The Kinin System of Human Plasma
VI. The Action of Plasmin[1] (36027)

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The role of plasmin in the activation of the kinin system has been debated for some time. The early studies of Beraldo (1) and Lewis(2) clearly demonstrated that plasmin can release kinin from plasma. These investigators and several others subsequently believed that plasmin is the main kinin-forming enzyme in plasma. Vogt (3) showed that plasmin may act directly on kininogen (heated plasma), but this is a slow reaction requiring high concentrations of plasmin. Since kinin was formed rapidly when plasmin was added to fresh plasma, Vogt concluded that plasmin acted on prekallikrein, converting it to kallikrein, which then acted on the kinin substrate. Recent findings (4, 5) indicate that the rapid action of plasmin on fresh plasma requires both activated Hageman factor (factor XIIa) and prekallikrein, plasmin acting presumably on factor XIIa, releasing prekallikrein activator.

Materials and Methods. Preparation of plasminogen and plasmin. Euglobulin precipitates were prepared from 880 ml of ACD human plasma (Canadian Red Cross). The plasma was defibrinated by adding 88 ml of 4% calcium chloride; allowed to clot and the serum was diluted 16 times its original volume with cold distilled water and acidified to pH with 5% acetic acid (6). After standing at 4° for 1 hr, the precipitate was collected by centrifugation at 3000*g* for 30 min, and redissolved in 0.008 *M* phosphate buffered isotonic saline (PBS, pH 7.4), recentrifuged, and PBS was added to the precipitate until it dissolved. The euglobulin solution was lyophilized and stored at 4°.

Aliquots of the lyophilized euglobulin were dissolved in distilled water and 150 ml were applied to a 10 × 100 cm column (K 100/100, Pharmacia, Montreal, Quebec) of Sephadex G-200 superfine and recycled through a second similar column. In the first run, the Sephadex was equilibrated with 0.1 *M* Tris HCl (pH 8.0) containing 0.1 *M* NaCl. In the second run, the buffer consisted of 0.05 *M* Tris HCl, 0.02 *M* lysine monochloride, 0.1 *M* NaCl and 0.01 *M* epsilon amino caproic acid (pH 8.5). Flow rate was 25.0 ml/hr (10.0 ml/tube). Ten adjacent tubes were pooled; and aliquots were concentrated 10-fold by lyophilization. The redissolved material was dialyzed against PBS and tested for casein hydrolysis (7) and fibrinolysis (8) after activation with streptokinase. The streptokinase (Varidase, Lederle Laboratories, Pearl River, NY) used for caseinolysis contained 4000 units/ml, of which 0.05 ml was used in the reaction mixture. For fibrinolysis, the streptokinase was made up in a concentration of 200 units/ml, of which 0.05 ml was added to the reaction mixture.

Fractions exhibiting caseinolysis and fibrinolysis coincided and were pooled, concentrated by positive pressure with UM-10 "Diaflo" membranes (Amicon Corp., Cambridge, Mass.), and applied to DEAE-Sephadex A-50 columns (2.5 × 14 cm). The DEAE-Sephadex A-50 columns (2.5 × 14 cm) were equilibrated with 0.1 or 0.05 *M* Tris HCl (pH 8.5). Protein was eluted with a linear NaCl gradient or by increasing the salt concentration stepwise. Some active material was rechromatographed by DEAE-Sephadex and some was recycled through three interconnected columns of Sephadex G-200 fine (5 ×

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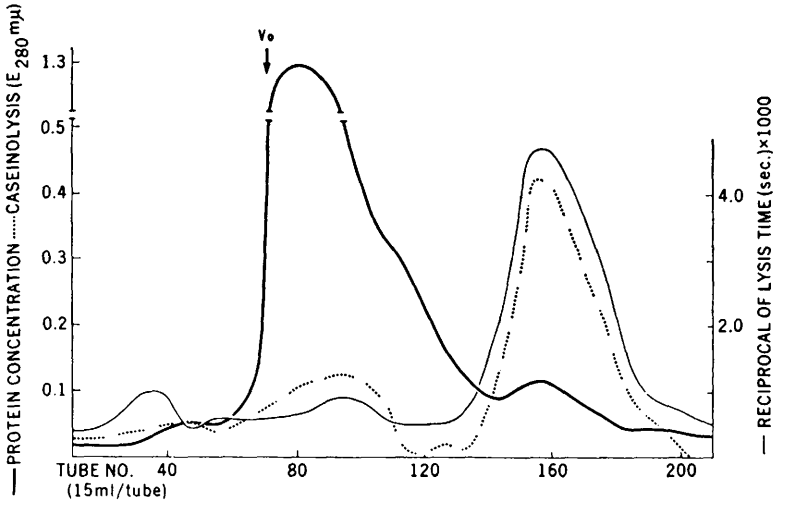

FIG. 1. Chromatography of 150 ml of euglobulin (OD 280 mμ: 3810) on a 10 × 100 cm column of Sephadex G-200 superfine, followed by recycling on a second similar column: Flow rate was 25.0 ml/hr (10.0 ml/tube). Caseinolysis and fibrinolysis-inducing fractions eluted in a small peak, following the main protein peak.

93, 5 × 89, 5 × 86 cm.).

Preparation of factor XIIa. Factor XIIa was prepared from ACD plasma, by adsorption with aluminum hydroxide (Amphogel, unflavored, John Wyeth, Philadelphia, PA), followed by chromatography through QAE-Sephadex A-50, Sephadex G-200 and CM-Sephadex as described in other publications (9–11). The end product corrected the clotting defect of plasma congenitally deficient in factor XII, but not that of plasma deficient in factor XI. The partially purified preparation of factor XII was partly activated after the initial anion exchange chromatography; but after gel filtration, addition of kaolin induced only a small increase in clot-promoting activity. Thus, the material used in these studies was activated Hageman factor or factor XIIa.

Preparation of prekallikrein. Partially purified prekallikrein was obtained by QAE-Sephadex A-50 chromatography, as described before (12) and further purified material was prepared by rechromatography through SP-Sephadex C-50 and Sephadex G-200 (5, 13).

Isoelectric focusing in polyampholines (LKB-Productor, Stockholm, Sweden) was carried out according to the instructions of the manufacturer and as described in an earlier publication (12). The pH range was 3–10.

Hydrolysis of BAEe (benzoyl arginine ethyl ester) was assayed by the method of Trautschold and Werle (14), that of TAMe (tosyl arginine methyl ester) and of LMe (lysine methyl ester) by the method of Siegelman *et al.* (15). Enhanced vessel permeability was tested as described by Udaka *et al.* (16). Kinin was assayed on the estrus rat uterus as described before (12). Analytic polyacrylamide electrophoresis was based on the method of Davis (17), as described elsewhere (18).

Results. The bulk of the caseinolytic and fibrinolytic activity eluted in a single peak, following the major protein peak (Fig. 1). The fractions exhibiting activity were pooled, concentrated, and applied to a DEAE-Sephadex column and eluted first with the equilibrating buffer (0.1 *M* Tris HCl, pH 8.5) and then with a gradient, the limiting buffer being 0.2 *M* NaCl. The results are shown in Fig. 2. Fibrinolytic activity eluted with the gradient, reaching a peak in the last third of the gradient. Enhanced vascular permeability was tested with, and without, addition of streptokinase (200 units/ml). The excluded fraction contained some kallikrein, which induced enhanced vessel permeability with, and without, the addition of streptokinase; also showed spontaneous BAEe hydrolysis and liberated

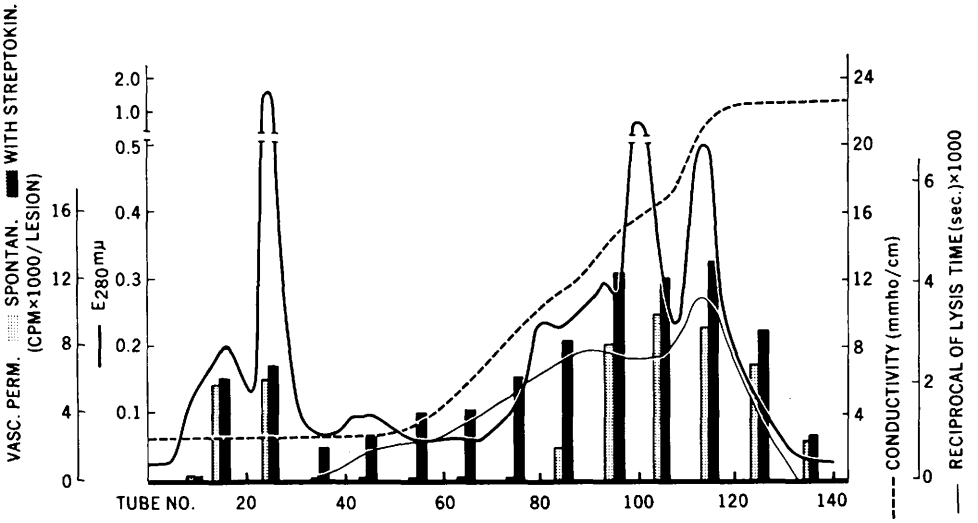

FIG. 2. DEAE-Sephadex A-50 chromatography of 50.0 ml (OD 280 mμ: 110) of crude plasminogen obtained by Sephadex G-200 (Fig. 1): Column size was 2.5 × 14 cm; flow rate, 15.0 ml/hr, 8.0 ml being collected per tube. Fibrinolysis was detected between tubes 40 and 130. Enhanced vascular permeability without streptokinase activation was present in the excluded peak and in the highly anionic fractions. Fractions eluting with the first half of the gradient induced enhanced vessel permeability only when treated with streptokinase.

kinin from heated plasma. The mid portion of the column (*i.e.*, tubes 40–80) enhanced vessel permeability only on addition of streptokinase. The latter fractions were pooled, concentrated, and used as a source of crude plasminogen. Fractions eluting with high anionic strength buffer (tubes 80–140) induced enhanced vessel permeability, which was enhanced by addition of streptokinase. This was more or less paralleled by BAEe, TAMe, and LMe hydrolysis. These latter fractions contained some thrombin, which upon addition to fibrinogen converted it to fibrin (18). Furthermore, these same fractions contained trace amounts of prekallikrein activator (10, 12, 19), which induced enhanced vessel permeability and activation of prekallikrein without the addition of streptokinase. The activator was demonstrable as a faint prealbumin band by disc electrophoresis. In order to obtain functionally pure plasminogen from these anionic fractions, tubes 80–140 were pooled and DFP was added in a final concentration of 10^{-4} *M*. The fractions were subsequently concentrated and dialyzed. The concentrate no longer contained thrombin-like activity, nor did it have prekallikrein-activating activity, but did induce caseinolysis upon incubation with streptokinase. The material was rechromatographed on DEAE-Sephadex equilibrated with 0.05 *M* Tris HCl (pH 8.5). Elution with the starting buffer was followed by steps of increasing NaCl concentration. Most of the plasminogen eluted when the NaCl was raised to 0.12 *M* (Fig. 3). Additional material obtained by Sephadex G-200 was first treated with DFP and then chromatographed as shown in Fig. 3. Some of this partially purified plasminogen was again passed through Sephadex G-200 as shown in Fig. 4. Despite these several steps of purification, the plasminogen still showed several bands in the β-region in disc gels. Its isoelectric point by isoelectric focusing lay between pH 4.7 and 5.5.

Our first impression was that plasmin acts on prekallikrein, converting it to kallikrein. However, the preparation used contained some prekallikrein activator (PKA), which probably induced the activation of prekallikrein. In all subsequent experiments, plasmin free of PKA was used and no direct activation of prekallikrein was observed.

While studying the action of plasmin on heated plasma (61° for 1 hr) (20, 21), it was found, in confirmation of earlier work (3, 22),

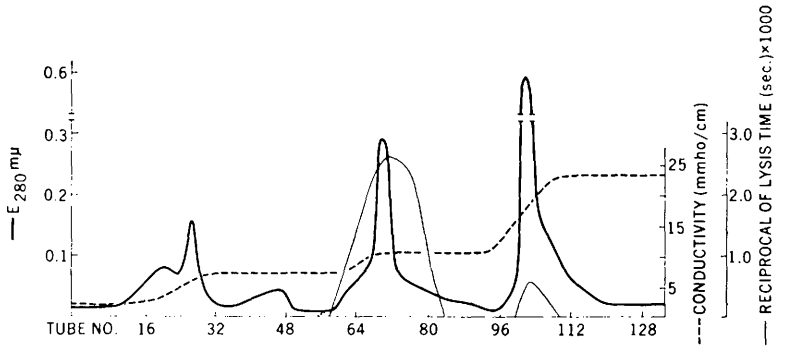

FIG. 3. Rechromatography of the fractions with marked fibrinolytic activity (tubes 80–140) obtained with the column described in Fig. 2: The material was first treated with DFP (10^{-4} *M*), dialyzed against the equibrating buffer (0.05 *M* Tris HCl, pH 8.5), and of this 48 ml (OD 280 mμ: 59) was applied to a 2.5 × 14 cm column of DEAE-Sephadex A-50. Stepwise elution was carried out as shown; plasminogen eluting between tubes 60–80.

that plasmin acts directly on kininogen (heated plasma), thereby releasing kinin. When plasmin was incubated with fresh plasma, without inactivating antiplasmins, but adding the kinase inhibitor *o*-phenanthroline (10^{-4} *M* final conc), less kinin was released from factor XII-deficient plasma (Fig. 5 and Table I). Following 1 min incubation, negligible amounts of kinin were released from factor XII-deficient plasma, compared to normal plasma, from which over 40 ng/ml was released. Upon longer incubation, some kinin was released from XII-deficient plasma, but more than twice that amount was liberated from normal fresh plasma. It was assumed that the larger quantity of peptide released upon longer incubation was due to the direct action of plasmin on kininogen. This is shown in the right side of Table I, using heated plasma, *i.e.*, kininogen free of preenzymes. These findings rule out the possibility that the difference seen with short incubation was due to insufficient kininogen in the factor XII-deficient plasma. However, more antiplasmin in the XII-deficient plasma could have accounted for the difference in the kinin release obtained with short incubation of fresh plasma. We therefore turned to experiments with fac-

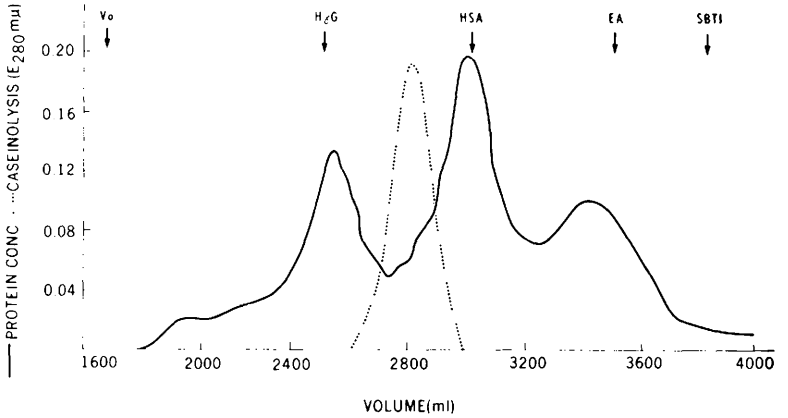

FIG. 4. Gel filtration of material obtained from a DEAE-Sephadex column eluted as shown in Fig. 3: The partially purified plasminogen was passed through three columns (5 × 93, 5 × 89, and 5× 86 cm) of Sephadex G-200 fine. Flow rate was 25.0 ml/hr (10.0 ml/tube). caseinolysis-inducing fractions eluted between the Ve of human γ-globulin and human serum albumin. The other two markers were egg albumin and soy bean trypsin inhibitor.

TABLE I. Action of Plasmin on Normal and Factor XII-Deficient Plasma.[a]

Incubation time (min)	Kinin (ng) liberated from 1.0 ml of plasma					
	Fresh			Heated (61° 60 min)		
	XII-Def.	Normal	Normal/ XII-Def.	XII-Def.	Normal	Normal/XII-Def.
1	<10	45	<4.5	—	—	—
5	—	—	—	200	125	0.625
40	92	210	2.2	800	500	0.625

[a] Two tenths millimeter of streptokinase activated plasmin was incubated with 0.2 ml of normal or factor XII-deficient fresh plasma (containing 10^{-4} *M* *o*-phenanthroline HCl) or plasma heated to 61° for 60 min. The mixture was boiled for 15 min, and the supernatant was tested on the estrus rat uterus, standardized with synthetic bradykinin.

tor XIIa, which had been isolated in a partially purified form in our laboratory (9–11).

When plasmin was first added to the factor XIIa preparation, the BAEe hydrolyzing activity of plasmin was partially inhibited. This finding was interpreted to mean that the partially purified factor XIIa preparation contained an "antiplasmin," presumably $C\bar{1}$-inactivator. Following acidification of the factor XIIa, this preparation retained its clot-promoting activity, having lost its plasmin-inhibiting activity. The acidified factor XIIa preparation was mixed with plasmin and the latter was inactivated with LBTI. This mixture was added to prekallikrein and this preenzyme was converted to its active form—kallikrein. The kallikrein hydrolyzed BAEe (Fig. 6, Table II). It also liberated kinin from heated plasma (Table III) and induced enhanced vascular permeability when injected intradermally into guinea pigs. Acidification had little effect on the clot-promoting activity (Table II). The interaction of plasmin with factor XIIa gave rise to a prealbumin band demonstrable by disc electrophoresis, similar to the prealbumin bands described before (9, 10, 23, 24). Development of PKA upon incubation of plasmin with factor XIIa occurred very rapidly (Fig. 7).

Discussion. It was known for some time that plasmin releases kinin from both fresh and heated plasma (1, 2, 22, 25–27). Some investigators presented evidence that plasmin releases kinin primarily by acting on kininogen (2, 26, 28–30). Webster and Pierce (31) were the first to consider the possibility that plasmin may activate prekallikrein. Vogt (3)

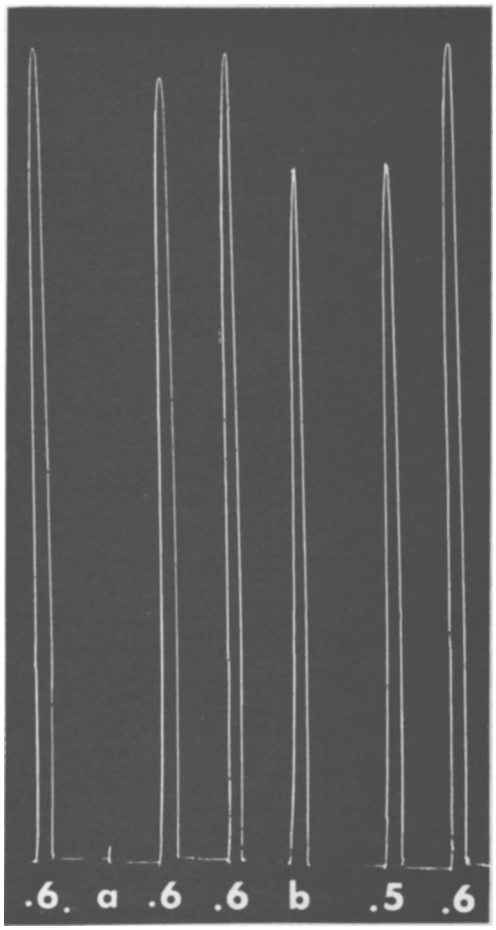

FIG. 5. Kinin assay on the rat uterus; kymograph tracing: (numerals) represent nanograms of bradykinin per milliliter added to the bath. At (a) a mixture of factor XII-deficient fresh plasma and plasmin was applied to the rat uterus (1:500). At (b) an incubation mixture of normal fresh plasma and plasmin was added to the bath (1:1000).

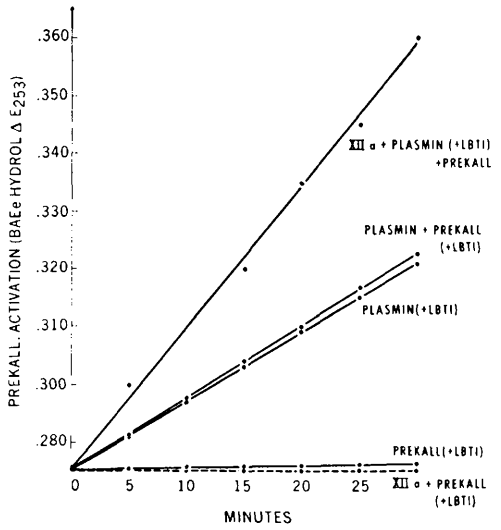

FIG. 6. BAEe hydrolysis of a mixture of factor XIIa, plasmin, lima bean trypsin inhibitor, and prekallikrein and that of appropriate controls. Three tenths milliliter of streptokinase activated plasminogen (plasmin) was incubated first with 0.3 ml of factor XIIa, then the plasmin was inhibited with 12 μg of LBTI followed by incubation with 0.5 ml of prekallikrein. The mixture was then assayed for BAEe hydrolysis.

and more recently Seidel *et al.* (32) have proposed that plasmin acts on prekallikrein, converting it to kallikrein which in turn liberates kinin from kininogen. They based this on the observation that, in the presence of a plasmin stabilizing agent, more kinin was released from fresh than from heated plasma. They concluded that "plasmin is both a kininogenase and a kallikrein activator. However, under natural conditions, in human plasma, only the activator can be efficient." These findings were confirmed by Buluk and Malofiejew (33).

The role of plasma kallikrein vs plasmin in the *in vivo* formation of kinin has been debated for some time, but now there seems to be general agreement that kallikrein forms kinin more rapidly than plasmin and in *in vivo* conditions probably more kinin is formed by kallikrein (27). However, recently, Budnitskaya *et al.* (34) showed that *in vitro* plasmin formed twice as much kinin from fresh plasma as did plasma kallikrein. Plasmin formed more kinin from heated than from fresh plasma.

Based on some of our findings (Tables II and III), the conclusions of Seidel *et al.* are correct. However, the mechanism by which kallikrein is activated by plasmin is an indirect one, *i.e.*, via formation of PKA from factor XIIa. This view is apparent from the data described in this paper and the findings of Kaplan and Austen (4). The question raised by Budnitskaya *et al.* (34) is complicated by the fact that the purity of their plasmin and kallikrein preparations are difficult to assess. It is likely that, on a weight basis, their partially purified plasmin was purer than their kallikrein. Our data presented in Table III would indicate that kalli-

TABLE II. Effect of Plasmin on Factor XIIa.[a]

Sample	Prekallikrein activation (nmoles of BAEe hydrol)	Clotting XII-deficient plasma (sec)
Acidified XIIa + LBTI	0	154
Acidified XIIa + plasmin + LBTI	17.3	290
Plasmin + LBTI	6.2	—
Buffer (VBIS)	—	884

[a] A partially purified preparation of factor XIIa (see Materials and Methods) was acidified by adding 0.5 ml of 0.33 *M* HCl to 1.0 ml of factor XIIa. The pH of this mixture was approx 3.0. After standing at room temperature for 15 min, it was neutralized with 0.5 ml of 0.33 *M* NaOH, followed by dialysis against PBS for 2 hr. Three tenths millimeter of acid-treated factor XIIa, was mixed with 0.3 ml of streptokinase activated plasmin and after 20 min incubation, the mixture and controls were incubated with 0.5 ml of prekallikrein. After (partial) inactivation of the plasmin with 12 μg of LBTI, the mixture was assayed for its ability to hydrolyse BAEe. For clotting tests the incubation mixtures were dialyzed against 0.2 *M* Veronal buffered isotonic saline (pH 7.35) and tested by the partial thromboplastin test as described before [Ref. (18)], using factor XII-deficient plasma for substrate.

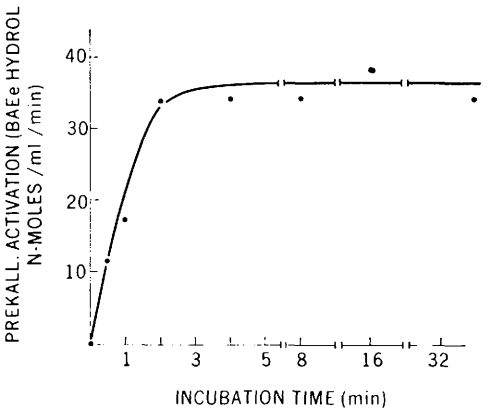

FIG. 7. Time curve of the action of plasmin on factor XIIa: Incubation time of the two reactants is shown on the abscissa. The total reaction mixture was the same as that described in Fig. 6. The ordinate shows the hydrolysis of BAEe.

krein is a more efficient kinin-forming enzyme. Furthermore, while plasmin liberates kinin from kininogen very slowly, liberation of PKA from factor XIIa is maximal within 2 min (Fig. 7). PKA activates prekallikrein very rapidly and in turn the activated kallikrein generates kinin within 30–60 sec (24). However, it still remains to be shown how much kinin can be released from a certain amount of kininogen by plasmin or kallikrein isolated from a given amount of plasma.

Summary. Plasminogen was isolated from acid euglobulin by gel filtration through Sephadex G-200 and anion exchange chromatography on DEAE-Sephadex A-50. Nonspecific esterases were inactivated with DFP and the plasminogen was rechromatographed.

Plasmin (streptokinase activated plasminogen) was shown to act on kininogen (heated plasma), from which it released kinin. This was a slow process. Rapid kinin formation was achieved when plasmin liberated prekallikrein activator from partially purified activated Hageman factor (factor XIIa), which in turn acted on prekallikrein. The active kallikrein enhanced vascular permeability when injected intradermally into guinea pigs, hydrolyzed BAEe, and rapidly liberated kinin from heated plasma substrate.

It is concluded that plasmin in high concentrations liberates kinin directly from kininogen. However, in low concentrations (which prevail *in vivo*), when it functions as an activator, it acts not directly on prekallikrein, as postulated repeatedly, but probably by liberating prekallikrein activator from factor XIIa.

TABLE III. Effect of Plasmin on Factor XIIa.[a]

Sample added to heated plasma	Liberation of kinin (ng/ml of heated plasma)
Acidified XIIa + LBTI + prekallikrein	0
Acidified XIIa + plasmin + LBTI + prekallikrein	240
Plasmin + prekallikrein	<10[b]

[a] Sample was incubated with heated plasma for 1 min. For further details see Tables I and II.

[b] Contractions fell below the dose response given by synthetic bradykinin.

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