# The Human Plasma Kinin System
## II. Contact Activation of Plasma Prekallikrein and Factor XI in Factor XII-Deficient Plasma[1] (35890)

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Kallikrein exists in normal plasma in form of a zymogen or precursor referred to as kallikreinogen or prekallikrein. The latter term has been recommended by a committee on nomenclature (1). Plasma thomboplastin antecedent (PTA, factor XI) is likewise present in plasma in an inactive state.

Prekallikrein can be isolated from rabbit (2), guinea pig (3), and human (4, 5) plasma, eluting in the cationic or excluded fraction during anion exchange chromatography Until very recently attempts to obtain functionally pure factor XI were unsuccessful (6); but in the past year Kingdon (7) purified bovine and Harpel (8), human factor XI.

When one adsorbs rabbit, guinea pig, or human plasma to antigen-antibody precipitates or Celite, and elutes the adsorbed protein and chromatographs it, one obtains plasma kallikrein, its activator, and some of the blood clotting factors (3–5, 9–12). It is generally believed that, during contact activation, kallikrein is activated by Hageman factor (factor XII) but there is no agreement concerning the mode of action of factor XII, direct or indirect (6, 13, 14).

The original aims of this experiment were (a) to obtain crude factor XI by adsorption to Celite, followed by desorption; and (b) to explore the possible existence of a precursor of prekallikrein activator. To our surprise we obtained highly active kallikrein in addition to activated factor XI. The unexpected results led us to further experimentations, which are described below.

*Materials and Methods.* Citrated plasma was obtained from a patient (E. W. H.) with severe factor XII deficiency.[3] One liter of blood was collected into plastic bags (Fenwall Blood Pack Unit, Code No. JC-2, Fenwall Laboratories, Morton Grove, Ill.) under sterile conditions. The bags contained 50 ml of 4% trisodium citrate. Five hundred milliliters of blood were collected in each bag, with continuous gentle agitation to ensure thorough mixing of the blood with the anticoagulant. The plasma was separated by centrifugation and the packed red cells were transferred to the patient. Platelet-poor plasma (PPP) was obtained by recentrifugation at 3020*g* for 30 min at 4°. The pH of the PPP was 7.65. It was then separated into varying sized aliquots, quick frozen in a mixture of dry ice and acetone, and kept either at —70° in a deep freeze or in liquid nitrogen until used. Siliconized glassware was used throughout.

Celite ("Hyflo", Super-Cel, Johns-Manville, Port Credit, Ontario) was suspended and thoroughly mixed with 0.001 *M* Tris HCl (pH 8.0), centrifuged, and the supernatant buffer was discarded. The wet Celite was suspended in 100 ml of PPP (150 mg of Celite/ml of plasma); the plasma–Celite mixture was stirred for 10 min at room temperature (23°) with a plastic rod; and then the plasma was separated by centrifugation (15 min at 3020*g*). The plasma was again exposed to Celite, stirred, and centrifuged.

[1] Supported by the Atkinson Charitable Foundation and the Ontario Heart Foundation.

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[3] The plasma had been tested against that of Mr. Hageman by Dr. O. D. Ratnoff and found to be free of Factor XII-activity.

The Celite from the two runs was mixed and washed repeatedly at 4° with 0.001 *M* Tris HCl (pH 8.0) until the OD 280 mμ readings of the washings were less than 0.05. These washings were discarded. Subsequently, the Celite was washed repeatedly with 0.1 *M* Tris HCl (pH 8.0) containing 2.0 *M* NaCl, again until the optical density readings reached less than 0.05. The washings obtained with 2.0 *M* NaCl were combined and thoroughly dialyzed against 0.1 *M* Tris HCl (pH 8.0) until the conductivity of the washings were equal to that of the dialyzing buffer (4.5 mmho/cm).

The dialyzed washings (Celite eluates) were concentrated to 15.0 ml; and of this, 12.0 ml were applied to QAE-Sephadex A-50 column (2 × 11 cm) equilibrated with 0.1 *M* Tris HCl. Elution was carried out with 0.1 *M* Tris HCl, followed by stepwise elution, *i.e.,* the starting buffer containing 0.08, 0.2, and 0.5 *M* NaCl, respectively. Flow rate was 9 ml/hr (7.5 ml/tube). Six adjacent tubes were pooled, concentrated to ¼ of the applied volume (3.0 ml). Aliquots of this material were dialyzed against either 0.02 *M* phosphate buffered isotonic saline, pH 7.4 (PBS) or 0.02 *M* Veronal buffered isotonic saline, pH 7.35 (VBIS). The pools were tested by the various assays described below.

Fractions exhibiting kallikrein-like and clot-promoting activities were pooled, and 17.0 ml were applied to a Sephadex G-200 fine column (5 × 80 cm) and recycled through two additional columns of the same size. The Sephadex was equilibrated with 0.1 *M* NaCl in 0.1 *M* Tris HCl (pH 8.0). Flow rate was 20 ml/hr (10.8 ml/tube). Ten adjacent tubes were concentrated to ½ the volume of the applied sample and tested as after QAE-Sephadex chromatography.

*Sucrose density gradient ultracentrifugation* was done by layering 0.5 ml of sample over a gradient of 5–20% sucrose in 0.1 *M* Tris HCl and centrifuging for 24 hr in a B-60 International preparative ultracentrifuge (Fisher Scientific, Toronto, Ont.), as described in an earlier publication (10). The crude Celite eluates, the chromatographic fractions and those obtained by ultracentrifugation were dialyzed against VBIS for clotting studies and against PBS for the other assays.

*Acrylamide disc gel electrophoresis.* This technique was based on that of Davis (15). A 7% running gel (pH 8.9) and a 2.5% stacking gel (pH 6.7) were used. Samples were run for 2 hr at 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 and destained electrophoretically.

*Hydrolysis of benzoyl-l-arginine ethyl ester (BAEe)* was measured by the method of Trautschold and Werle (16). The estimation of the degree of *enhanced vascular permeability* was based on the method of Udaka *et al.* (17). The *kinin assay* on the estrus rat uterus and the two assays just mentioned are described in detail in a recent publication (5).

*Factors XII and XI activities.* Partial thromboplastin time, using plasmas congenitally deficient in factors XII or XI or artificially depleted of factor XI, was carried out by the method of Ratnoff and Davie (18). As our only modification, we omitted kaolin from the reaction mixture, having observed earlier that kaolin inhibits clot formation when the reaction mixture contains factors XII and/or XI in fully active form. According to the method of Nossel (19) normal PPP was artifically depleted of factor XI by adsorbing with 6 mg of Celite-512 (Johns-Manville, Port Credit, Ont.)/ml of plasma. Unlike Nossel, we incubated the supernatant plasma for 6 hr at 37° instead of 18 hr.

Samples were also analyzed for factor VIII activity and IX activity, by measuring their ability to correct the prolonged clotting time of plasmas congenitally deficient in these factors. The method of Breckenridge and Ratnoff (20) was followed.

The following were used as controls for the above described clotting tests: (a) the test sample was replaced by VBIS; and (b) the test sample was replaced by normal PPP diluted 1:5 with VBIS.

Assay for factor X activity was likewise performed, based on Denson's (21) modification of the method originally described by Bachmann *et al.* (22). Charcoal-treated ox

plasma for this assay was obtained from Diagnostic Reagents Ltd., Thames, Oxon. England.

*Conversion of fibrinogen to fibrin* was tested as described previously (23), using grade L human fibrinogen (A. B. Kabi Stockholm, Sweden) further purified by the method of Shamash and Alexander (24). The fibrinogen preparation obtained in this manner contains trace amounts of factor VIII, but no detectable II, V, VII, IX, X, XI, and XII activities.

*Results and Discussion.* Crude Celite eluates enhanced vascular permeability when injected into guinea pig skin. They hydrolyzed the arginine ester BAEe and liberated kinin from fresh and heated plasma substrates. One milliliter of crude eluate liberated about 0.64 $\mu$g of bradykinin equivalent from 1.0 ml of heated plasma and about 0.5 $\mu$g from fresh plasma. One milliliter of eluate hydrolyzed 390 nmoles of BAEe/min. The clotting tests with the crude Celite eluates are shown in Table I.

Following anion exchange chromatography, most of the vascular permeability-enhancing, BAEe-hydrolyzing, kinin-liberating, and clot-promoting activities eluted with the excluded large protein peak. (Fig. 1). Because we had only a limited amount of plasma congenitally deficient in factor XI, only some of the fractions (pools) obtained by anion exchange chromatography were tested, using this plasma as substrate, as shown in Table II. The excluded protein peak (fractions 1–5) was free of factors IX, VIII, and X activities and did not induce conversion of fibrinogen to fibrin. The bulk of the protein in this first peak was immunoglobulin-G. Unlike that obtained with Celite eluates from normal human plasma (12), little protein and negligible activity eluted after the excluded peak. In fact no activity at all was detected with fractions obtained with 0.2 *M* NaCl, the salt concentration which elutes prekallikrein activator when Celite eluates of normal plasma are chromatographed (5, 12). Prekallikrein activator partially corrects the clotting defect of factor XII (but not factor XI)-deficient plasma. In the present experiment anionic fractions eluting with 0.2 *M* NaCl induced

TABLE II. Factor XI Activity—Partial Thromboplastin Time.[a]

| Sample | Clotting time (sec) |
|---|---|
| VBIS (control) | 392 |
| Crude Celite eluate | 132 |
| Fraction 1 | 83 |
| 2 | 94 |
| 3 | 129 |
| 4 | 163 |
| 5 | 174 |
| 7 | 201 |
| 9 | 302 |
| 11 | 312 |

[a] Partial thromboplastin time was carried out as described in Table I, using plasma congenitally deficient in factor XI as substrate. Fractions refer to those obtained with QAE-Sephadex chromatography, as shown in Fig. 1.

TABLE I. Partial Thromboplastin Time of Crude Celite Eluates.

| | Clotting time (sec)[a] | | |
|---|---|---|---|
| | Fact. XII-deficient plasma | Fact. XI deficient plasma | Fact. XI-depleted plasma |
| Celite eluates | 121 | 132 | 173 |
| VBIS | 626 | 392 | 295 |
| PPP (1:5) | 189 (109)[b] | 175 | 203 (123) |

[a] One-tenth milliliter of sample was incubated with 0.1 ml of crude 0.1% soy bean phosphatide (Centrolex P, Chemurgy Division, Central Soya, Chicago, Ill.) in an uncoated unused Pyrex tube. The mixture was incubated at 37° for 2 min, before adding 0.1 ml of substrate plasma, followed by an additional incubation for 8 min. Finally, 0.1 ml of calcium chloride (0.025 *M*) was added and the stopwatch was started. The tubes were tilted about every 15 sec until a solid clot formed. The data represent the mean of two determinations.

[b] Values in parentheses represent clotting time obtained in the presence of kaolin (acid washed, American Standard, Fisher Scientific), 10 mg/ml of 0.1% soy bean phosphatide.

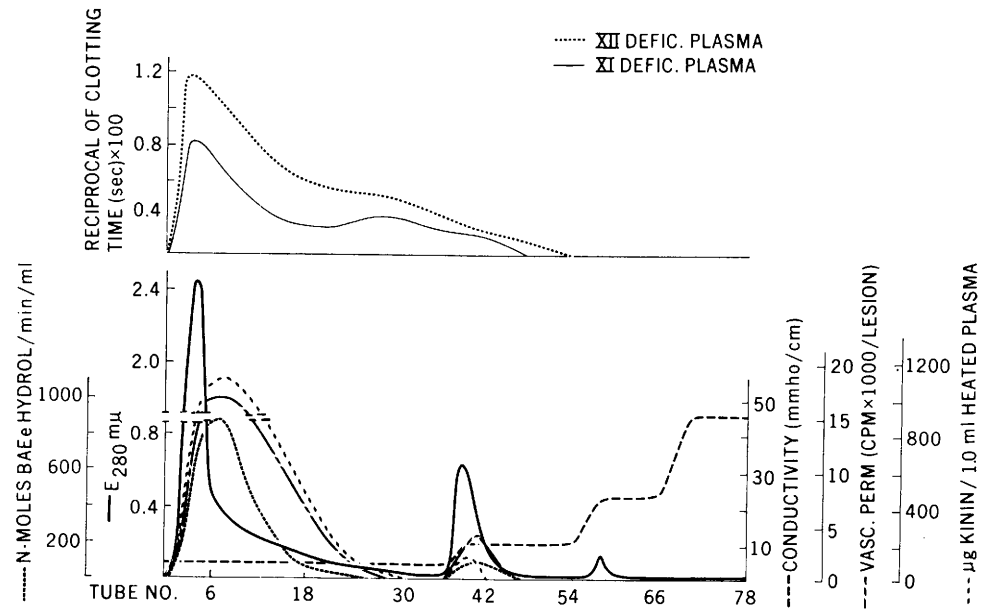


FIG. 1. QAE-Sephadex chromatography of 100 ml of celite eluates of factor XII-deficient plasma eluates dialyzed against the equilibrating buffer (0.1 *M* Tris HCl, pH 8) was applied to a 2 × 11-cm column of QAE-Sephadex A-50 and eluted first with the equilibrating buffer and then with increasing concentration of NaCl. Six adjacent tubes were pooled and concentrated to 3.0 ml. Thus 13 pools (fractions) were obtained and these were used for the assays shown; (upper) the clotting times obtained when chromatographic fractions were added to either plasma congenitally deficient in factor XII or made artificially deficient, *i.e.*, depleted of factor XI (see Materials and Methods).

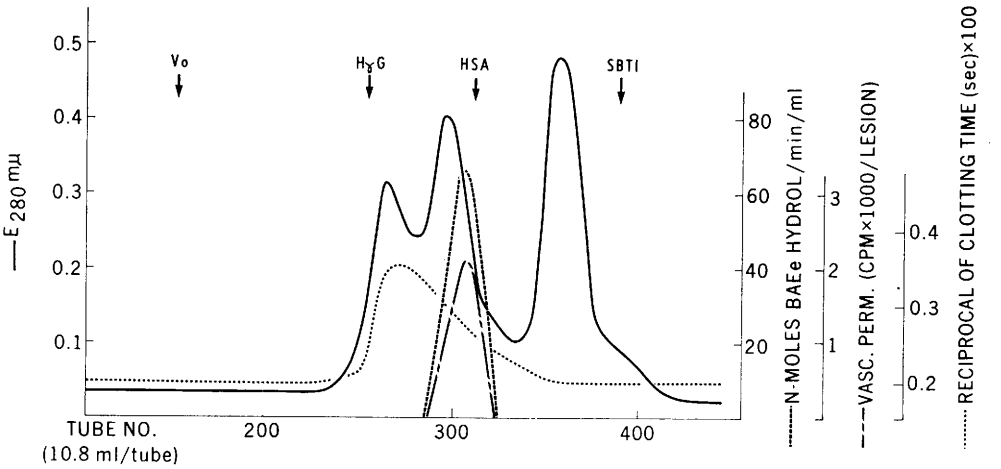


FIG. 2. Sephadex G-200 chromatography of material obtained in the first 36 tubes shown in Fig. 1. The material was passed through a 5 × 80-cm column and recycled through 2 additional columns of the same size. Nine adjacent tubes were pooled and concentrated to 8.5 ml. The pools obtained in this manner were used for testing. Coagulation data refer to results obtained with factor XII-deficient plasma.

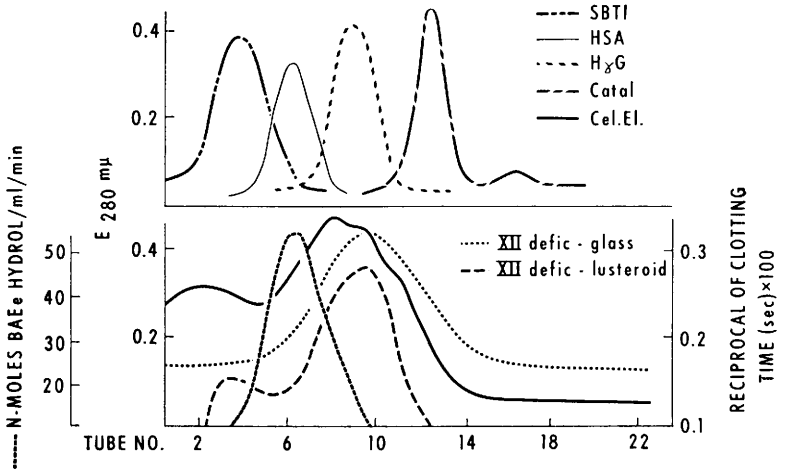


FIG. 3. Sucrose density gradient ultracentrifugation of Celite eluates (Cel.El.). Procedures are described in the text. Marker proteins: SBTI = soy bean trypsin inhibitor (mol wt 21,500); HSA = human serum albumin (mol wt 69,000); HγG = human γ-globulin (mol wt 160,000); Catal = beef liver catalase (mol wt 225,000). The fractions were tested against factor XII-deficient plasma in both glass and lusteroid tubes.

slight, but insignificant, correction of factor X-depleted ox plasma and factor IX-deficient plasma. They converted fibrinogen to fibrin in the presence of calcium, but this reaction required 20 hr to develop, indicating trace amounts of thrombin-like activity.

Upon gel filtration of the excluded peak obtained by anion exchange chromatography, the clot-promoting activity more or less paralleled the γ-globulin peak (Fig. 2). The arginine esterase and vascular permeability-enhancing activities eluted with an elution volume intermediate between human γ-globulin and human serum albumin. Using the method of Andrews (25), the molecular weight of the clot-promoting component was estimated to be about 170,000, whereas the component possessing the properties of kallikrein was estimated to be approximately 90,000. These data were confirmed by sucrose density gradient ultracentrifugation (Fig. 3). Figure 4 shows the electrophoretic patterns of the components obtained by sucrose density gradient ultracentrifugation. The kallikrein did not hydrolyze lysine methyl ester and had no caseinolytic activity. It liberated kinin from heated plasma (Fig. 5).

The physiochemical and biological properties of the kallikrein obtained from factor XII-deficient plasma were found to be identical to plasma kallikrein we obtained from normal plasma (5, 12). To obtain active plasma kallikrein from plasma lacking factor XII was an unexpected finding because, as stated in the introduction, it is generally held that Hageman factor is essential for the activation of prekallikrein to kallikrein, when the activation is induced by contact (6, 13). Three mechanisms may explain the activation of prekallikrein in the present experiment: First, trace amounts of factor XII may have been present in the factor XII-deficient plasma. This is difficult to rule out with absolute certainty. However, despite 26-fold concentration of each chromatographic pool over the volume of the applied plasma, no vascular permeability inducing, prekallikrein-activating or factor XII-deficiency correcting activities eluted in the anionic fractions by QAE-Sephadex. The same anionic fractions contain these activities when the Celite elutates are obtained from normal plasma (5, 12). Second, the prekallikrein may have become activated by plasmin (26, 27), which in turn may have become activated during contact activation of the plasma. However, plasminogen activation to plasmin likewise seems to require factor XII, in addition to other factors (28); finally (a

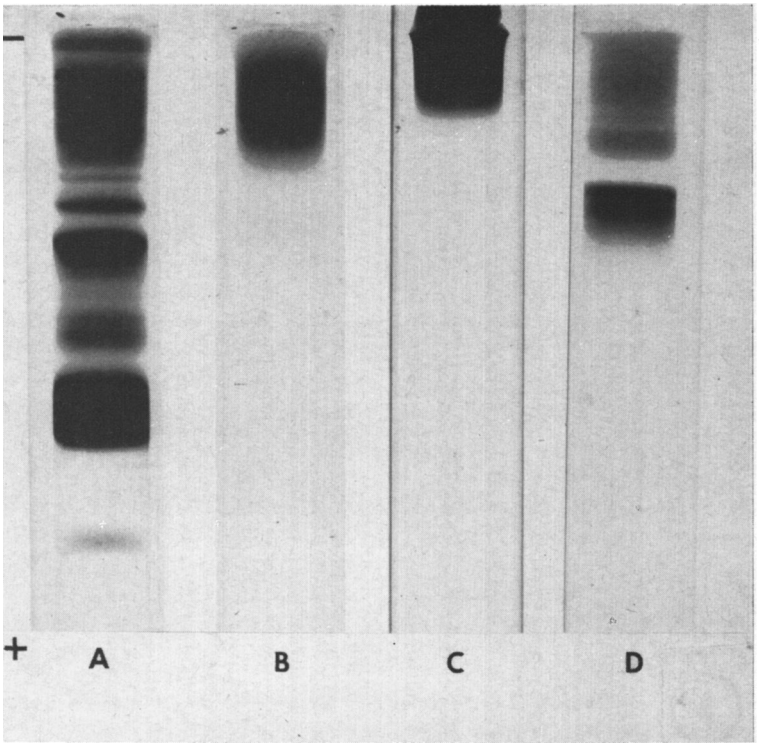


FIG. 4. Disc gel electrophoresis of normal human plasma (A); human γ-globulin (B); tubes 4–8 (C); and tubes 9–12 (D) obtained by sucrose density gradient ultracentrifugation (Fig. 3).

more likely possibility), massive contact with large quantities of a "surface active material" activated prekallikrein to kallikrein, by inducing "configurational changes" in the zymogen.

As stated in the introduction, an original aim of this experiment, was to isolate factor XI by adsorption to Celite (in the absence of factor XII), followed by a one- or two-step fractionation of the Celite eluates. In our experience, both factors adsorb to Celite when we used normal plasma, and we recovered the bulk of the clot-promoting activity in the excluded or cationic fraction during anion exchange chromatography. Schiffman *et al.* (29) also reported that factor XI eluted in the excluded fraction when non-contact plasma was chromatographed on DEAE-cellulose. By gel filtration on Sephadex G-200 the molecular weight of contact activation product was found to be about 170,000 (12). We have referred to this material as "contact activation product," in the sense of a XII–XI complex, as suggested by several investigators (19, 30–33). Other investigators hold that this clot-promoting activity is due to activated factor XI (6, 34, 35). Contrary to reports that factor XI cannot be activated by contact in the absence of factor XII (36), we were able to obtain clot-promoting material from factor XII-deficient plasma, with the physicochemical properties identical to material obtained from normal plasma (12). Thus, it appears that massive contact exposure of factor XII-deficient plasma to Celite is followed by adsorption and activation of both kallikrein and factor XI. Furthermore, since the clot-promoting materials obtained from normal and factor XII-deficient plasma have identical properties, the material in "contact activation product" represents activated factor XI, as suggested by Ratnoff *et al.* (34, 35). Our preparation of factor XI differs from that of Kingdon (7), who reported that his had a molecular weight of 50,000–60,000. However, Kingdon's preparation was obtained from bovine plasma.

Whether activation by extensive contact

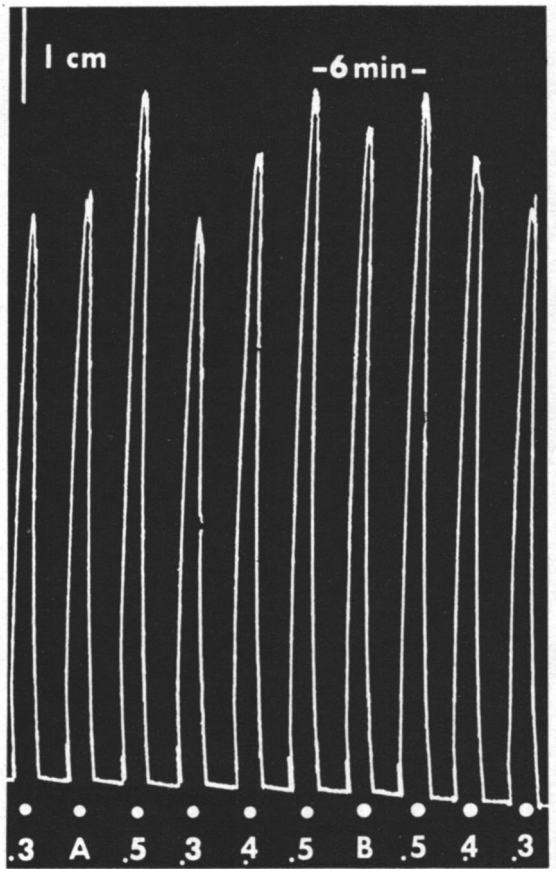


FIG. 5. Kymograph tracing of contractions given by a rat uterus suspended in de Jalon solution. Numerals represent nanograms per milliliter of synthetic bradykinin. At (A), a 150-fold and at (B) a 100-fold dilution of a mixture of kallikrein and heated plasma were added to the bath.

has any *in vivo* significance is open to doubt. Situations under which such a process conceivably could take place in the intact organism are sudden formation of antigen–antibody precipitates as in aggregate anaphylaxis and exposure to collagen in vessels denuded of their endothelium.

Finally, a negative finding in our experiments was the inability to isolate a prekallikrein activator or its precursor, by the procedures used. It should be added, however, that no such precursor could be activated with trypsin, when factor XII-deficient whole plasma was chromatographed (37).

*Summary.* Noncontacted factor XII-deficient human plasma was mixed with Celite, the protein adsorbed to the Celite was eluted with a concentrated salt solution, and then chromatographed on QAE-Sephadex A-50, followed by Sephadex G-200.

The first (excluded) peak obtained by anion exchange chromatography contained substances with physicochemical and biological properties indistinguishable from plasma kallikrein and "contact activation product." Material obtained with the excluded peak enhanced vascular permeability, hydrolyzed arginine ester, liberated kinin from both fresh and heated plasma, corrected the prolonged clotting time of plasmas congenitally deficient in factors XII or XI, artificially depleted of XI, but did not enhance the clotting of plasmas deficient in factors IX, VIII, and X. On gel filtration the molecular weight of kallikrein was estimated to be about 90,000 and that of the clot-promoting material was about 170,000. The latter was confirmed by sucrose density gradient ultracentrifuga-

tion.

We conclude that prekallikrein and factor XI can be activated directly by massive exposure to an active surface. This activation does not seem to require factor XII. It is further concluded that the clot-promoting activity of contact activation product is due to factor XI. Finally, we could obtain no prekallikrein-activating material from factor XII-deficient plasma.

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