

PLASMA PREKALLIKREIN: ISOLATION, CHARACTERIZATION,
AND MECHANISM OF ACTIVATION*

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Kinins are released from their plasma stores by enzymes derived from plasma or other fluids such as saliva, urine, or pancreatic secretion (1, 2). Indirect evidence suggests that activation of kallikrein, the kinin-releasing enzyme in plasma, is an intermediate step in a cascade reaction. However, the number of components reacting in the sequence and their order of participation has been uncertain. In addition, the participation of the kinin-forming system in inflammatory injury has not been defined. In order to gain an understanding of these unknown factors we have chosen to prepare each component of the system in precursor form. In this way, the number, sequence of interaction, and mode of activation may be assessed directly. We will detail here the preparation and characteristics of one of these components, the kinin-generating enzyme, as a single, stable precursor. Comparison of physical parameters of this precursor with active kallikrein shed some light on the mode of activation of this plasma proenzyme.

Materials and Methods

Rabbit Blood.—Rabbits were bled from the ear artery with siliconized needles. The blood was collected in polypropylene centrifuge tubes containing acid-citrate dextrose anticoagulant, pH 6.8. The cells were removed by centrifugation at 3500 rpm for 40 min. Plasma globulins were treated with neutralized and saturated ammonium sulfate containing 0.001 M ethylenediaminetetraacetic acid (EDTA) and hexadimethrine bromide 50 μ g/ml at 4°C. Globulins soluble at 33% saturation and insoluble at 50% saturation were obtained for further study.

Hexadimethrine Bromide.—Hexadimethrine bromide (HBr,¹ Aldrich Chemical Co., Inc.,

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¹ *Abbreviations used in this paper:* AGLMe, acetyl glycyl lysine methyl ester; ALA, acetyl lysine amide; ALMe, acetyl lysine methyl ester; BAFe, benzoyl arginine ethyl ester; BLMe, benzoyl lysine methyl ester; CBZLMe, carbobenzoxy lysine methyl ester; DFP, diisopropyl fluorophosphate; HBr, hexadimethrine bromide; LBTI, lima bean trypsin inhibitor; LMe,

Milwaukee, Wis.) was stored at 4°C under vacuum with desiccation and was dissolved in distilled water to make a stock solution of 10 mg/ml. HBr 50 µg/ml was used to treat surfaces with which the plasma globulins for preparative chromatography were exposed. Dialysis membranes, anion exchangers, gel filtration media, and Pevikon (Pharmacia Fine Chemicals, Uppsala, Sweden) were rinsed thoroughly with HBr for 1 hr and then washed thoroughly.

Glassware.—All glassware was treated with a siliconizing agent (Siliclad, Clay-Adams, Inc., Parsippany, N. J.) and oven dried. Plastic test tubes, columns, vessels, and pipettes were employed whenever possible.

Standard Buffers.—Commonly used isotonic buffers were 0.15 M sodium chloride buffered with 0.01 M phosphate, pH 7.4 (phosphate-buffered saline, PBS), or buffered with 0.01 M tris (hydroxymethyl) aminomethane (Tris), pH 7.4 (Tris-buffered saline, TBS).

Anion Exchange Chromatography.—Diethylaminoethyl (DEAE)-Sephadex A-50 (Pharmacia) 3.5 ± 0.5 mEq/g was hydrated, charged, and equilibrated with 0.0075 M phosphate and 0.001 M EDTA, pH 8. The final ionic strength was 0.01 M. The anion exchanger was packed into plastic 3.7 × 75-cm columns by gravity.

Protein solutions for chromatography were dialyzed, applied to the column, and 100 ml of buffer was allowed to flow in. The rate of flow was maintained with a pump (LKB Varierpex, Bromma, Sweden) at 50 ml/hr. After excluded proteins eluted, a gradient of sodium chloride in phosphate-EDTA buffer was then commenced and the effluent collected.

Cation Exchange Chromatography.—Carboxymethyl (CM)-Sephadex C-50, 4.5 mEq/g was hydrated and equilibrated with citrate-phosphate buffer, pH 6, $\mu = 0.01$. The sample was applied to a 3.1 × 30 cm column of the gel and washed. Proteins were eluted with a linear gradient which utilized 0.25 M sodium chloride buffered with citrate-phosphate in the terminating vessel.

Pevikon-Block Electrophoresis.—Electrophoresis in Pevikon (Pharmacia) was performed in a standard fashion with 0.05 M phosphate, pH 8.0 (3). A constant voltage of 400 v was applied for 20 hr. The block was then sectioned into 1-cm segments and proteins were recovered by centrifugation.

Gel Filtration.—Molecular sieving was carried out on 4 × 100-cm columns of hydrated Sephadex G-200 (Pharmacia). The void volume was determined by blue colored 2 × 10⁶ mol wt dextran and the internal volume of the gels calibrated with marker substances whose diffusion coefficients and molecular weight were known. The eluting buffer was PBS with a constant flow rate of 8–12 ml/hr.

Isoelectric Focusing.—Isoelectric point determinations were performed in siliconized 110-ml columns designed for this purpose (LKB). Appropriate ranges of polyampholytes establishing a range of 2 pH units, usually pH 5–7, were distributed in a 0–45% sucrose gradient. The final ampholyte concentration was 2 g/100 ml. A constant voltage of 500–600 v was applied for 30–48 hr; the temperature was held constant at 10°C. Fractions were collected, the pH determined at 22°C, and the pH of aliquots adjusted by the addition of an equal volume of 0.1 M Tris buffer, pH 7.5, and when necessary, a drop of 0.5 N NaOH.

Ultracentrifugation.—Preparative and analytical ultracentrifugation was carried out in linear gradients of sucrose dissolved in PBS or TBS as described elsewhere (4).

Disc-Gel Electrophoresis.—Polyacrylamide-gel electrophoresis was carried out with materials and buffers described by Davis (5). Gels were stained for protein with Coomassie brilliant blue

lysine methyl ester; OMTI, ovomucoid trypsin inhibitor; PAS, periodic acid-Schiff; PBS, phosphate-buffered saline; PKA, prekallikrein activator; PMSF, phenyl methyl sulfonyl fluoride; Rm, relative mobility; SBTI, soya bean trypsin inhibitor; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TLCK, tosyl lysine chloromethyl ketone; TLMe, tosyl lysine methyl ester; U, unit.

in 10% trichloroacetic acid (TCA). In some instances, proteins were recovered from the gel by eluting successive 2-mm segments of an unstained gel in TBS 0.2 ml overnight at 4°C.

Sodium Dodecyl Sulfate (SDS)-Acrylamide-Gel Electrophoresis.—Molecular weight determinations of prekallikrein incubated with buffer or the prekallikrein activator were determined by SDS-acrylamide-gel electrophoresis (6). Acrylamide 10% and cross-linker 0.2% were used without disulfide reducing agents. After electrophoresis at 8 ma/tube for 4 hr, the gels were stained with Coomassie brilliant blue (7) or placed on dry ice until firm and cut with a gel slicer into 1.3-mm segments.

Glycoprotein (PAS) Staining Procedure.—After electrophoresis in SDS-acrylamide gels, glycoprotein was demonstrated by the periodic acid-Schiff (PAS) method. Proteins were fixed in the gel by several changes of 7% acetic acid: 50% methanol (1:1) for 24 hr. After further washing with 7% acetic acid, the gel was placed in 0.2% periodic acid for 1 hr. The gel was again washed with three changes of acetic acid for 6 hr and placed in the Feulgen reagent for 30 min. The gel was then photographed and the relative mobility (R_m) of PAS-reactive bands was measured.

Radioiodination.—Prekallikrein was trace radiolabeled with ¹²⁵I by a modification of the chloramine-T method (8).

Kinin Assay.—Quantitation of kinins was determined by bioassay on the estrus rat uterus. The amplitude of contractions of unknown samples was recorded on a moving chart (Phipps & Bird, Inc., Richmond, Va.) and compared to given quantities of synthetic bradykinin (BRS 640, Sandoz Ltd., Basel) in Tyrode's solution containing 0.1% gelatin. Samples which contained kinins were also treated with chymotrypsin (Nutritional Biochemicals Corp., Cleveland, Ohio) 10 μg/ml, or carboxypeptidase B (Worthington Biochemical Corp., Freehold, N. J.) 10 μg/ml, for 15 min at 37°C and reassayed.

Kininogen Assay.—Quantitation of kininogen was determined by the amount of kinin released and expressed as equivalents of synthetic bradykinin as measured by bioassay. Aliquots of 0.25 ml containing kininogen were buffered at pH 7.5 with 0.1 M Tris, treated with trypsin 25 μg, and incubated for 1 hr at 37°C; the volume was then brought to 2 ml with buffer and assayed.

Separate tests were performed on 0.25-ml aliquots of kininogen to determine whether kininase or inhibitors of trypsin or kallikrein were present in the preparation. Isolated kininogen was free of these interfering substances. During preparation of kininogen, however, they could be measured to varying degree. When necessary, kininase was inhibited by treatment of the test mixture with 8-hydroxyquinoline to a final concentration of 1 mg/ml. An excess of trypsin was used when trypsin inhibitors were found.

Kallikrein Assay.—The kinin-releasing enzyme was measured by release of kinin from isolated kininogen or hydrolysis of synthetic amino acid esters. (a) The kinin-generating activity of kallikrein was determined with purified kininogen (9). To 0.25 ml of kininogen containing 250- to 1000-ng equivalents, buffered to pH of 7.5, 0.1 ml of kallikrein was added. After incubation at 37°C for 30 min, the reaction was stopped by addition of 100 μg of soya bean trypsin inhibitor (SBTI), the volume brought to 2 ml, and assay for free kinin carried out. Controls included reagents mixed but not allowed to incubate, as well as each reagent incubated alone.

(b) A rapid, accurate quantitative determination of kallikrein was performed by differential spectrophotometry (10) with benzoyl arginine ethyl ester (BAEe). Samples were added to 3.0 ml of a solution containing 1 mmole BAEe/liter in 0.15 M sodium chloride buffered with 0.01 M Tris, pH 7.6. After incubation at 37°C for specific time intervals, the increased absorbance at 253 nm due to hydrolysis of BAEe was compared to known quantities of benzoyl-L-arginine. The amount of enzyme giving hydrolysis of 1 μmole/min at 37°C was taken as 1 unit (U).

The capacity of kallikrein to hydrolyze certain synthetic substrates was investigated by

high voltage paper electrophoresis. In all instances, the amino acids were of the L-configuration and substitution was in the *N*-alpha position when pertinent. The following substrates were purchased (Cyclo Chemical Corp., Los Angeles, Calif., or Mann Research Chemicals, New York): acetyl lysine amide (ALA), acetyl glycyl lysine methyl ester (AGLMe), lysine methyl ester (LMe), acetyl lysine methyl ester (ALMe), benzoyl lysine methyl ester (BLMe), carbo-benzoxy lysine methyl ester (CBZLMe), and tosyl lysine methyl ester (TLMe).

Synthetic substrates 0.05 M were prepared in 0.1 M phosphate buffer, pH 7.5. To 10 μ l substrate was added 0.5 μ g kallikrein in 50 μ l PBS. The reaction mixture was allowed to incubate at room temperature (22°–25°C) and 10 μ l spotted on Whatman chromatography paper 46 \times 57 cm after varying intervals of time. The paper was moistened with pyridine-acetate buffer, pH 6.4, and electrophoresis carried out to the cathode at 1200 v for 75 min. The paper was dried at 65°C and stained with a solution of ninhydrin 2% in acetone. Comparison of the degree of hydrolysis at standard time intervals, usually 1, 3, and 5 hr allowed a relative comparison of the rate of hydrolysis with different substrates.

Prekallikrein Assay.—Prekallikrein required activation for enzymatic detection. Quantitative estimation of this proenzyme, when activated, was the same as for kallikrein.

Activation of Prekallikrein.—Activation was carried out with the prekallikrein activator isolated from plasma or with trypsin. The prekallikrein activator (PKA) was isolated in highly purified form from rabbit or human plasma (11, 12). PKA in TBS, 0.1 ml, was added to aliquots of prekallikrein, incubated at 37°C for 30 min, and the reaction stopped by addition of lima bean trypsin inhibitor (LBTI) 100 μ g in 0.1 ml. As PKA does not hydrolyze BAEe or directly release kinin from kininogen, this activator did not interfere in these tests for kallikrein.

Trypsin (2 \times crystallized, Worthington Biochemicals) was dissolved in 0.001 M HCl at 1 mg/ml. Before use, the trypsin was diluted in 0.1 M Tris buffer, pH 7.5. In a typical experiment, 0.1–0.3 ml aliquots of a sample or fraction believed to contain prekallikrein, 0.1 ml trypsin (1–4 μ g) at pH 7.5 were incubated at 37°C for 15 min. At the end of incubation, lima bean trypsin inhibitor (Worthington Biochemicals) 100 μ g in 0.1 ml was added to inhibit the trypsin. Controls included trypsin alone, trypsin with LBTI, and untreated prekallikrein.

Other substances investigated for the ability to activate prekallikrein were bovine thrombin (Parke, Davis & Co., Detroit, Mich.) and chymotrypsin (Worthington Biochemicals). Human C1s was a gift from Dr. Neil Cooper.

Hydrolysis of Protein Substrates.—Solutions of ¹²⁵I-labeled proteins 4 mg/ml were mixed with kallikrein or trypsin 2 μ g/ml or buffer and incubated at 37°C for 24 hr. The protein was then precipitated with TCA 10% and the ¹²⁵I measured in precipitate and supernatant.

RESULTS

Isolation of Plasma Prekallikrein.—Rabbit arterial blood was freed of cells and salted out with ammonium sulfate. The globulin fraction, soluble at 30% saturation and precipitated at 50% saturation, was dialyzed and applied to a plastic column containing DEAE-Sephadex A-50. The elution characteristics of prekallikrein obtained by a gradient of NaCl are shown in Fig. 1. Prekallikrein eluted entirely in the precursor form since, before activation, fractions failed to liberate kinin from functionally purified rabbit kininogen or to hydrolyze synthetic substrates. After activation by trypsin or the plasma PKA, a single peak of kallikrein was observed in fractions eluting at $\mu = 0.09$. The prekallikrein was pooled and concentrated by precipitation with 60% ammonium sulfate. After overnight dialysis, the proteins were chromatographed on DEAE-Sephadex A-50 at pH 6.6. Fractions were again eluted by a sodium chloride

gradient in phosphate-EDTA buffer. Although the specific activity was increased only slightly by rechromatography on DEAE-Sephadex (Table I), the requirement for hexadimethrine bromide no longer became necessary.

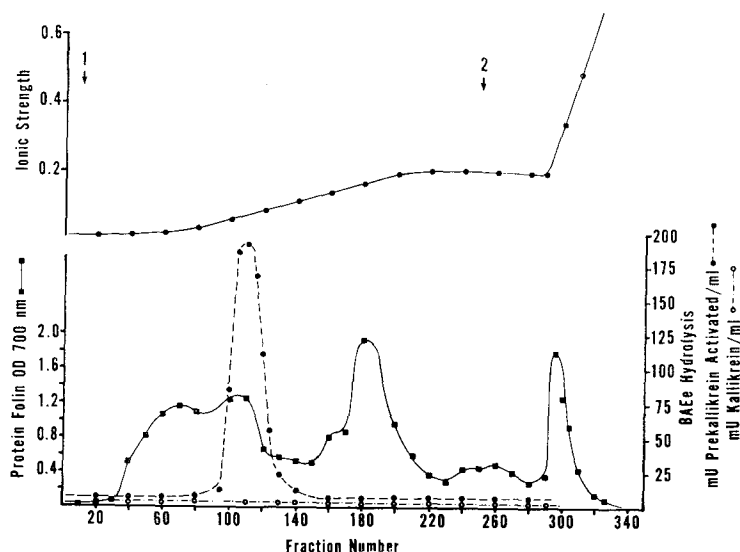


FIG. 1. Chromatography of ammonium sulfate-precipitated globulins on DEAE-Sephadex A-50. A gradient of NaCl to 0.25μ was developed in the starting buffer of phosphate-EDTA 0.01μ beginning at arrow 1. The ionic strength was increased to 1.0μ with NaCl beginning at arrow 2. After activation, kallikrein was quantitated by BAEe hydrolysis.

TABLE I
Isolation of Rabbit Plasma Prekallikrein: Specific Activity, Degree of Purification, and Per Cent Yield

Prekallikrein step	Volume	BAEe hydrolysis	Protein	Specific activity	Purification	Yield
	ml	U/ml	mg/ml	U/mg		%
Plasma	1400	1*	80.0	0.013	1*	100
DEAE-Sephadex I	93	12.6	30.8	0.41	32	83.7
DEAE-Sephadex II	144	5.8	12.4	0.47	36	59.6
CM-Sephadex	176	2.6	0.2	13.0	1000	31.3
Pevikon	14.6	5.7	0.07	81.0	6200	5.9

* Estimated, 1 U/ml.

The pooled fractions of prekallikrein were dialyzed against citrate-phosphate buffer and applied to a column of CM-Sephadex C-50 equilibrated at pH 6.0. The elution of prekallikrein from the cation exchanger is given in Fig. 2. The kallikrein precursor eluted in a single peak at an ionic strength of $\mu = 0.14$. The specific activity increased approximately 25-fold (Table I).

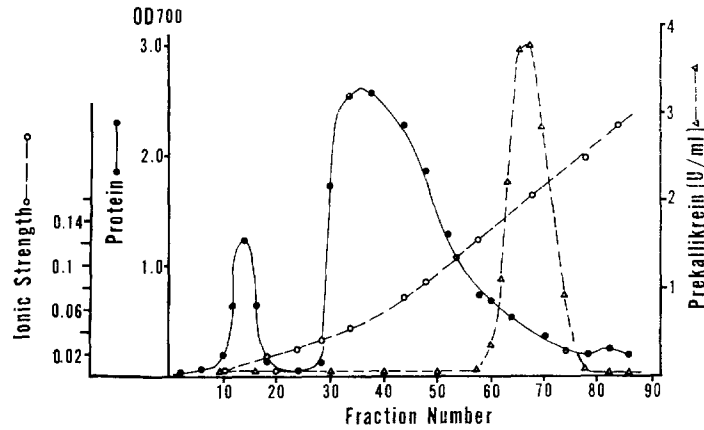


FIG. 2. Chromatography on CM-Sephadex C-50 of prekallikrein pooled from anion exchange chromatography. After excluded proteins eluted, a gradient of NaCl to 0.25μ was developed in the starting buffer of citrate-phosphate of 0.01μ . After activation, kallikrein was quantitated by BAEe hydrolysis and expressed in units per milliliter.

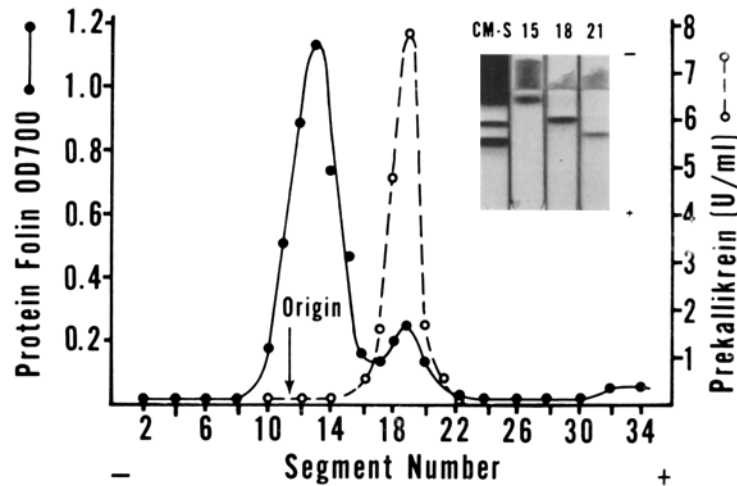


FIG. 3. Pevikon-block electrophoresis of prekallikrein pooled and dialyzed after cation exchange chromatography. After treatment with the prekallikrein activator or trypsin (see Materials and Methods), kallikrein was found to correspond to the smaller peak of protein. The insert shows 7.5% acrylamide-gel electrophoresis of the sample applied (CM-S) and fractions 15, 18, and 21.

The fractions containing prekallikrein from CM-Sephadex were concentrated to 6 ml by ultrafiltration, dialyzed, and subjected to the final purification procedure, electrophoresis in Pevikon. The results are depicted in Fig. 3. The specific activity increased to 81 BAEe U/mg protein (Table I). Prekallikrein

was freed from the major contaminating protein, gamma globulin, and corresponded to a small protein peak of γ_1 electrophoretic mobility. Analysis of the fractions containing prekallikrein by analytical acrylamide-gel electrophoresis revealed a single major band of protein in these fractions. Successive 2-mm cuts from a gel were eluted in buffer overnight and assayed before and after incubation with PKA with BAEe or functionally purified kininogen substrates. Kinin release and ester hydrolysis corresponded to the stained protein band (Fig. 4).

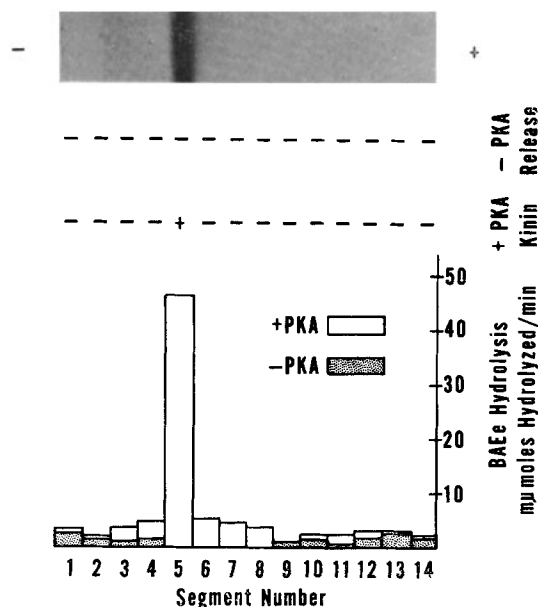


FIG. 4. Polyacrylamide-gel electrophoresis 7.5%, pH 8.3, of isolated prekallikrein. The upper gel, stained for protein with Coomassie blue in trichloroacetic acid, was cut at the tracking dye; a parallel gel was cut in 2-mm segments, eluted, and tested for kallikrein and esterase activity with or without the addition of the prekallikrein activator.

The prekallikrein remained stable at 4°-8°C for several months in its highly purified form. No spontaneous activation occurred. Addition of PKA or trypsin activated predictable amounts of kallikrein activity.

Activation of Prekallikrein.—Direct activation of prekallikrein was performed by treatment with (a) highly purified PKA and (b) bovine pancreatic trypsin. When kallikrein activity was generated, two activities were readily measured, namely, the hydrolysis of synthetic amino acid esters and the capacity to release a kinin from purified kininogen. As ester hydrolysis was rapidly and quantitatively performed spectrophotometrically, kallikrein was quantitated by this means (*vide infra*). When a solution of isolated prekallikrein 68 $\mu\text{g/ml}$ was

TABLE II
Activation of Plasma Prekallikrein

Substance added	Amount added	Preallikrein*	Incubation	BAEe hydrolysis
			<i>min</i>	<i>μmoles/min</i>
None		+	0	2.4
			12	2.4
PKA‡	1.1 μg	+	0	3.6
			6	103.6
			12	196.4
PKA	0.55 μg	+	0	3.6
			6	64.3
			12	121.4
PKA	1.1 μg	—	12	0.0
Trypsin‡	1 μg	—	20	0.0
Trypsin	1 μg	+	0	2.6
			5	52.4
			10	74.5
			20	96.9
Chymotrypsin‡	2 μg	—	30	0.0
	2 μg	+	30	0.0
	0.5 μg	+	30	0.0
Thrombin§	2.5 U	—	30	33.3
Thrombin	2.5 U	+	30	31.4
	1.3 U	+	30	8.1
C1 esterase§	10.6 μg	—	30	47.9
C1 esterase	10.6 μg	+	30	40.4
	5.3 μg	+	30	25.3
	2.7 μg	+	30	14.7

* Prekallikrein was added (+) at 68 μg/ml, or substituted with TBS (—). All incubations performed at 37°C, pH 7.5.

‡ After incubation with the prekallikrein activator (PKA), trypsin, or chymotrypsin, the reaction was stopped by addition of 100 μg LBTI 0.1 ml.

§ BAEe hydrolysis was determined 30 min after addition of thrombin or human C1 esterase. No inhibitor was added. Ester hydrolysis did not increase over control values after incubation with these agents.

treated with varying quantities of PKA containing 0.5–1.0 μg in 50 μl and the reaction stopped by transferring an aliquot to 100 μg of LBTI, kallikrein was generated as shown in Table II. The reaction was carried out at pH 7.4, ionic strength of 0.1, at 37°C. If the LBTI was added immediately after mixing,

kallikrein generation was not observed. If the inhibitor was added after approximately 30 min, full activation could be measured, showing that LBTI was not inhibiting kallikrein. Hydrolysis of BAEe or kinin release from kininogen was not observed in controls of PKA incubated with buffer.

The second substance shown to activate prekallikrein was trypsin. Addition of 1 μg of trypsin in 0.1 ml to prekallikrein rapidly generated activity in the preparation. The interfering effects of trypsin in hydrolysis of BAEe or release of kinin were eliminated by addition of LBTI or ovomucoid trypsin inhibitor (OMTI) 1 mg/ml. These inhibitors had no effect on the detection of kallikrein once formed. The time course of activation of a solution containing 60 μg of prekallikrein/ml by 1 μg of trypsin is shown in Table II.

Other enzymes were investigated to determine whether they could activate prekallikrein (Table II). These included (a) bovine chymotrypsin, (b) bovine thrombin, and (c) human C1s. These substances failed to activate the proenzyme.

The possibility of autoactivation was investigated by incubating 0.5 μg of kallikrein with 5 μg of prekallikrein; the mixture was sampled at 1, 2, and 6 hr. The reaction was held at neutral pH in PBS at 22°C; autoactivation was not observed.

Characterization of Plasma Prekallikrein.—Physical properties of prekallikrein and its active product were determined by gel filtration, sucrose gradient ultracentrifugation, and isoelectric focusing in polyampholytes. The two substances were subjected to these procedures both singly and simultaneously without apparent difference. When tested together, a mixture of prekallikrein and kallikrein was prepared so as to contain a 3- to 4-fold greater quantity of the proenzyme. The active enzyme was assayed first; then the precursor was activated and measured. When necessary, the values due to kallikrein were subtracted to determine the contribution for the proenzyme alone.

Molecular sieve chromatography: When prekallikrein and kallikrein were co-chromatographed on a calibrated 4 \times 100 cm column of Sephadex G-200, the precursor consistently emerged approximately 8 ml before the kallikrein (Fig. 5). IgG, bovine albumin, and equine heart cytochrome C, substances used to calibrate the column, were used to determine the diffusion coefficients of prekallikrein and kallikrein (Fig. 6). When the log molecular weight was plotted as a function of the elution volume (13), the kallikrein had a mol wt approximately 10,000–15,000 daltons less than its precursor.

Ultracentrifugation: Simultaneous ultracentrifugation of kallikrein and prekallikrein was performed in linear gradients of sucrose 5–25% in PBS. A sample 0.2 ml to which internal markers IgG globulin-¹²⁵I and bovine albumin-¹²⁵I were added was centrifuged at 45,000 rpm for 15 hr. Based on known sedimentation rates for the marker substances of 6.8 and 4.45, respectively, the kallikrein was observed to sediment at 4.0S and the prekallikrein at 4.5S (Fig. 7). Assuming a partial specific volume of 0.73, the molecular weights of the

PLASMA PREKALLIKREIN

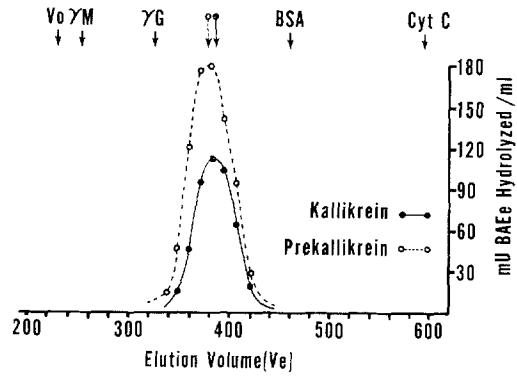


FIG. 5. Gel filtration on Sephadex G-200 of a mixture of prekallikrein and kallikrein. The peak of elution of the marker substances is indicated: \bar{V}_o , blue dextran; γ M, IgM globulin; γ G, IgG globulin; BSA, bovine albumin; and Cyt C, horse heart cytochrome c.

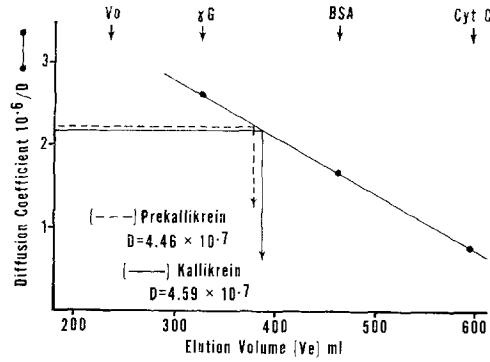


FIG. 6. Diffusion coefficients of prekallikrein and kallikrein determined by gel filtration on Sephadex G-200. Abbreviations as in Fig. 5; D is diffusion coefficient in cm^2/sec .

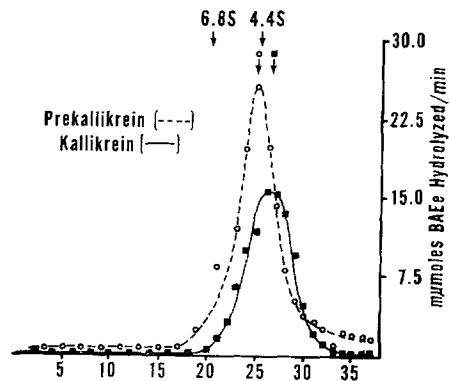


FIG. 7. Sucrose gradient ultracentrifugation of prekallikrein and kallikrein cosedimented with the internal markers IgG globulin- ^{131}I and bovine albumin- ^{125}I .

two substances were calculated from their diffusion and sedimentation coefficients (Table III). For rabbit prekallikrein, the molecular weight was 99,800 daltons; the kallikrein was 86,300 daltons.

Isoelectric focusing: Prekallikrein and kallikrein had an isoelectric point between 5 and 6 when these substances were individually focused in polyampholytes. Analysis of a mixture of the two substances containing 100 μg of prekallikrein and 35 μg of kallikrein is shown in Fig. 8. The isoelectric point was determined to be 5.9–6.05 for prekallikrein and 5.35–5.4 for kallikrein.

Chemical Properties of Prekallikrein.—Kallikrein and its precursor readily

TABLE III
Physical and Chemical Properties of Prekallikrein and Fragments Released by PKA

	Prekallikrein	Kallikrein	Fragment
Electrophoretic mobility	γ_1	γ_1	ND*
Sedimentation coefficient	4.5	4.0	0.9
Diffusion coefficient ($\times 10^{-7}$ cm ² /sec)	4.46	4.59	13.0
Molecular weight: (a) ‡	99,900	86,300	6,900
(b)	100,000	88,000	11,000
(c)	102,000	91,000	11,600
Isoelectric point	5.9	5.4	ND
BAEe, U/mg protein	81	92	0

* ND, not determined.

‡ (a) Calculated from S and D, v assumed 0.73; (b) SDS-gel electrophoresis; (c) determined on Sephadex G-150 by the method of Andrews (13).

stained with the protein dyes Coomassie blue and Buffalo black and absorbed in the ultraviolet region characteristic for the peptide bond. To determine whether prekallikrein-¹²⁵I contained carbohydrate, 5- μg quantities were subjected to SDS gel electrophoresis and stained by the PAS method. A single band which stained with this procedure corresponded (R_m 0.22) to a similar band in a separate gel stained with Coomassie blue and to the peak of ¹²⁵I label as determined in a third gel cut in 1.3-mm segments.

Mechanism of Prekallikrein Activation.—In order to study the mechanism whereby the prekallikrein activator generates kallikrein from its precursor, products of this interaction were resolved by SDS-acrylamide-gel electrophoresis and by ultracentrifugation. Aliquots of prekallikrein-¹²⁵I 5 μg in 0.1 ml were incubated with buffer or PKA 25 μl containing approximately 0.5 μg

of protein for 40 min at 37°C. A 10 μ l aliquot was then withdrawn from each tube to determine that activation had taken place. The samples were then mixed with bromphenol blue, glycerine, and SDS 0.1% and electrophoresed.

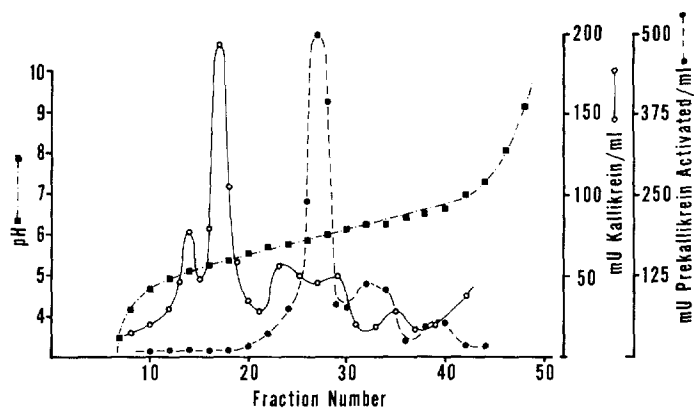


FIG. 8. Isoelectric focusing of prekallikrein and kallikrein; 0°C, pH 5-7, 500 v, 40 hr.

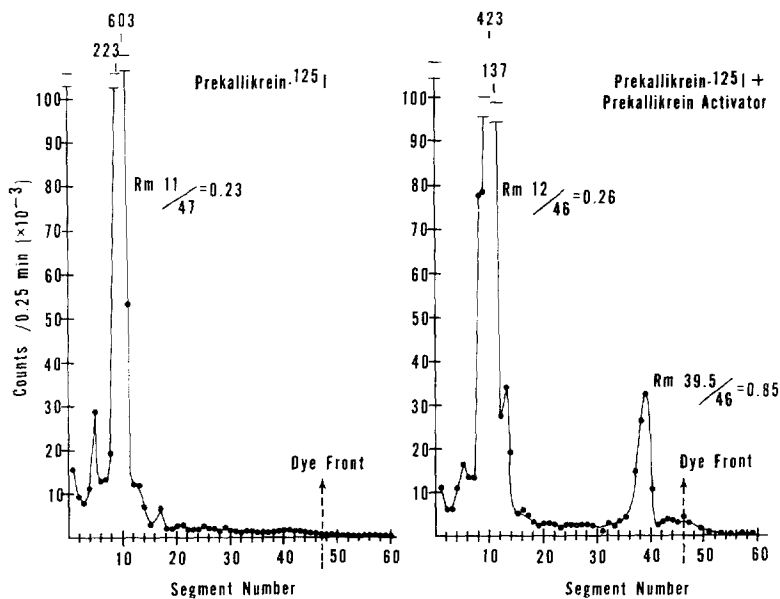


FIG. 9. SDS-acrylamide-gel electrophoresis of prekallikrein- 125 I incubated with buffer (*left panel*) or PKA (*right panel*).

When the gels were cut and the 125 I counts determined, the precursor was observed to be cleaved into two labeled moieties containing 90.5 and 9.5% of the label, respectively (Fig. 9).

When the log molecular weight was plotted as a function of the Rm and compared with substances of known molecular weight (Fig. 10), kallikrein and the fragment released by PKA had mol wt of 88,000 and 11,000, respectively.

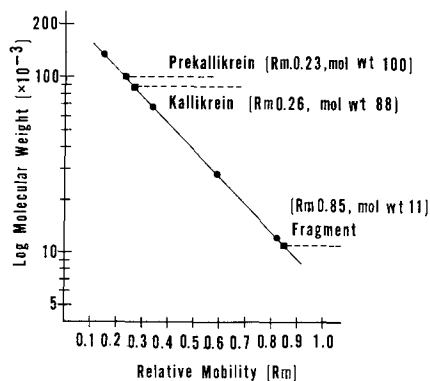


FIG. 10. Molecular weights of prekallikrein and fragments released by PKA plotted as a function of relative mobility. The Rm of marker substances cytochrome c, egg white trypsin inhibitor, bovine albumin, and albumin dimer is the mean of four determinations.

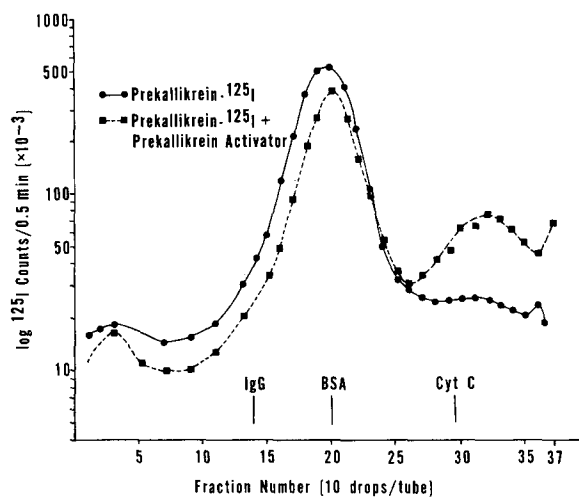


FIG. 11. Sucrose gradient ultracentrifugation of prekallikrein-¹²⁵I incubated with buffer or PKA. A third gradient contained marker substances IgG, albumin, and cytochrome c.

Additional direct evidence for limited proteolysis of rabbit prekallikrein by PKA was obtained by gradient ultracentrifugation in sucrose. Prekallikrein-¹²⁵I 10 μ g preincubated with buffer or with PKA was applied to the top of separate gradients of sucrose and sedimented. A third gradient contained marker substances. The results are given in Fig. 11. After activation, two labeled frag-

ments sedimenting at 4.2S and 0.9S were observed. Kallikrein activity was associated with the larger fragment. Unactivated prekallikrein sedimented at 4.5S.

Hydrolysis of Synthetic Substrates.—Isolated rabbit plasma kallikrein hydrolyzed synthetic amino acid substrates of arginine and lysine at pH 7.6. Product formation was measured spectrophotometrically or by high voltage electrophoresis.

The methyl or ethyl esters of *N*- α -benzoyl-substituted arginine were superior substrates for quantitation of rabbit kallikrein. Linear initial velocities were obtained when 0.63–6.25 μ g of kallikrein was added to BAEe 0.001 M at 25°C,

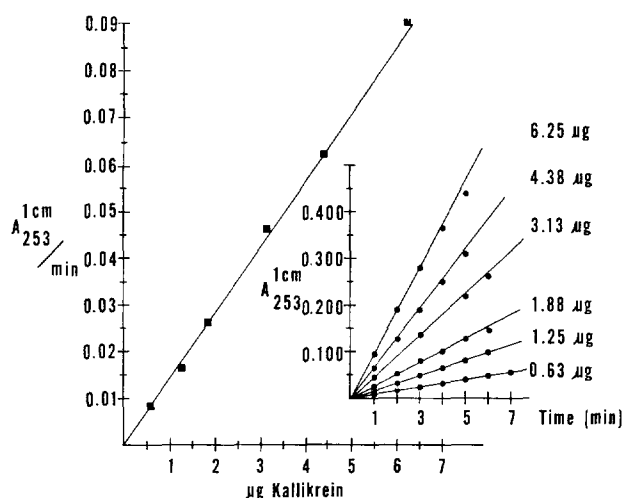


FIG. 12. Hydrolysis of mM BAEe pH 7.6, 25°C by varying amounts of rabbit plasma kallikrein. Initial velocities determined at minute intervals are shown in the insert. The molar extinction coefficient for BAEe is 840.

μ 0.15, at pH 7.6. Under these conditions, the velocity of substrate hydrolysis bore a linear relationship to the quantity of enzyme added (Fig. 12). At 25°C, 1 mg of rabbit kallikrein hydrolyzed 51.4 μ M BAEe/min.

The hydrolysis of certain synthetic substrates, not conveniently measured by spectrophotometry, were observed by high voltage electrophoresis on paper. Aliquots of substrate alone or substrate to which 0.5 μ g of kallikrein was added were subjected to electrophoresis at 1, 3, and 5 hr (Fig. 13). When the rates of hydrolysis were compared, the following rank order was observed: AGLMe, CBZLMe > BLMe, ALMe > TLMe > LMe. ALA was not cleaved under these conditions.

Inhibition of Kallikrein.—The effect of certain trypsin inhibitors on the esterolytic activity of rabbit kallikrein is given in Table IV. The trypsin in-

hibitors diisopropyl fluorophosphate (DFP), Trasylol (FBA Pharmaceuticals, New York) and SBTI were effective inhibitors of kallikrein at low molarity. By contrast, other inhibitors of trypsin, namely, LBTI, egg white trypsin inhibitor (OMTI), tosyl lysinechloromethyl ketone (TLCK), and phenyl methyl sulfonyl fluoride (PMSF), were not kallikrein inhibitors. Each inhibitor was separately tested with trypsin to verify its potency.

Hydrolysis of Protein Substrates.—After prolonged incubation of protein solutions with kallikrein, small quantities of TCA-soluble ^{125}I -labeled peptides were released from albumin (2.5%), gamma globulin (2.0%), or hemoglobin (< 1%). By contrast, 61% of labeled gamma globulin was TCA soluble after incubation with trypsin under identical conditions.

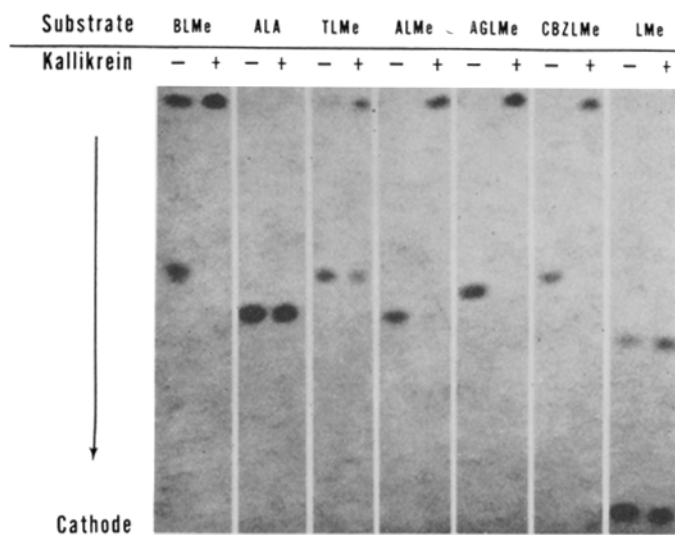


FIG. 13. High voltage electrophoresis of synthetic substrates incubated with buffer or plasma kallikrein for 5 hr at room temperature.

DISCUSSION

The importance of these investigations of prekallikrein in rabbit plasma are as follows. (a) The glycoprotein was isolated from other members of the kinin-forming system and purified approximately 6000-fold. The final product was free from detectable contaminants, yielding a single band in polyacrylamide-disc electrophoresis. (b) Only one prekallikrein was found in plasma that could be activated by the prekallikrein activator derived from purified Hageman factor. (c) Prekallikrein was activated by PKA through enzymatic cleavage into two polypeptide fragments measuring 88,000 and 11,000 daltons. (d) The larger fragment, kallikrein, behaved as a trypsin-like enzyme with restricted specificity.

The Kallikrein Precursor.—In early attempts to purify precursor components of the kinin system, apparently spontaneous and complete activation of pre-kallikrein took place. The difficulty was overcome by use of plastic vessels throughout and pretreatment of containers and separatory media with hexadimethrine bromide. Presumably, one of the initial components of the kinin-

TABLE IV
Inhibition of Rabbit Kallikrein by Trypsin Inhibitors

Substance tested	Final concentration	BAEe hydrolysis*	Inhibition
	moles/liter	μ moles/min	%
None	—	196	—
DFP	1×10^{-3}	36	81
	5×10^{-4}	69	65
	2.5×10^{-4}	105	46
	1.25×10^{-4}	132	32
Trasylol	2.5×10^{-6}	13	93
	1.25×10^{-6}	51	74
	6.25×10^{-7}	115	41
	3.12×10^{-7}	159	20
SBTI	2.5×10^{-6}	71	64
	1.25×10^{-6}	86	56
	6.25×10^{-7}	107	44
	3.12×10^{-7}	146	25
LBTI	5×10^{-5}	192	2
OMTI	5×10^{-5}	199	0
TLCK	5×10^{-5}	199	0
PMSF	5×10^{-5}	185	5

* Initial velocity of 0.1 ml of kallikrein incubated 15 min with buffer or inhibitor 0.1 ml and assayed on mM BAEe, pH 7.6, 25°C, μ 0.15.

forming system is easily activated. From the pioneer work of Margolis (14) and Ratnoff and Miles (15), we suspected that blood-clotting factor XII (Hageman factor) might be the labile or easily activated substance. Hexadimethrine bromide was selected as a known inhibitor of the surface activation of Hageman factor (16, 17). Using the techniques described in this report and in abridged form elsewhere (11, 4), it has been possible to prepare prekallikrein consistently from rabbit and human plasma.

Others have obtained a partial purification of prekallikrein or a mixture of

the precursor and its activated moiety. Prekallikrein (kallikreinogen, prokininogenase) has been partially purified from guinea pig serum and bovine plasma. Davies and Lowe (18) and Davies, Holman, and Lowe (19) obtained guinea pig prekallikrein in semipurified form by a one-step method on DEAE-cellulose. Their final product consisted of a mixture of kallikrein and prekallikrein. The activity was augmented by incubation with aged albumin-rich serum fractions. Nagasawa et al. purified bovine plasma prekallikrein by chromatography on DEAE- and CM-Sephadex. The bovine prekallikrein was specifically activated by bovine Hageman factor (20).

Characterization of Prekallikrein.—The proenzyme behaved as a single homogenous molecular species throughout all preparative and analytical techniques. The molecular weight of the precursor was estimated by three methods. There was close agreement between the results obtained by molecular sieve chromatography (98,000–102,000 daltons), by calculation from sedimentation and diffusion rates (99,900 daltons), and by SDS-acrylamide-gel electrophoresis (98,000–106,000 daltons). Prekallikrein migrated in Pevikon at pH 8.1 with γ_1 -globulins. The electrophoretic mobility of prekallikrein in various supporting media was in keeping with its isoelectric point measured to be 5.9–6.05 in polyampholytes.

The periodic acid-Schiff method was used to detect carbohydrate in prekallikrein after electrophoresis in SDS-acrylamide gels. A single PAS-positive band was found at the same relative mobility as the band staining with Coomassie blue dye and with the peak of ^{125}I label in cut gels (Rm 0.22). Rabbit prekallikrein is thus a glycoprotein.

Comparison of Human and Rabbit Prekallikrein.—We have applied the methods described here to the isolation of human prekallikrein. The isolated proenzyme again corresponded to a small peak of protein migrating anodally to gamma globulin after electrophoresis in Pevikon similar to that in Fig. 3. After activation with human PKA, esterolytic activity corresponded exactly to this protein peak. In contrast to rabbit prekallikrein, human prekallikrein had a sedimentation coefficient of 5.2 S and a calculated mol wt of 107,000 by SDS-acrylamide gel electrophoresis. The isoelectric point was 7.7. After activation, there was no observable difference in sedimentation coefficient or molecular weight.

Other studies support the concept of a single kallikrein despite activation of the molecule in plasma. Human plasma kallikrein, purified by Webster (21) from acetone-activated plasma, appeared to be a single substance by its elution from DEAE-cellulose and Amberlite IRC-50. Porcine plasma kallikrein was investigated by Habermann and Klett (22). The isolated enzyme had a calculated mol wt of 86,000 and migrated as a $\gamma_1\text{-}\gamma_2$ globulin. The enzyme rapidly hydrolyzed BAEe (54 $\mu\text{g}/\text{min}$ per mg) at 37°C. By contrast, Colman, Mattler, and Sherry (23), in studies of β_2 -kallikrein in human plasma, described three

substances behaving as a kallikrein. The three kallikreins of these authors differed in charge, molecular weight, and their capacity to cleave synthetic esters. The possibility that at least two of the kallikreins were related was shown immunologically. Our findings based on an analysis of the precursor prekallikrein in both rabbit and human plasma revealed only one kallikrein in plasma, corresponding most closely to kallikrein I of Colman et al. (23).

The Activation of Prekallikrein.—We believe the present report provides the first direct evidence that prekallikrein is activated by a process of limited proteolysis. This conclusion is based on the finding of distinct physical changes in the molecule upon activation and upon the appearance of a split fragment. An increased diffusion coefficient, decreased average Stoke's radius, and decreased sedimentation coefficient were found to accompany activation. Treatment of highly purified prekallikrein, radiolabeled with ^{125}I , with the prekallikrein activator resulted in cleavage of the molecule into two labeled fragments. The fragments were resolved by gradient ultracentrifugation, gel filtration, and SDS-acrylamide-gel electrophoresis. The parent molecule was found to be cleaved into fragments of approximately 88,000 and 11,000 daltons. The larger fragment possessed kallikrein activity. The biologic importance of the smaller fragment is being investigated currently.

Additional evidence supporting the postulation that prekallikrein is activated by limited proteolysis stems from an analysis of its activator, PKA. PKA has been isolated and characterized in this and other laboratories (4, 11, 12, 24–26). As will be reported in detail separately, PKA is a 2.6S acidic pseudoglobulin with an isoelectric point of 4.6 and a calculated mol wt of 31,800 daltons. We have recently isolated the precursor of the prekallikrein activator directly from human and rabbit plasma (27). In agreement with the findings of Kaplan and Austen (24, 28), this molecule was identified as Hageman factor (27). The enzymatic nature of PKA was supported by: (a) the effect of temperature and substrate concentration on the kinetics of its activation of prekallikrein (29); (b) its inhibition by DFP and certain trypsin inhibitors; and (c) ability to hydrolyze synthetic amino acid esters.

Kallikrein Substrates and Inhibitors.—Kallikrein generated from isolated rabbit prekallikrein hydrolyzed esters of *N*-substituted lysine or arginine. Indeed, rabbit kallikrein hydrolyzed BAEe (92 $\mu\text{moles}/\text{min}$ per mg) at 37°C more efficiently than trypsin (31 $\mu\text{moles}/\text{min}$ per mg). However, the action of purified kallikrein was restricted. This was best demonstrated by the finding that kallikrein failed to liberate substantial peptides from gamma globulin while trypsin cleaved large amounts of peptides in the same period of incubation.

The spectrum of substances inhibitory for kallikrein was restricted to the trypsin inhibitors, DFP, SBTI, and Trasylol. Four other trypsin inhibitors, LBTI, OMTI, TLCK, and PMSF, were without effect on kallikrein. Rabbit

kallikrein is thus a trypsin-like enzyme of restricted specificities for substrates and inhibitors.

SUMMARY

The precursor of the kinin-forming enzyme, prekallikrein, was isolated from rabbit plasma protected from activation during preparatory procedures. Prekallikrein was shown to be a 4.5S γ_1 -glycoprotein with an isoelectric point of 5.9 and a mol wt of 99,900. The proenzyme was activated at neutral pH by an enzyme from rabbit or human plasma we have termed prekallikrein activator (PKA) or by trypsin. Prekallikrein was activated by PKA by a process of enzymatic scission. This resulted in the appearance of two fragments; the larger of these possessed kallikrein activity.

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