation of results in such studies has been made difficult by the extremely short life-span of kinins and by the instability of precursor components. Once activated, the components react sequentially during the preparation of samples, and are rapidly affected by inhibitors in the plasma.

In an assessment of the role that members of this system might play in the pathogenesis of various diseases, it has become necessary to isolate each component of the plasma kinin-forming system in precursor or inactive form. In so doing, one may be able to determine the exact number of components in the system, to study their interactions and mechanisms of activation, and to see if potentially important side products result during the process of activation. With precursor components available, their turnover times and participation in pathologic states could be assessed. In addition, since the kinin-forming system and intrinsic clotting system are apparently related, the role of the clotting system in inflammation will be subject to investigation.

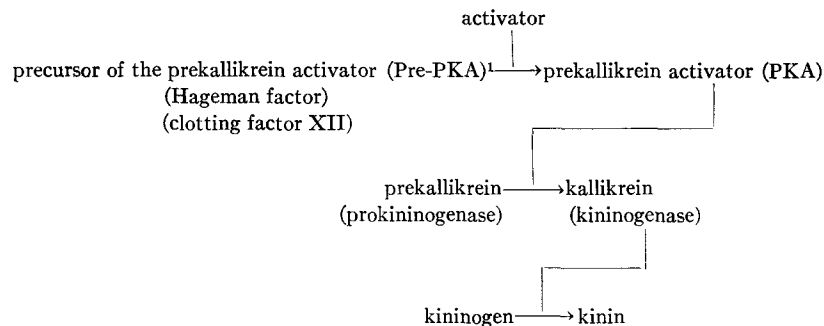
The present article describes the preparation and characterization of the first component of the kinin-forming system in rabbit and human plasma. The process of activation is analyzed.

We have selected the following terminology on the basis of enzyme-sub-

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strate reactions assigning functional names to each component:



Materials and Methods

Blood was obtained from rabbits, normal human volunteers, and an individual² known to be deficient in clotting factor XII (Hageman factor). The factor XII-deficient plasma failed to correct the clotting defect of other factor XII-deficient plasma, but did correct the defect in factor XI-deficient plasma (for assays of clotting activity, see below). The blood was collected through siliconized needles from the ear artery of rabbits or the anti-cubital vein of human beings directly into plastic tubes containing acid-citrate anticoagulant (ACD). Generally, 1 liter of rabbit blood or 500 ml of human blood were collected at one time. The formed elements were centrifuged away at 3500 rpm for 40 min and the plasma fractionated with saturated neutral ammonium sulfate at 4°C. The globulin fraction precipitated at 55% saturation of ammonium sulfate and soluble at 25% was obtained and dialyzed against 0.01 M sodium phosphate buffer at pH 8.0 containing sodium ethylenediaminetetraacetate (EDTA) 0.001 M and hexadimethrine bromide (H Br, Aldrich Chemical Co., Inc., Milwaukee, Wis.) 50 µg/ml, before use. Plastic vessels were employed throughout.

Chromatography.—Diethylaminoethyl (DEAE)-Sephadex A-50 and carboxymethyl (CM)-Sephadex C-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) were prepared as directed in literature obtained from Pharmacia. The DEAE-Sephadex was hydrated and charged and then rinsed thoroughly in H Br, 50 µg/ml, and packed into a plastic column of 3.7 × 75 cm when 1 liter starting volume of plasma was used, or 3.1 × 62 cm when 500 ml was used. The gel was equilibrated with 0.01 M sodium phosphate buffer, pH 8.0 (starting buffer). The first chromatographic separation was performed using 1800 ml starting buffer in an open mixing Erlenmeyer flask and 1800 ml terminal buffer comprising starting buffer and sufficient NaCl to yield 0.5 M concentration in an open Erlenmeyer flask. EDTA 0.001 M was included in both buffers. The two flasks were connected by a siphon tube. Samples of protein were applied to the column and rinsed in with starting buffer before beginning the gradient of NaCl. The second chromatographic separation consisted of DEAE-Sephadex A-50, prepared

¹ Abbreviations used in this paper: ACD, acid-citrate anticoagulant; BAEe, benzoyl-L-arginine ethyl ester H Cl; BSA, bovine serum albumin; H Br, hexadimethrine bromide; HGG, human gamma globulin; OMTI, ovomucoid trypsin inhibitor; PKA, prekallikrein activator; Pre-PKA, precursor of the prekallikrein activator; TAME, tosyl L-arginine methyl ester; TBS, Tris containing NaCl, 0.15 M.

² We are indebted to Mrs. Gunda Hiatt for her generous contributions of blood.

and equilibrated as above in a plastic column of 2.6×35 cm. The same starting buffer (1800 ml) was employed, but 1800 ml 0.15 M NaH_2PO_4 containing 0.20 M NaCl and 0.001 M EDTA was used as terminal buffer. The third chromatographic separation was carried out on G-200 Sephadex, employing a column of 5×100 cm. The fourth separatory procedure involved CM-Sephadex C-50, packed in a plastic column of 2.6×35 cm. A buffer of citric acid and Na_2HPO_4 , pH 5.85, $\mu = 0.01$, was employed as starting buffer and a citric acid- Na_2HPO_4 buffer, pH 7.4, $\mu = 0.037$, containing 0.3 M NaCl , as terminal buffer. Pooled fractions, rich in Pre-PKA, were concentrated after the first two steps by addition of sufficient ammonium sulfate to yield 60% saturation. The concentrated protein was then dialyzed against the starting buffer of the next chromatographic procedure.

Electrophoresis.—Disc electrophoresis on polyacrylamide gel (7 and 12%) was conducted in gel cylinders of 70×6.5 mm or when larger recoveries were required, 110×9 mm according to the methods of Davis (3). Bromphenol blue and, when noted, bovine serum albumin (BSA)- ^{125}I were incorporated as markers. At the termination of electrophoresis, the gels were removed from the siliconized glass tubes and either stained in Coomassie blue in 20% trichloroacetic acid or sliced in 2 mm sections for elution in tris(hydroxymethyl)aminomethane buffer (Tris) containing 0.15 M NaCl , (TBS) for 24 hr.

Isoelectric Fractionation.—Performed with polyampholytes (LKB Instruments, Inc., Bromma, Sweden) as previously reported (4).

Ultracentrifugation.—Performed in linear gradients containing 5–20% sucrose in TBS. Gradients of sucrose were prepared in a Buchler Instruments, Inc. (Fort Lee, N. J.), apparatus. Centrifugation was carried out in a Beckman Spinco L-4 ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 65,000 rpm for 6 hr, or at 40,000 rpm 15 hr in an L-1 ultracentrifuge. Internal markers of human gamma globulin (HGG)- ^{131}I and BSA- ^{125}I were included in the sample when sedimentation values of unknown proteins were calculated.

Activation of the Precursor of Prekallikrein Activator (Pre-PKA, Hageman Factor, Factor XII).—The activation of Pre-PKA was performed in TBS buffer, pH 7.5. Pre-PKA was exposed to particles such as kaolin, diatomaceous earth, or to ellagic acid (K & K Laboratories Inc., Plainview, N. Y.). The particles were all previously washed in TBS and suspended at 5 mg/ml. 500 μg of kaolin was generally employed to activate preparations of Pre-PKA. The particles were shaken together with Pre-PKA at room temperature for 20 min. This was found in preliminary observations to be optimal in time and temperature for binding all detectable Pre-PKA over the ordinary range of Pre-PKA concentration. When plasma kallikrein inhibitor was known to be present in the Pre-PKA preparation (generally until the second separation on DEAE-Sephadex A-50 was performed), the incubated preparations were centrifuged. The supernatant (containing the inhibitor) was removed and the sedimented particles were then assayed for bound Pre-PKA which was now in its active form, PKA, and for clot-promoting activity as outlined below.

Trypsin was also used to activate Pre-PKA. 5 $\mu\text{g}/\text{ml}$ trypsin was incubated with Pre-PKA in TBS, pH 7.5, for 20 min at 37°C . To inhibit the trypsin, but not subsequent components of the kinin-forming system, ovomucoid trypsin inhibitor (OMTI, Worthington Biochemical Corp., Freehold, N. J.), 50 $\mu\text{g}/\mu\text{g}$ trypsin, was added. Completeness of the activation of Pre-PKA was assessed by testing for any remaining Pre-PKA which would bind to kaolin, since in preliminary assays it was found that although Pre-PKA bound completely to kaolin, PKA would not.

Preparation of Prekallikrein.—Briefly described (4, 5) and will be given in detail in a separate publication.³ Unless specified, rabbit or human prekallikrein was employed after chromatography on DEAE-Sephadex A-50 twice.

³ Wuepper, K. D., and C. G. Cochrane. 1971. Plasma prekallikrein: isolation, characterization, and mechanism of activation. Submitted for publication.

Preparation of Kininogen.—Performed as noted previously (6), by chromatography twice on DEAE-Sephadex A-50, followed by CM-Sephadex C-50 and gel filtration chromatography on Sephadex G-200.

Assays of the Activated Products of Pre-PKA (Hageman Factor).—Activated Pre-PKA, either bound to kaolin or in solution after treatment with trypsin, was assayed by its ability (a) to activate isolated prekallikrein, or (b) to promote clotting of factor XII-deficient human plasma.

(a) After activation of Pre-PKA in the kaolin or trypsin, the product, PKA, was measured by adding prekallikrein. The mixture incubated at 37°C for 40 min. This converted prekallikrein to its active enzyme form which was measured by its ability to cleave kinin from isolated kininogen or to hydrolyze 1 mM benzoyl-L-arginine ethyl ester HCl (BAEe, Mann Research Labs Inc., New York) as described previously.³ The hydrolysis of BAEe was measured by increasing absorption of the liberated benzoyl arginine at 253 nm.

Quantitation of PKA activity was performed in duplicate with dilutions of PKA. TBS was used as diluent. PKA (0.1 ml) was added to an excess of prekallikrein (100 milliunits in 0.1 ml). After an incubation period of 30 min, 37°C, 3.0 ml BAEe was added at 37°C. After 20 min, the tubes were rapidly cooled and the OD at 253 nm recorded. If necessary, the tubes could be brought back to 37°C and incubated for another interval of 20 min. Preliminary assays showed that the concentration of prekallikrein was sufficient to yield a linear plot of activation by PKA over a range of PKA concentration. The hydrolysis of BAEe by kallikrein was found to be linear over a range of approximately 0.075–0.300 OD units. A milliunit of PKA activity was defined as that amount in 0.1 ml that activated enough prekallikrein to yield an increase in OD at 253 nm of 0.001/min under the conditions noted above.

The release of kinin from kininogen was assayed in a 5 ml muscle bath in Tyrode's-glucose solution at 37°C, using estrus rat uterus. Quantitative values were obtained by comparing the amplitude of contraction of the experimental samples with those produced by known quantities of synthetic bradykinin (Sandox, Inc., Hanover, N. J.).

(b) Clotting assays were also performed after activation of Pre-PKA with kaolin. These tests were carried out in plastic tubes, except where noted, and using factor XII-deficient human plasma. 100 µg kaolin (in 0.1 ml) that had been exposed to Pre-PKA were mixed with rabbit brain cephalin (0.05 ml of 1/50 dilution) obtained as a gift from Dr. Cecil Hougie, and 0.05 ml factor XII-deficient plasma in ACD. After incubation at 37°C for 8 min, 0.05 ml 0.05 M CaCl₂ was added and a timer started. The tubes were tilted until the formation of a firm clot was noted. The elapsed time was recorded. 100 µg untreated kaolin was used as control. Human plasmas deficient in clotting factors XII, XI, and IX were generously contributed by S. I. Rapaport.

Quantitative clotting assays were performed using dilutions of the clot-promoting material in TBS. 0.1 ml of the dilutions were added to cephalin and 0.05 ml factor XII-deficient or normal plasma as outlined above. A log-log plot was then drawn of the clotting time vs. the dilution used. 1 unit of clot-promoting activity was defined as that quantity in 0.1 ml yielding a decrease of 25% of control values.

Ultrafiltration.—Performed in an Amicon cell using UM-10 membranes (Amicon Corp., Lexington, Mass.). The cell and membrane were rinsed with H Br, 50 µg/ml, before use and the functional capacity of the membrane tested with cytochrome C.

Radioisotopes.—Labeling of proteins with ¹²⁵I or ¹³¹I was performed according to the technique of McConahey and Dixon (7).

RESULTS

Isolation of the First Component of the Kinin-Forming System. (Precursor of the Prekallikrein Activator, Pre-PKA, Hageman Factor, Clotting Factor XII).—Globulins of human and rabbit plasma were obtained and treated as noted in

Materials and Methods. Analyses revealed the bulk of Pre-PKA activity to be in the protein fraction soluble at 25% and precipitable at 55% saturation of ammonium sulfate.

The elution of the globulin from the first column of DEAE-Sephadex A-50 is shown in Fig. 1. Pre-PKA in aliquots of eluted fractions were activated with kaolin and, as noted in the figure, eluted when the ionic strength reached 0.13–0.15. No activity was detected when kaolin was omitted from the test system, or when the kaolin was pretreated with H Br (50 μ g/500 μ g kaolin), indicating that the molecule was in precursor form. The Pre-PKA was pooled and concentrated by precipitation with ammonium sulfate at 60% saturation

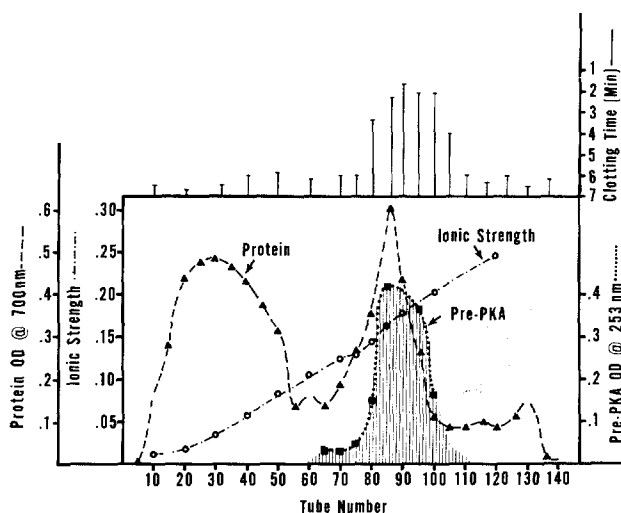


FIG. 1. Chromatographic separation of human plasma globulins on DEAE-Sephadex A-50. The pH was maintained at 8.0 and a gradient of NaCl was used to elute the proteins. The tubes containing Pre-PKA are marked by cross-hatching. Prekallikrein eluted with the initial peak of protein.

and applied to a second column of DEAE-Sephadex A-50. The elution of Pre-PKA, shown in Fig. 2, indicated that Pre-PKA separated from the bulk of contaminating proteins by this second elution from the anionic exchange medium. Pre-PKA-containing fractions were then pooled, concentrated as before by precipitation with ammonium sulfate, and applied to a column of G-200 Sephadex. A typical separation is shown in Fig. 3. Analysis of eluted fractions in 7% polyacrylamide gel electrophoresis revealed that for both rabbit and human Pre-PKA, several anodally migrating contaminants were removed by this procedure. The contaminants eluted both before and after the bulk of Pre-PKA. There remained three contaminants. These were removed by chromatography on CM-Sephadex C-50.

The elution of Pre-PKA from CM-Sephadex C-50 is shown in Fig. 4. Pre-PKA eluted when the effluent reached pH 6.9 and the ionic strength 0.20. By analysis on polyacrylamide gel electrophoresis, the Pre-PKA was found to be separated from the cathodal and anodal contaminants, leaving a single protein

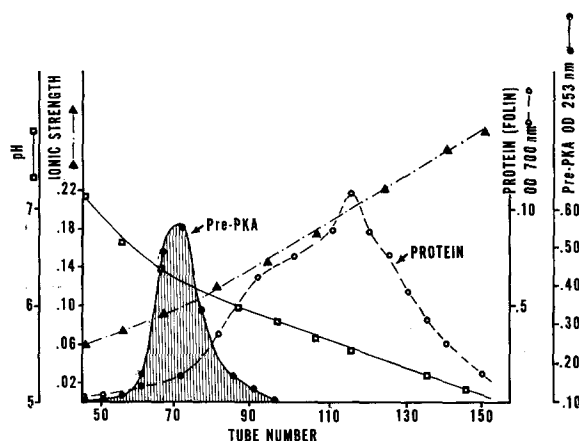


FIG. 2. Chromatographic separation on DEAE-Sephadex A-50 of fractions containing Pre-PKA. The fractions were obtained from the first DEAE-Sephadex column as noted in Fig. 1. Both a gradient of NaCl and NaH_2PO_4 were employed to elute the proteins.

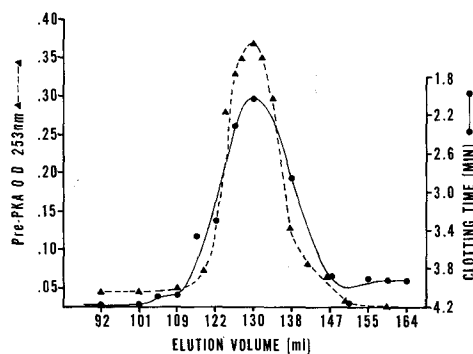


FIG. 3. Gel filtration chromatography of fractions rich in Pre-PKA obtained from the second separation on DEAE-Sephadex A-50 as noted in Fig. 2. By selecting fractions from the central portion of the peak, contaminants migrating in the anodal portion of an analytical polyacrylamide gel were eliminated.

band (Fig. 5). Separate electrophoretic preparations were cut and eluted and, upon activation with trypsin, revealed Pre-PKA activity associated with the band. This was found in both human and rabbit Pre-PKA.

The Pre-PKA so purified, upon treatment with kaolin, was found capable

of activating prekallikrein leading to release of kinin from kininogen. Pre-PKA without treatment with kaolin was ineffective and addition of prekallikrein was essential for the release of kinin. Quantitative data on the release of kinin will be published separately.⁸ The yield of Pre-PKA was found to be 15% of the starting quantity.

At this point, the Pre-PKA molecules became unstable and at times acti-

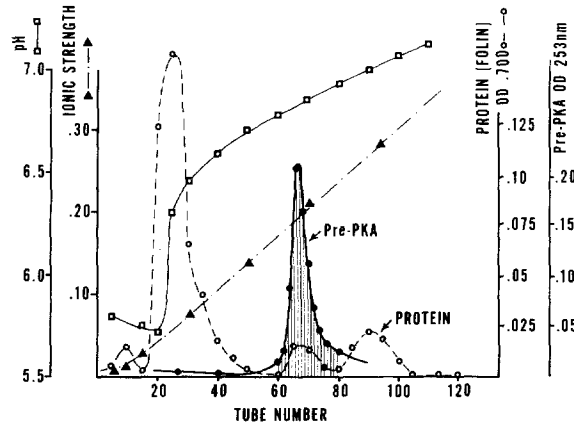


FIG. 4. Cation exchange chromatography of CM-Sephadex C-50 of Pre-PKA rich proteins. The proteins were obtained from gel filtration isolation of Pre-PKA as noted in Fig. 3.

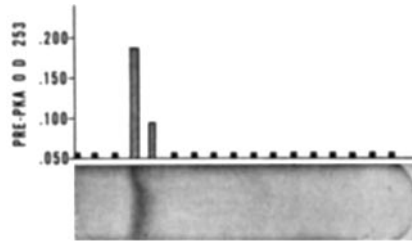


FIG. 5. Analysis of human Pre-PKA (Hageman factor) on analytical polyacrylamide gel. The gel below was stained with 0.05% Coomassie blue, while a similar gel was sliced and the proteins eluted. An assay for the presence of Pre-PKA is shown.

vated spontaneously. This was most prevalent with human Pre-PKA. Concentration by ultrafiltration, negative pressure through dialysis tubing, or lyophilization were all found to promote activation of unstable preparations. Nevertheless, by conducting separations rapidly, it was often possible to achieve concentration by ultrafiltration, retaining the bulk of Pre-PKA in precursor form.

Clot-Promoting Factor Associated with Pre-PKA; Its Relationship to Clotting

Factor XII (Hageman Factor).—The first component of the kinin-forming system is purportedly associated with clot-promoting activity. In order to examine the possibility that Pre-PKA possessed potential clot-promoting activity, the eluates of each separatory column were assayed for clot-promoting ability with and without treatment with kaolin. Upon addition of kaolin, clot-promoting activity was detected (using factor XII-deficient plasma for clotting assays) in areas corresponding to the presence of Pre-PKA (Fig. 1)

TABLE I
Specificity of Clotting Activity of the First Component of the Kinin-Forming System (Pre-PKA)

Pre-PKA*	Clotting plasma	Clotting time
<i>ml</i>		<i>min</i>
0.01	Factor XII-deficient	2.83
0.025		2.58
—		19.50
0.01	Factor XI-deficient	5.39
0.025		5.28
—		5.45
0.01	Factor IX-deficient	19.50
0.025		19.70
—		19.70
0.01	Fibrinogen 0.25%	>38
0.025		>38
—		>38
Thrombin IU		1.20

* Human Pre-PKA activated with kaolin, 25 μ g, 20 min at 22°C. Pre-PKA-kaolin mixture then added to deficient plasma, 0.05 ml, and cephalin, 0.05 ml. Incubation with factors XII- and IX-deficient plasma carried out for 8 min at 22°C, and with factor XI-deficient plasma 1 min before adding 0.05 ml CaCl₂ (0.05 M). Interval between addition of CaCl₂ and the development of a firm clot was recorded. Factor XI-deficient plasma clotted in 2.2 min upon addition of partly purified human factor XI.

This was noted for both human and rabbit Pre-PKA. A similar association was found through each step in the isolation procedure. Activity was not found when kaolin was omitted. Purified human Pre-PKA was then activated with kaolin and assayed for its capacity to promote clotting of human plasmas genetically deficient in factors XI and IX as well as purified human fibrinogen. The results are given in Table I. They indicate that activated Pre-PKA promoted clotting of plasma deficient in factor XII, but not XI or IX or fibrinogen. Rabbit Pre-PKA behaved similarly.

Further evidence indicating a relationship of Pre-PKA and factor XII de-

rived from the finding that Pre-PKA activity was absent in factor XII-deficient plasma obtained from two different individuals. Globulins soluble at 25% saturation of ammonium sulfate but precipitable at 50% were prepared from human plasma deficient in factor XII or factor XI. These were then sedimented in sucrose density gradients, and activation of Pre-PKA attempted with trypsin. Pre-PKA activity was readily detected in the sedimented globulins of factor XI- but not factor XII-deficient plasma (Fig. 6). In these tests, the samples used were deliberately excessive in quantity in order to allow detection of even small quantities of Pre-PKA. The apparent rapid sedimentation of Pre-PKA in the factor XI-deficient plasma is probably explained, as noted below, by the presence of kallikrein inhibitors in the 4-4.5S region. Similarly, no Pre-PKA was detected in factor XII-deficient plasma chromatographed on DEAE-Sephadex A-50.

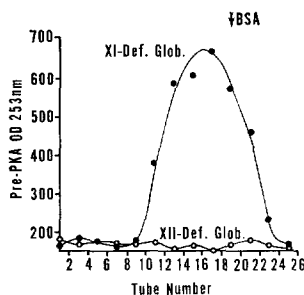


FIG. 6. Assay of Pre-PKA activity in human plasma globulins deficient in clotting factors XII and XI. Globulins were obtained by precipitation from plasma with ammonium sulfate, and sedimented in a sucrose gradient, 40,000 rpm, 16 hr. Pre-PKA was detected in globulins of Factor XI-but not factor XII-deficient plasma.

The data suggest an identity of Pre-PKA and Hageman factor. Physical characterization of the two activities was then performed to assess this further.

Physical Characterization of Pre-PKA and Clot-Promoting (Hageman Factor) Activities.—Assays of physical characteristics were performed by gel filtration, isoelectric fractionation on polyampholytes, and sedimentation in density gradients of sucrose. The results are shown in Figs. 7-9. As indicated, the eluting patterns of Hageman factor activities in each case were superimposed revealing identical properties. When the volume of elution of Pre-PKA and factor XII activities from G-200 Sephadex (Fig. 7) were plotted as a function of the log molecular weight or diffusion coefficient (8), a molecular weight of 110,000-120,000 and a diffusion coefficient of 4.25×10^{-7} cm²/sec could be assigned to each. Sedimentation in sucrose density gradients indicated a sedimentation rate of 4.5-4.6S (Fig. 8). The values were true of both rabbit and human proteins. Isoelectric fractionation revealed an

isoelectric point of 6.1S for rabbit Pre-PKA (Fig. 9). Pre-PKA and Factor XII activities could, therefore, not be separated by these techniques. Activation of both Pre-PKA and Factor XII was achieved with either kaolin or trypsin.

It should be noted that when sedimentation in sucrose gradients was performed with whole plasma or globulins, the measured sedimentation rate of Pre-PKA was 6–7S. In experiments designed to discern the reason for this rapid sedimentation rate, an inhibitor(s) of kallikrein was (were) found in the

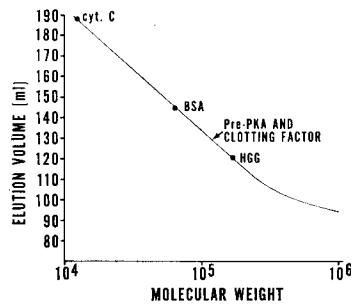


FIG. 7. Gel filtration of human Pre-PKA (Hageman factor) through G-200 Sephadex. The volume of elution was plotted as a function of the log molecular weight. The column was calibrated with markers as shown.

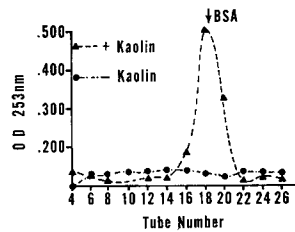


FIG. 8. Sedimentation of human Pre-PKA (Hageman factor) in a gradient of sucrose, 5–20%. Sedimentation was conducted at 50,000 rpm for 6 hr.

crude plasma or globulins that sediments in the 4–4.5S region. This inhibitor prevented detection of Pre-PKA in that region leaving measurable activity only in the more rapidly sedimenting zones. Recentrifugation of the rapidly sedimenting Pre-PKA revealed a 4.5S rate of sedimentation. The inhibitor(s) was (were) found to block the activity of kallikrein, trypsin, and thrombin.

The possibility was considered that the superimposable elution profiles of Pre-PKA and factor XII could be explained on the basis that the clot-promoting factor was first activated by treatment with kaolin or trypsin, and this then activated the Pre-PKA. The Pre-PKA activity would, therefore, correspond precisely to the position of clot-promoting factor. To test this possibil-

ity, definite quantities of protein from each shoulder of the elution profile of the column of G-200 Sephadex, shown in Fig. 3, were mixed with protein from the center of the peak or from either shoulder region. Activation was then obtained with kaolin and the quantity of Pre-PKA was measured. The values were expressed in units of PKA activity. These values were compared with those expected from the arithmetical sums of the units of each when combined. Presumably, if the clotting factor was required to activate the Pre-PKA, a marked excess of measured activity over that expected would result. As shown in Table II, the measured and expected values were comparable. This indicated that the two activities eluted from the gel filtration column together.

Since the evidence accumulated strongly indicates that the precursor mole-

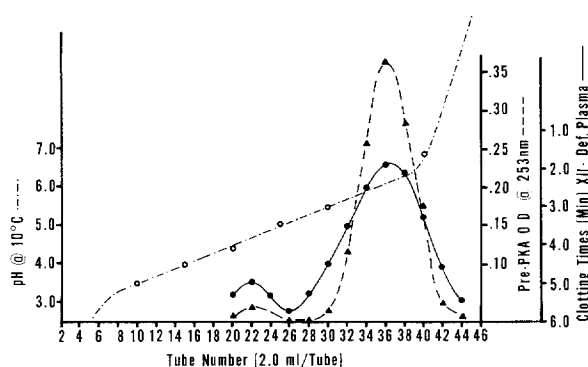


FIG. 9. Isoelectric fractionation of rabbit Pre-PKA in polyampholytes. Separation was performed over a 30 hr period.

cule of the prekallikrein activator is Hageman factor (clotting factor XII), the more commonly used term Hageman factor will be employed hereafter.

Molecular Changes Associated with the Activation of Hageman Factor (Pre-PKA).—In previous studies in this laboratory (9), diatomaceous earth was used to bind and activate Hageman factor from whole human or rabbit plasma. The two activities were then eluted, after washing of the particles, with 2 M NaCl; however, exposure of isolated Hageman factor to kaolin or diatomaceous earth yielded activation of the PKA and clotting activities, but the active moieties were so firmly bound to the particles as to resist elution with 2 M NaCl or mild acid conditions. Attention was turned, therefore, to other methods of activating the first component: (a) activating Hageman factor by treatment with trypsin, and (b) allowing spontaneous activation to occur.

(a) *Activation by treatment with trypsin:* 50 ml of human or rabbit Hageman factor was obtained from the peak tubes of activity of a column of CM-Sepha-

dex, i.e., from the fourth step of purification. The protein was concentrated approximately tenfold by ultrafiltration. One-half of the protein was then treated with trypsin as noted in Materials and Methods, and the other half was kept for analysis of Hageman factor. 2 ml of the trypsin-treated portion was reacted with OMTI for assays of PKA activity, while the remainder was examined for migration of the split products of Hageman factor in polyacrylamide gel electrophoresis (OMTI contains proteins that migrate in the anodal region of polyacrylamide gels and it was therefore omitted). Even though abundant PKA activity was present after treatment with trypsin, it would not bind to kaolin as evidenced by the lack of hydrolysis of prekallikrein and its inability to promote clotting of factor XII-deficient plasma after washing of the kaolin in TBS. All PKA activity resided in the supernatant. This indicated that trypsin activated the Hageman factor completely. The trypsin-

TABLE II
Comparison of the Measured and Expected Quantity of Pre-PKA Obtained by Combining Various Fractions Eluting from G-200 Sephadex

Tube numbers combined*	PKA expected	PKA measured
	<i>milliunits</i>	<i>milliunits</i>
147 + 155	82	73
147 + 162	115	105
155 + 162	93.5	100

* The gel filtration column is that shown in Fig. 3. The tube numbers correspond to the following elution volumes: tube 147, 122 ml; tube 155, 130 ml; tube 162, 145 ml. Volumes used in the assay: tube 147, 0.1 ml; tube 155, 0.01 ml; tube 162, 0.2 ml.

treated Hageman factor, after addition of OMTI, was examined in three ways for the presence of split products: 1 ml was fractionated by gel filtration on a column of G-200 Sephadex; 0.2 ml was assayed by ultracentrifugation in a gradient of sucrose; and 0.1 ml was tested in duplicate in polyacrylamide gel electrophoresis. The fractions were assayed for PKA and clotting activities.

The results of gel filtration are shown in Fig. 10. Both activities were found in the same fractions yielding superimposable patterns of elution. The volume of elution of these activities was plotted as a function of log molecular weight, proteins of known molecular size having been used to calibrate the column. PKA and clot-promoting activities eluted at a molecular weight of 30,000–32,000. Ultracentrifugation of the trypsinized Hageman factor revealed that the active PKA and clot-promoting fractions sedimented together at 2.6S. The patterns of sedimentation and gel filtration of PKA were identical to those previously reported from this laboratory (4, 9, 10).

Electrophoresis in polyacrylamide gels showed that the Hageman factor had been cleaved so that a protein band no longer appeared in the cathodal

section of the gel. Instead, four new and fainter bands appeared in the anodal region between BSA- ^{125}I and bromphenol blue tracking dye (Fig. 11). Another band was apparent in the midportion of the gel. Not infrequently, only three anodal protein bands appeared. Elution of a gel that was processed in parallel and cut into 1.5 mm sections showed both PKA and clot-promoting activities in the far anodal region. The results using human and rabbit Hageman factor were similar.

(b) *Spontaneous activation of Hageman factor:* It was essential to know if the activation of Hageman factor isolated from human or rabbit plasma could be achieved by means other than trypsinization since fragments obtained by this method may not be similar to those achieved by more physiologic activation. As noted previously, Hageman factor activated spontaneously at times during

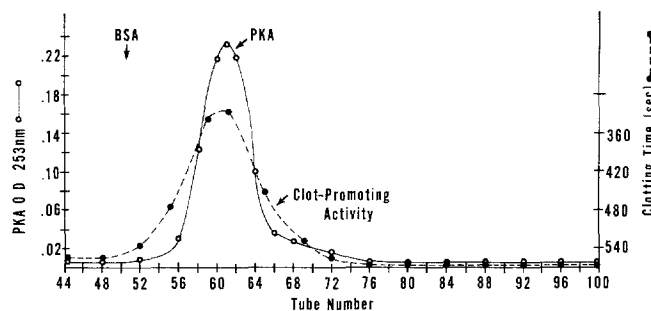


FIG. 10. Gel filtration of fragments of activated rabbit Hageman factor (Pre-PKA) on G-200 Sephadex. The elution of fractions containing PKA and clot-promoting activity are shown eluting after BSA marker. The molecular weight of the two activities was noted to be 32,000 when the elution volume was plotted as a function of the log molecular weight.

concentration by ultrafiltration after chromatography on CM-Sephadex. Accordingly, 50 ml of human and rabbit Hageman factor obtained from CM-Sephadex were allowed to activate spontaneously at 4°C. The three assays performed in the previous section were also employed, i.e. gel filtration, ultracentrifugation, and electrophoresis in polyacrylamide gel. The results were quite similar to those noted in the previous section. PKA and clot-promoting activities eluted together in the 30,000 molecular region in chromatography on Sephadex G-200, and both sedimented in the 2.6S region. Two or three anodal bands in the prealbumin region were generally found by acrylamide gel electrophoresis, one of them coincident with PKA and clot-promoting activity. Activation was never complete, however, and a small amount of Hageman factor was generally found in the appropriate fractions (i.e., as defined earlier in this article).

Quantitative Changes in Clot-Promoting Activity Occurring during Activation

of *Hageman Factor*.—In previous studies (4, 19), we have reported that the activation product of Hageman factor, i.e. PKA, possessed little detectable clot-promoting activity in the face of abundant PKA activity. The PKA had been obtained by exposure of chelated plasma to diatomaceous earth followed by elution of PKA with 2 M NaCl. The parent molecule on the other hand was rich in clot-promoting activity when activated by kaolin. To examine this question further, rabbit Hageman factor obtained from the fourth step of the purification process, i.e. from CM-Sephadex, was activated with kaolin. The clotting and PKA capacities of the activated Hageman factor were compared with the same properties of purified PKA. The PKA was obtained from spontaneously activated Hageman factor, the PKA being purified by disc electrophoresis in polyacrylamide. When compared on the basis of equal quantities of prekallikrein activating capacity (measured in milliunits), the clot-pro-

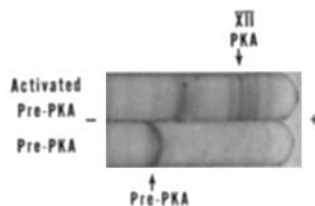


FIG. 11. Analytic polyacrylamide gel electrophoresis of activated human Hageman factor (Pre-PKA) (upper gel). Comparison is made with unactivated Hageman factor (lower gel). The positions of PKA and clotting factor are noted in the upper gel corresponding to a band of protein. These activities were obtained from a duplicate gel carried in parallel, sliced, and eluted. While four prealbumin bands are shown, more frequently only two or three were observed.

moting activity of the kaolin-treated Hageman factor was found to be about 50-fold greater than that of the fully cleaved product, PKA.

DISCUSSION

Studies are presented on the isolation, characterization, and activation of the precursor form of the first component of the kinin-forming system in rabbit and human plasma. Functionally, this molecule is the precursor of the activator of prekallikrein (Pre-PKA) which, as the evidence has shown, is identical to the initial component of the intrinsic clotting system, Hageman factor (factor XII). The protein was found to be a molecule of 110,000–120,000 mol wt as determined by gel filtration, to sediment at 4.5–4.6S, and to possess electrophoretic charge characteristics of β -globulin with an isoelectric point of 6.1. The discrepancy between sedimentation velocity and gel filtration data may be explained on the basis of molecular asymmetry and/or carbohydrate content. Present information does not allow for a distinction between these

possibilities. Isolation of this component was made possible by preventing contact of the plasma and subsequent fractions with anything but plastic vessels, by pretreating all surfaces and separatory media (except cation exchange gels) with H Br, and by reducing the time of preparation to a minimum. Of considerable interest is the finding that human and rabbit Hageman factor bind firmly to an anion exchange medium (DEAE-Sephadex A-50) and to a cation exchange medium (CM-Sephadex C-50) as well. Presumably, the molecule possesses strong charge densities, both positive and negative, and advantage was taken of this property in the isolation procedure.

Pre-PKA and Clotting Factor XII.—Evidence strongly suggested an identity of Pre-PKA and clotting factor XII (Hageman factor). The highly purified Pre-PKA was found upon activation to promote clotting of factor XII—but not Factor XI— or Factor IX—deficient human plasma or fibrinogen. Pre-PKA bound readily and completely to kaolin, ellagic acid, or diatomaceous earth in 0.01 M EDTA and acquired the clot-promoting activity in the process. Of particular note, Pre-PKA was not detectable in plasma of two individuals genetically deficient in factor XII. Pre-PKA and clot-promoting activities eluted identically from anion and cation exchange media and were found to have an identical isoelectric point (6.1). The volume of elution in gel filtration (= mol wt 110,000–120,000) and the sedimentation rates (4.5–4.6S) were the same.

The dual nomenclature appears redundant in the face of such evidence, and even though immunologic techniques have not yet been employed to identify each form of the molecule, one is justified in equating the two. The commonly used name, Hageman factor, has therefore been applied.

A link between the first component of the kinin-forming system and factor XII has been known for years. Margolis and Bishop (11–14) and Webster and Ratnoff (15) demonstrated that plasma from individuals genetically deficient in factor XII (Hageman factor) would not generate kinins upon exposure to glass. In addition, Kaplan and Austen (16, 17) have demonstrated Factor XII activity associated with the activator of prekallikrein isolated by electrophoresis in polyacrylamide gels.

Activation of Hageman Factor.—The process of activation of the molecule was associated with fragmentation into several daughter molecules. Activation was achieved by treatment with trypsin, exposure to kaolin, glass, diatomaceous earth, or by allowing Hageman factor in highly purified form to activate spontaneously at 4°C. After fragmentation, two active properties were acquired: (a) the capacity to convert prekallikrein (prokininogenase) to its enzymatically active state (PKA activity), and (b) the capacity to promote clotting of factor XII—but not Factor XI— or Factor IX—deficient plasma. These two activities could not be separated by disc gel electrophoresis, gel filtration chromatography, or by ultracentrifugation in a gradient of sucrose.

Thus, their physical characteristics were identical: 32,000 mol wt, 2.6S sedimentation velocity, and a prealbumin electrophoretic mobility in acrylamide gels.

It was of interest that exposure of Hageman factor to surfaces such as that provided by kaolin generated immense clot-promoting activity relative to that of the PKA fragment. A comparison of the quantity of clot-promoting capacity of Hageman factor activated by kaolin and of the fragment PKA revealed the former to possess more than 50-fold greater clotting capacity than the latter. In support of this finding is the observation (unpublished) that enzymatic cleavage of Hageman factor to PKA in solution generates full PKA activity but very little clot-promoting activity. It would appear that the capacity to promote clotting is diminished when the PKA fragment is generated. This may be explained by a loss of functional activity in clotting on the PKA fragment, or by inhibition of the majority of clot-promoting sites associated with a different fragment.

The description and physical parameters of PKA have been reported previously from this laboratory (4, 9, 10). The association of clot-promoting activity with PKA was observed first by Kaplan and Austen (16). Movat et al. (18-20) have also reported the presence of a prekallikrein activator in the prealbumin region of acrylamide gels after electrophoresis. We have recently described the enzymatic nature of PKA (21) which will be dealt with in a separate article.³ The possibility that the other anodal fragments possess biologic activity is of great interest.

While the sedimentation rate of Hageman factor in the present studies is close to that (4.5-5.5S) reported by Donaldson and Ratnoff (22), the sedimentation of active Hageman factor is different. Using ellagic acid to activate Hageman factor, these authors found a rapid sedimentation rate, the activated molecule reaching the bottom of the tube of a sucrose gradient of 10-40% in less than 100 min at 100,000 *g*. The rapid sedimentation was noted even when the preparation was centrifuged in 5% bovine albumin, 0.5 M NaCl, or 5 M urea. The reason for the discrepancy is not clear, but may be related to the different methods of activation of Hageman factor. Schoenmakers et al. (23) and Haanen et al. (24) have reported a sedimentation rate of 7.1 for activated bovine Hageman factor. The Hageman factor was prepared by eluting the active material from glass, followed by further purification. In acrylamide gels, the clot-promoting activity moved slowly toward the anode as opposed to the prealbumin migration of the Hageman factor fragment reported in the present study. The bovine clotting factor (23, 24) was also associated with BAEe esterase activity which was not true of activated human and rabbit Hageman factor in the present studies. The bovine protein activated prekallikrein (25). This, together with its BAEe and TAME hydrolytic properties, indicated that the Hageman factor was already active. Its activity was inhibited by lima

bean trypsin inhibitor. Thus, the physical properties and the data on inhibition of the bovine protein are markedly different from those of the human and rabbit active product, PKA. We have also noted that a clot-promoting factor could be obtained by elution from diatomaceous earth that was exposed to whole plasma (26). After purification, this factor possessed BAEe hydrolytic activity, sedimented at about 7S, and migrated in acrylamide gel at the same position as that of Schoenmakers et al. (23, 24). These two clot-promoting substances share many features, including method of preparation, the only difference being that the 7S protein in our hands would clot factor XI- as well as factor XII-deficient plasma. This has also been noted by Movat et al. (20). Of particular interest, the clot-promoting fractions eluted from diatomaceous earth as noted above were found by us to activate Hageman factor, releasing from it PKA (26). It will be interesting to find whether this Hageman factor activator in plasma is related to plasmin, found recently by Kaplan and Austen (17) and by Burrowes (27) to generate PKA from its precursor.

SUMMARY

The isolation and characterization of the first component of the kinin-forming system in human and rabbit plasma are presented. Functionally, the molecule is the precursor of the activator of prekallikrein (Pre-PKA) and evidence is presented that it is identical with Hageman factor (clotting factor XII). The component from each plasma possessed similar characteristics. This molecule was found to have a mol wt of 110,000 and sedimentation rate of 4.6S. It migrated in electrophoresis as a β -globulin, having an isoelectric point of 6.1. Upon activation with glass, kaolin, diatomaceous earth, ellagic acid, or trypsin, the activated molecule converted purified prekallikrein (prokininogenase) to the active enzyme.

Clot-promoting activity was associated with the capacity to activate prekallikrein through each procedure of isolation. The clot-promoting factor was in precursor form, requiring treatment with kaolin or trypsin to gain activity. Evidence indicated that the protein was Hageman factor (factor XII): it promoted clotting of factor XII-deficient, but not Factor XI- or IX-deficient plasma, and did not convert fibrinogen to fibrin. It bound to and was activated by kaolin or other negatively charged particles in the presence of chelating agents; the activation by kaolin could be prevented by pretreating the kaolin with hexadimethrine bromide (H Br); prekallikrein-activating and clot-promoting activities were identical in their physical properties; and the prekallikrein activator could not be detected in Hageman factor-deficient plasma.

Activation of Hageman factor was accompanied by cleavage of the molecule into several fragments, one of which possessed prekallikrein-activating (PKA) and clot-promoting properties. The PKA fragment sedimented at 2.6S and by gel filtration was found to have a molecular weight of 32,000. The

PKA possessed only $\frac{1}{50}$ the clot-promoting capacity of the freshly activated native molecule.

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