The Kinin System of Human Plasma
V. The Probable Derivation of Prekallikrein Activator from Activated Hageman Factor (XIIa)[1] (36026)

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In the preceding publications of this series, we have shown that a small molecular weight anionic substance acts as prekallikrein activator (PKA) (1), that PKA can be obtained from Celite eluates of whole plasma (2), but that a shunt-like pathway may operate in the activation of prekallikrein, since the latter can be activated to kallikrein in factor XII-deficient plasma (3). We have furthermore presented some evidence that PKA may derive from activated Hageman factor (factor XIIa), but these studies were mitigated by the presence of thrombin in almost all the fractions, which interfered with clotting studies (4).

In present study, a functionally pure factor XIIa was obtained and, as shown below, some of it seemed to convert to PKA by treatment with trypsin or by adsorption onto Celite.

Materials and Methods. Collection and preparation of plasma. Blood was collected from healthy (25–45 year old) donors into plastic syringes containing 4% trisodium citrate (pH 7.4). The ratio of blood to citrate was 9:1. The plasma was separated by centrifugation at 3000*g* for 30 min, followed by recentrifugation at the same speed for an additional 30 min, both at 4°. Plastic containers or siliconized glassware was used throughout these experiments.

Ion exchange chromatography and gel filtration. These procedures were done essentially as described in detail before (1). The plasma was dialyzed against 0.08 *M* Tris HCl (pH 8.0, conductivity 4.5 mmho/cm) containing 0.001 *M* ethyldiamine-tetraacetate (EDTA). The QAE-Sephadex (Pharmacia Fine Chemicals, Montreal, Quebec) columns were equilibrated with the above buffer. In a pilot study, 30.0 ml of plasma was fractionated on a 2.5 × 18 cm column. In the main experiment, 225.0 ml of plasma was fractionated on a 5 × 38 cm (K50/50, Pharmacia) column. The total 280 mμ OD of the applied sample was 13,000. Fractions were collected at the rate of 60 ml/hr (10 ml/tube). After washing with the initial buffer, the adsorbed protein was eluted stepwise by increasing the NaCl.

Aliquots of the fractions were concentrated by lyophilization and dialyzed against 0.2 *M* Veronal buffered isotonic saline (VBIS, pH 7.35) for clotting assays or against 0.2 *M* phosphate buffered saline (PBS, pH 7.4) to test esterase and vascular permeability-enhancing activity. The excluded fractions were examined also for the presence of prekallikrein.

Fractions obtained after the excluded peak that corrected the clotting defect of congenitally factor XII-deficient plasma, were pooled, concentrated by freeze-drying and applied to three interconnected columns of Sephadex G-200 fine (5 × 93, 5 × 89, 5 × 86 cm) equilibrated with 0.1 *M* NaCl in 0.1 *M* Tris HCl (pH 8.0). Flow rate was 25.0 ml/hr (10.0 ml/tube). The sample applied to the Sephadex G-200 was 23.5 ml (OD 280 mμ: 13.4/ml).

Isoelectric focusing was carried out as described before (1), using a pH range of 3–10. The volume of the applied sample was 4.0 ml.

Polyacrylamide electrophoresis was based on the method of Davis (5), using a 7% running gel (pH 8.9) and a 2.5% stacking gel (pH 6.7). Samples were run for 2 hr at

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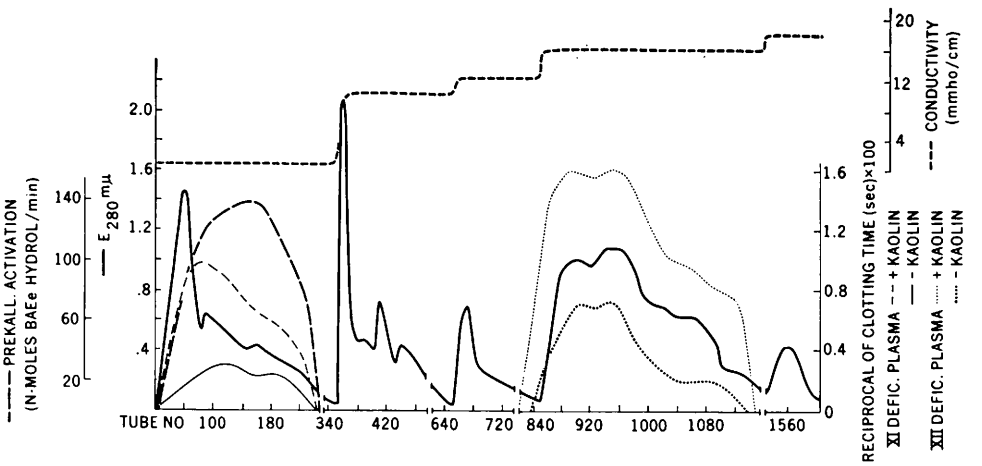

FIG. 1. QAE-Sephadex A-50 chromatography of Al $(OH)_3$ adsorbed human plasma as described under Materials and Methods: Prekallikrein and factor XI-activity eluted in the excluded peak and factor XII activity in the peak eluting with 0.14 *M* NaCl (conductivity 16.0 mmho/cm). Most of factor XI and factor XII was present in unactivated form and could be activated with kaolin.

100 V and 4–2.5 mA/tube. The chamber buffer was Tris–glycine, pH 8.3. The gels were stained with 1% amido black in 7% acetic acid. For preparative work, the gels were cut at 0.5 cm intervals, suspended in 1.0 *M* NaCl, squeezed repeatedly through a plastic 2.5 ml syringe, and agitated at 4° for 24 hr. After centrifugation, the supernatant was removed and the homogenized gel again was washed repeatedly with 1.0 *M* NaCl. The pooled supernatants were dialyzed against VBIS or PBS.

Hydrolysis of benzoyl arginine ethyl ester (BAEe), kinin assays, and tests for enhanced vascular permeability were performed as described in the first publication of this series (1).

Clotting studies. Partial thromboplastin time, using either congenitally factor XII deficient plasma or factor XI-depleted plasma were done as described by Özge-Anwar *et al.* (2, 3). To ascertain whether clot-promoting activity of individual fractions was due to unactivated factor XII or activated factor XII, partial thromboplastin time was done in plastic tubes in the presence or absence of kaolin.

Results. In the above-mentioned pilot study, it was found that fractions correcting the clotting deficiency of plasma congentially deficient in factor XII, eluted with 0.1 *M* Tris HCl containing 0.12 *M* NaCl. This activity was found in the descending portion of a peak containing most of the albumin. Based on this observation, the second, larger column was run in steps, using 0.1 *M* Tris HCl, followed by this buffer containing increasing concentrations of NaCl (0.06, 0.09, 0.14, and

TABLE I. Effect of Trypsin or Kaolin on Partially Purified Factor XII.[a]

Sample injected	Enhancement of vascular permeability[b] (CPM/lesion)
Factor XII + buffer	0
Factor XII + kaolin	12,900
Buffer + kaolin	7900
Factor XII + trypsin, 30 sec	17,550
Factor XII + trypsin, 120 sec	25,400
Trypsin + LBTI	950

[a] One millimeter sample (crude factor XII; see Fig. 1) was incubated with 100 μg of trypsin for 30 or 120 sec, followed by inactivation of the trypsin with 120 μg of lima bean trypsin inhibitor (LBTI). With kaolin the sample was incubated for 8 min.

[b] A 0.1 ml dose was injected intradermally into guinea pigs, as described under Materials and Methods.

TABLE II. Effect of Crude Factor XII and Celite Eluates of It (PKA) on Prekallikrein and Factor XII-Deficient Plasma.[a]

Sample	Prekallikrein activation (nmoles BAEe hydr./min/ml)	Clotting XII-def. plasma (sec)	
		—Kaolin	+Kaolin
Buffer + prekallikrein	0	—	—
Factor XII + prekallikrein	31.8	—	—
Factor XII + buffer	29.3	340	68
PKA + prekallikrein	67.3	—	—
PKA + buffer	13.0	1020	545
Buffer	—	1200	990

[a] Partially purified factor XII (see Fig. 1) was incubated with Celite (150 mg/ml of factor XII). After washing with 0.001 *M* Tris HCl, the PKA was eluted from the Celite with 2.0 *M* NaCl in 0.1 *M* Tris HCl (pH 8.0) and concentrated to the original volume of the incubated factor XII. Prekallikrein and clotting assays were done as described in Materials and Methods.

0.16). Prekallikrein and factor XI eluted in the excluded peak together with the bulk of immunoglobulin-G (Fig. 1). Using polystyrene tubes and plasma artificially depleted of factor XI (6), it could be shown that part of the factor XI was in active form, but that the clot-promoting activity could be further enhanced by adding kaolin. The same was true of factor XII, which eluted with 0.14 *M* NaCl. These latter fractions had no effect on plasma depleted of factor XI. Fractions which corrected the clotting defect of factor XII-deficient plasma (tubes 840–1160, Fig. 1) were pooled, concentrated, and tested for enhancement of vascular permeability and for their ability to active prekallikrein with and without trypsinization, followed by inactivation of the trypsin with lima bean trypsin inhibitor (LBTI). The results pertaining to enhancement of vessel permeability are shown in Table I, which show also results obtained when kaolin (10 mg/ml sample) was added to the sample. As shown in Table I, kaolin injected by itself induced some increase in vessel permeability, presumably by acting on factor XII present in the interstitial fluid and the plasma of the experimental animal. When 30 μg of trypsin was incubated for 2 min with partially purified (partially activated) factor XII, followed by neutralization of the trypsin with LBTI, and prekallikrein (tubes 10–260, Fig. 1) added to this mixture, kallikrein was generated. The kallikrein hydrolyzed BAEe at the rate of 105 n moles/ml/min. Activation of prekallikrein with more highly purified fractions is described in more detail below.

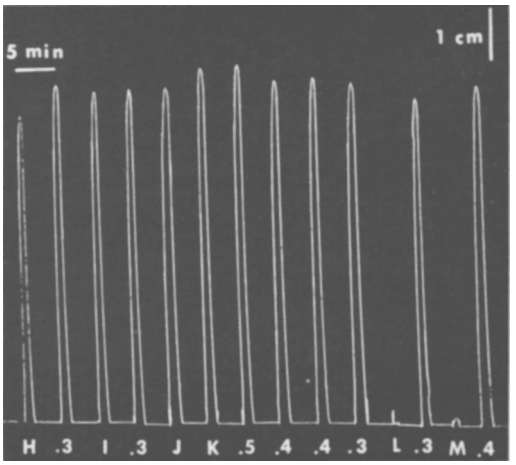

FIG. 2. Kymograph tracing; kinin assay on the rat uterus: (numerals) represent amount (ng/ml) of synthetic bradykinin added to the bath. (H) is PKA with prekallikrein 1:200; (I and J) the same mixture 1:150; and (K) 1:100; (L and M) factor XIIa and prekallikrein in dilution of 1:100 and 1:50, respectively.

In addition to the treatment with trypsin and kaolin, an aliquot of the partially purified factor XII preparation was incubated at room temperature for 10 min with constant stirring with 150 mg of Celite (Hyflo Super Cel, Johns-Manville, Port Credit, Ont.)/ml of sample. After washing, as described before (2), the Celite eluates were concentrated

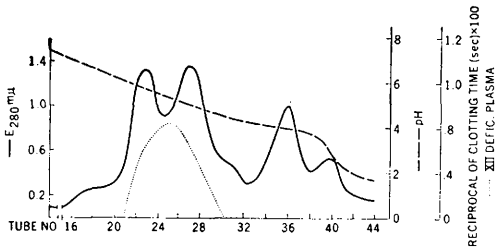

FIG. 3. Isoeletric focusing of partially purified factor XII (Fig. 1): The partial thromboplastin time was carried out with factor XII-deficient plasma, with kaolin in the mixture.

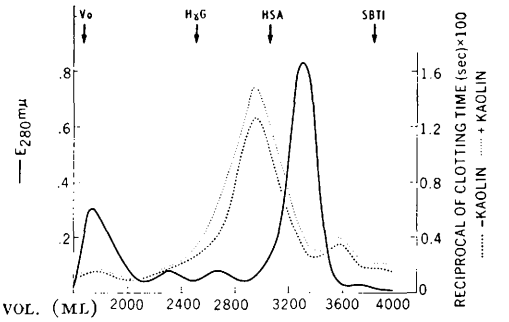

FIG. 4. Gel filtration of partially purified factor XII (Fig. 1) through 3 consecutive columns of Sephadex G-200 fine, as described under Materials and Methods: Fractions correcting a plasma deficient in factor XII eluted with a Ve intermediate between that of human γ-globulin and human serum albumin.

to the original sample volume. The Celite eluates exhibited prekallikrein activator (PKA)-activity and had lost part of the clot-promoting activity of the crude factor XII preparation (Table II). The PKA induced activation of prekallikrein, which liberated kinin (460 ng/ml) from plasma heated to 61° for 1 hr (Fig. 2).

The partially purified factor XII preparation had some spontaneous arginine esterase activity (Table II). This activity was inhibited by DFP (10^{-4}, 10^{-3} *M*). However, this treatment had no effect on the clot-promoting activity of factor XIIa, although it readily inhibited PKA-activity.

By preparative polyacrylamide electrophoresis clot-promoting activity (which corrected the prolonged clotting time of factor XII-deficient plasma) eluted as a β-globulin.

Finally, the isoelectric point was determined by isoelectric focusing, and the sedimentation coefficient and molecular weight by sucrose density gradient ultracentrifugation. The partially purified factor XII focused in the pH range of 5.0 to 5.5 (Fig. 3). The *S*-value was 5.5–6.0 and the molecular weight was estimated to be approximately 86,000.

Material demonstrating the above activities was applied to Sephadex G-200 column and recycled through 3 columns. The sample was tested before application to the Sephadex G-200 column. It clotted factor XII-deficient plasma, there being a difference when polystyrene tubes were used with kaolin (60 sec) and without kaolin (147 sec). VBIS control was 460 sec with kaolin and 1015 sec without kaolin. Thus, the applied sample consisted of a mixture of unactivated Hageman factor (XII) and activated Hageman factor (XIIa). Following gel filtration the recovered material seemed to be almost fully activated, eluting just ahead of serum albumin (Fig. 4), with an estimated molecular size of approximately 118,000.

Clotting studies with factor XII-deficient plasma, using serial twofold dilutions were carried out. Maximal correction of the clotting defect was observed with the undiluted material obtained by gel filtration and with 1:2 and 1:4 dilutions. Dilutions of 1:8–1:256 showed a linear relation between the clotting time and dilution (Fig. 5).

The factor XIIa preparation obtained by

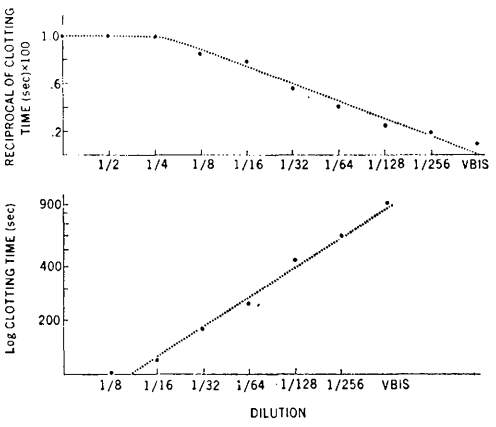

FIG. 5. Correction of the clotting defect of factor XII-deficient plasma, using serial twofold dilutions of factor XII (see Fig. 4).

TABLE III. Hydrolysis of BAEe by Prekallikrein Activator Obtained by Trypsinization of Factor XIIa.

Incubation mixture	Prekallikrein activation (nM BAEe/min/ml)
Factor XIIa + Trypsin + LBTI + Prekallikrein	170
Factor XIIa + Prekallikrein	0
Factor XIIa + Buffer	0
Prekallikrein + Buffer	0
Prekallikrein + Trypsin + LBTI	0

One milliliter sample (Factor XIIa, Fig. 4) was incubated with 25.0 μg of trypsin for 2 minutes, followed by inactivation with 30 μg of LBTI. This mixture was then incubated with prekallikrein for 20 minutes at 37°C, followed by estimation of BAEe hydrolysis.

gel filtration was trypsinized to obtain PKA. After inactivation of the trypsin with LBTI, the reaction mixtures were incubated with prekallikrein, followed by incubation with BAEe and the hydrolysis of the latter was determined, shown in Table III. In these studies, it was noted that excess LBTI inhibited the hydrolysis of BAEe. Since it has been determined in earlier studies that LBTI has no effect on BAEe hydrolysis induced by kallikrein, it was assumed that LBTI acts on PKA or on the activation of prekallikrein by PKA. Addition of 20 or 40 μg of LBTI,

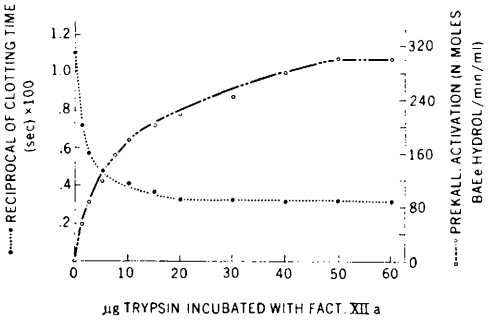

FIG. 6. Effect of trypsinization on factor XIIa: Increasing concentrations of trypsin were incubated with factor XIIa for 5 min, followed by neutralization of the trypsin with LBTI. Correction of the clotting defect of factor XII-deficient plasma decreased by about ⅔ of the original factor XIIa after adding 20 μg of trypsin, but further increase of the trypsin did not abolish this residual clot-promoting activity. PKA-activity leveled off after 50 μg of trypsin.

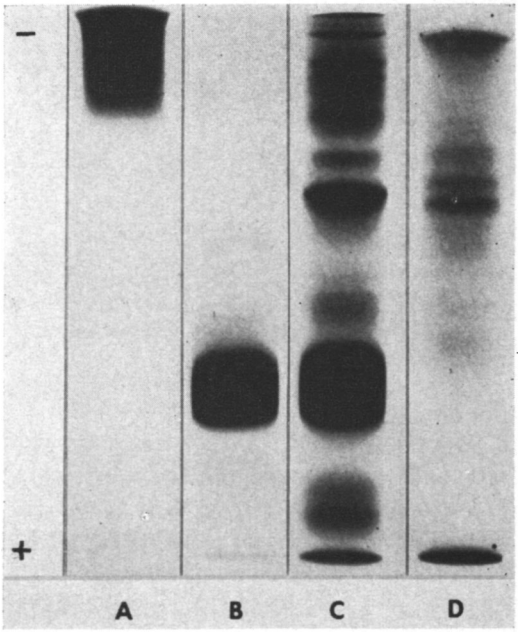

FIG. 7. Disc gel electrophoresis of partially purified human γ-globulin (A); commerical human serum albumin (B); partially purified factor XIIa treated with 30 μg of trypsin (C); and highly concentrated Celite eluates obtained from partially purified factor XIIa (D).

above that used for the neutralization of trypsin, induced a 12 and 25% inhibition, respectively.

Inceasing concentrations (2.5–60 μg/ml) of trypsin were added to factor XIIa. PKA-activity increased, reaching a peak with 50 μg of trypsin and the clot-promoting activity (correction of defect in factor XII-deficient plasma) decreased, leveling off after 20 μg of trypsin, but remaining constant thereafter (Fig. 6). The disc gel pattern is shown in Fig. 7.

Discussion. Three hypotheses have been advanced to explain the mode of activation of the kinin system and the role of activated Hageman factor (factor XIIa) in this process (7). Miles (8) and others have postulated that factor XIIa activates the precursor of a permeability globulin, which in its active form (Pf/dil) acts on prekallikrein and the latter releases kinin from kininogen. Nagasawa *et al.* (9) and Jahreis and Habermann (10) presented some evidence that factor XIIa acts

directly on prekallikrein. A prekallikrein activator distinct from factor XIIa and Pf/dil has been demonstrated first in guinea pig (11–13) and then in rabbit (14) and in human plasma (1, 15–17). It was Kaplan and Austen (15), who first proposed and presented some evidence that prekallikrein activator (PKA), a low molecular weight highly anionic substance, may represent a fragment of factor XIIa.

The present paper, while not entirely conclusive—since our factor XIIa preparation was not homogenous by polyacrylamide electrophoresis—adds additional evidence to the concept that PKA may derive from factor XIIa. As shown in Fig. 6 incubation of partially purified factor XIIa with increasing amounts of trypsin lead to the formation of increasing amounts of PKA and this went more or less hand in hand with partial loss of the clot-promoting activity of factor XIIa. Both PKA-activity and clot-promoting activity leveled off and addition of excess trypsin did not bring about a loss of the clot-promoting activity. Thus, it would appear that PKA, which is probably a fragment of factor XIIa, *i.e.*, XIIf (2), while retaining some of the clot-promoting property of the parent molecule, acquires a new property, that of activating prekallikrein. This could mean that a new active center has been exposed in the fragment. This hypotheses is supported by the finding that PKA is inhibited by DFP (1, 2, 4, 12, 13); whereas the clot-promoting activity of factor XIIa, as reported in this paper and as described repeatedly and discussed in detail by Ratnoff (18, 19), is resistant to DFP treatment. It should be stressed, however, that it is the prekallikrein activating capacity of XIIf which is DFP susceptible; whereas its ability to correct the defect in factor XII-deficient plasma is only partially inhibited by DFP (2, 20, 21).

PKA-activity could be generated in our factor XIIa preparation also by exposure to Celite. Massive exposure to a "surface-active agent" brings about activation and generation of PKA as well as kallikrein when plasma is incubated with Celite (2). One would assume that PKA activates prekallikrein to kallikrein in such experiments. However, prekallikrein can be activated also when factor XII-deficient plasma is exposed to Celite (3). Such massive contact exposure may induce fragmentation of kallikrein with the fragment retaining kallikrein-like activity (2).

Despite some discrepancies, it is now agreed upon by several groups of investigators that PKA or XIIf is a fragment of activated Hageman factor or factor XIIa (22). To ascertain that the natural serum activator of plasma kallikrein is indeed a fragment of factor XIIa very large quantities of plasma will have to be chromatographed, in order to obtain functionally and physiochemically pure factor XIIa. Such experiments, using over 10 liters of plasma as starting material, are currently conducted in our laboratory. The major contaminants interfering from a functional point of view are plasminogen, which, when activated to plasmin, is able to fragment factor XIIa to PKA (23–25). Furthermore, recently we encountered a natural inhibitor of kallikrein in some of our factor XIIa preparations of guinea pig plasma. It is as yet uncertain whether it acts on factor XIIa or PKA. It would appear that to date only Cochrane (17) succeeded in obtaining small amounts of factor XIIa from rabbit plasma, which migrated as a single band by the disc electrophoresis.

Summary. A partially purified preparation of activated Hageman factor (factor XIIa) was prepared by anion exchange chromatography and gel filtration (Sephadex G-200) of noncontact human platelet poor plasma. The factor XIIa preparation corrected the clotting defect of congenitally factor XII-deficient plasma, but not that of plasma depleted of factor XI. It had a pI of approximately 5.3 and an approximate molecular weight of 118,000 by gel filtration and 86,000 by sucrose density gradient ultracentrifugation.

When treated with trypsin or Celite, it developed prekallikrein-activating activity. No such activity could be induced with the factor XIIa preparation. The prekallikrein activator, as described before, was highly anionic and had a molecular weight of less than 40,000. It migrated as a prealbumin by disc electrophoresis. Multiple bands were

present in the factor XIIa preparation, which eluted as a β-globulin during disc electrophoresis.

Some evidence is presented that the prekallkrein activator is a fragment (XIIf) of the parent molecule (XIIa) and that the fragment loses some of the clot-promoting activity, while it acquires prekallikrein activating activity. Whereas the prekallikrein activator was susceptible to DFP, its clot-promoting activity was only partially inhibited by this serine esterase inhibitor. On the other hand, DFP in a concentration as high as 10^{-3} *M* had no inhibitory effect on intact factor XIIa.

The authors thank Dr. D. M. Wrobel of the Canadian Red Cross for a generous supply of human plasma. We also thank Dr. J. G. Scott for the factor XII-deficient plasma; and, last but not least, we acknowledge the skillful assistance of Mrs. Otti Freitag, Mrs. Anneliese Carré, and Miss Marica Michael.

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Received July 17, 1971. P.S.E.B.M., 1971. Vol. 138.