Studies on the Prekallikrein (Kallikreinogen)–Kallikrein Enzyme System of Human Plasma

I. ISOLATION AND PURIFICATION OF PLASMA KALLIKREINS

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ABSTRACT By measurement of its arginine esterase activity, plasma kallikrein was purified from fresh frozen ACD plasma. The steps involved alcohol fractionation, isoelectric precipitation, and carboxymethyl (CM) Sephadex and DEAE cellulose chromatography. Three enzymatically active fractions were finally isolated and termed plasma kallikreins I, II, and III; they represented purifications of 970,320- and 590-fold, respectively. All three kallikreins were active biologically; they increased vascular permeability in the guinea pig and released a kinin from human plasma, as measured in the rat uterus bioassay. Bradykinin and/or closely related kinins were identified in the kallikrein I plasma digest by radioimmunoassay.

Kallikreins I, II, and III had similar ratios of hydrolytic activity on a variety of arginine and lysine esters and were immunochemically related. However, differences were present on physicochemical characterization: kallikrein I had $S_{20,w}$ of 5.7, a mol wt of 99,800, and migrated as a slow gamma globulin; kallikrein II migrated as a fast gamma globulin with a mol wt of 163,000, but the evidence suggested that it was closely related, if not interconvertible, with kallikrein I. Kallikrein III, on the other hand, migrated as an alpha globulin and reacted quite differently with inhibitors.

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INTRODUCTION

Kallikrein (1) is the name of a class of proteolytic enzymes that effect the release of vasodepressor peptides or kinins from a plasma α_2-globulin substrate, termed kininogen. These peptides, including bradykinin, are extremely potent pharmacological agents. In nanogram quantities they not only produce profound vasodilation but act on other smooth msucles including the uterus and small intestine (2). The kinins also are known to increase capillary permeability, produce pain, and influence the migration of leukocytes (3). A mechanism for rapid inactivation unique for this group of pharmacologically active peptides involves the cleavage of the terminal arginine by a plasma carboxypeptidase (kininase I) or the terminal dipeptide phenylalanine-arginine by kininase II (4). In vitro, purified pancreatic carboxypeptidase B will also destroy the biological activity of the kinins (5) by the former mechanism.

Human pancreatic, urinary, and plasma kallikreins have been described but Webster, Emmart, Moriya, and Pierce have shown these to be different by immunological techniques (6), susceptibility to proteolytic inhibitors (7), ability to hydrolyze synthetic substrates (8), and electrophoretic mobility (9). Although the urinary and pancreatic enzymes have been partially purified, little progress has been made in the isolation and purification of plasma kallikrein, which normally exists in plasma in the form of an inactive precursor, prekallikrein or kallikreinogen.[1]

[1] The International Union of Biochemistry recommends the use of prekallikrein instead of kallikreinogen, however this term is not yet widely accepted. In this work we adhere to the more common usage, kallikreinogen.

Recently, Sherry, Alkjaersig, and Fletcher (10) described an arginine esterase in human siliconized plasma that is present only after glass contact or exposure to kaolin, and that did not evolve in Hageman factor (factor XII)–deficient plasma. Since Hageman factor has been shown to be necessary for release of plasma kinin (11), and plasma kallikrein is a known arginine esterase (8), they postulated that active factor XII was necessary for the activation of this arginine esterase, and suggested that the latter enzyme may be related to plasma kalikrein or the permeability factor (PF/dil). In order to ascertain the identity of the arginine esterase, we made an extensive investigation that establishes this activity as plasma kallikrein. The results of this and associated studies are reported in the present paper and the one following it.

The present article is concerned with the isolation, purification, and characterization of human plasma kallikrein(s). As will be shown, purification of this enzyme, based on its arginine esterase activity, resulted in its fractionation into three different components, each with kallikrein activity, as determined by the ability to release kinin from plasma when measured by bioassay and immunoassay. Since the purity of these preparations considerably exceeds levels previously obtained, characterization of the properties of this group of enzymes has been partially achieved. The second paper (12) consists of a detailed study of the activation and inhibition of the kaolin-activated arginine esterase enzyme system of plasma and the identification of this arginine esterase activity as plasma kallikrein(s). In addition, the properties of a closely related plasma arginine esterase, activated at low ionic strength and perhaps identical to PF/dil, were examined and the relation of this enzyme to plasma kallikrein further delineated.

METHODS

Substrates. Acetyl tyrosine ethyl ester (ATEe), acetyl tyrosine methyl ester (ATMe), and the following monohydrochloride salts of the substituted methyl esters of L-arginine and L-lysine were synthesized by Dr. H. Plaut (Cyclo Chemical Corp., Los Angeles, Calif.) : acetyl–arginine methyl ester (AAMe), benzoyl–arginine methyl ester (BAMe), tosyl–arginine methyl ester (TAMe), carbobenzoxy–arginine methyl ester (CBZ-AMe), acetyl–lysine methyl ester (ALMe), and tosyl–lysine methyl ester (TLMe). The substrates were homogeneous chromatographically, and all had an elemental analysis consistent with their formulas. All substrates were crystalline, except the acetyl-arginine derivative that was a thick oil. A dry standardized preparation of AAMe was prepared as previously described (13).

Inhibitors. Heparin sodium, USP 160,000 U/g;[2] hexadimethrine (Polybrene), 10 mg/ml;[3] diisopropyl fluorophosphate (DFP), 5.5 M;[4] tosyl-lysyl chloromethylketone hydrochloride (TLCK);[5] beef lung inhibitor (Trasylol), 5000 KI units/ml;[6] chicken ovomucoid;[7] and pancreatic trypsin inhibitor (PTI) and soybean trypsin inhibitor (SBTI), crystallized salt free,[8] were used in concentrations indicated in the text.

Chromatography. Diethylaminoethyl (DEAE) cellulose with a capacity of 0.9–1.0 meq/g was obtained from two sources.[9, 10] No differences were noted in the behavior of these two anion exchangers. The DEAE cellulose was washed and columns were packed as described by Sober, Gutter, Wyckoff, and Peterson (14) with buffers that are indicated in the legends to the figures. Sephadex G-200 beads, 40–120 μ,[11] and carboxymethyl (CM) Sephadex C-50, medium, capacity 4.5 meq/g,[11] were swollen in appropriate buffers for more than 72 hr and were packed by gravity according to the instructions of the manufacturer. Blue dextran 2000 [11] was used to measure the void volume of the G-200 column and to detect flaws in the packing. All column chromatography and gel filtration procedures were carried out at 2°–5°C. Concentration of the column eluates was accomplished by ultrafiltration under positive pressure, using a commercial device.[12] Protein concentration was determined by measuring the absorbancy of solutions at 280 mμ in a Beckman DU-spectrophotometer.

Antisera. Rabbit anti α_1-antitrypsin antiserum,[13] anti α_2-macroglobulin antiserum,[14] and goat polyvalent antiserum,[15] were used in the immunodiffusion and immunoelectrophoresis experiments.

Plasma fractionation. Fresh frozen pooled human plasma that was anticoagulated with acid citrate dextrose solution in plastic packs was used, about 700-ml in a typical experiment. Fraction IV-1 was prepared by the method of Cohn and associates (15). Further fractionation of fraction IV-1 was accomplished by the method of Kominz (16) that was originally designed for the purification of ceruloplasmin: fraction IV-1, a paste containing 3.5 g protein in 40 ml, was suspended in 200 ml (5 volumes) of 0.06 M NaCl. The precipitate was removed by centrifugation and the supernatant was adjusted to pH 4.8 with an acetate buffer (pH 4, $\mu = 0.8$). Ethanol was added to a final concentration of 15% as the temperature was reduced to −5°C. The blue green precipitate served as starting material for chromatographic procedures. Lyophilization was performed with 10–15% loss of activity.

Measurement of esterase activity. The assay used is described in detail in the accompanying paper (12). With plasma, or in the early stages of purification, all preparations were activated with kaolin for 1 min before assay. After contact with the ion exchangers used for chromatography, all fractions became fully active without further exposure to kaolin; in fact, the addition of kaolin resulted in loss of activity, presumably due to adsorption of the active enzymes. Kaolin activation was omitted with these latter fractions. All assays were done with 0.05 M TAMe, except comparing of substrate ratios where a substrate

[2] Fisher Scientific Co., Fairlawn, N. J.
[3] Abbott Laboratories, Chicago, Ill.
[4] Aldrich Chemical Co., Inc., Milwaukee, Wis.
[5] Cyclo Chemical Corp., Los Angeles, Calif.
[6] Farbenfabriken Bayer AG, Leverkusen, Germany.
[7] Pentex, Inc., Kankakee, Ill.
[8] Worthington Biochemical Corp., Freehold, N. J.
[9] Schleicher and Schuell Co., Keene, N. H.
[10] Whatman, W. R. Batson, Ltd., England.
[11] Pharmacia, Piscataway, N. J.
[12] Amicon Corporation, Cambridge, Mass.
[13] Hoecht Pharmaceuticals, Inc., Cincinnati, Ohio.
[14] Immunology, Inc., Lombard, Ill.
[15] Kindly prepared by Dr. C. K. Osterland.

concentration of 0.015 M was employed. Michaelis constants were determined by conventional methods (17).

Other procedures. C'1 esterase activity was tested by hydrolysis of acetyl-tyrosine ethyl ester, using the Hestrin ester method as modified by Roberts (18), or by the hydrolysis of acetyl-tyrosine methyl ester[16] previously described (13). Caseinolytic activity was measured by the method of Alkjaersig, Fletcher, and Sherry (19). Clotting factors II, V, and VII/X were measured by the method of Owren and Aas (20); factor X by the method of Bachman, Duckert, and Koller (21); and factor VIII, IX, and XII by a modification of the partial thromboplastin time, with kaolin (22) and specifically deficient plasmas.[17]

Bioassay of permeability activity was kindly performed by Doctors D. Wong and E. Becker by the method of Ratnoff and Miles (23).

The identification of the esterases as biologically active kallikreins was conducted on the isolated rat uterus as previously described (24).[18] In these experiments 0.50 ml human plasma and the appropriate concentration of the purified preparations were mixed in the uterine bath to induce kinin formation, and either the height of the contraction or the time of the contraction, whichever was most quantitative was compared with a partially purified preparation of acetone-activated human plasma kallikreins (24). The results are reported in arbitrary units for comparative purposes. Release of bradykinin from human plasma by kallikrein fractions was measured directly by the immunoassay method of Spragg, Austen, and Haber (25). Starch gel electrophoresis (26), disc electrophoresis (27), immunoelectrophoresis (28), starch zone electrophoresis (29), ultracentrifugation (30), and immunodiffusion (31) were performed according to established methods. Estimation of molecular weight was accomplished by analysis of sedimentation equilibrium patterns (32).

RESULTS

Purification of the kaolin-activated arginine esterase. The procedures used in the purification of the kaolin-activated plasma arginine esterase are shown in the flow diagram (Fig. 1). The initial steps in the purification and the results of a typical experiment involving the fractionation of 700-ml of plasma are shown in Table I. Despite precautions to insure minimum contact with wettable surfaces, the enzyme gradually activated during the purification procedure; for this reason all purification ratios are compared with the total activity elaborated in the starting plasma by kaolin activation.

The three-step procedure (Table I) resulted in a 12-fold purification with a rise in specific activity from 0.017 to 0.203 μM TAMe hydrolyzed/min per mg. As with many methods involving alcohol fractionation, the over-all yield was low (12.5%). Additional enzymatic activity was recovered in Cohn fraction IV-4, but because of the tendency for spontaneous activation and the relatively low specific activity, this fraction was not employed further in these studies.

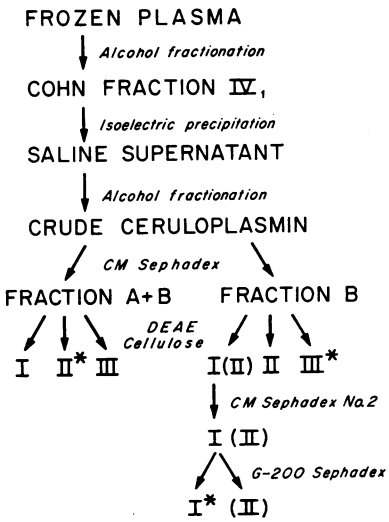

FIGURE 1 Flow diagram of purification procedures for the isolation of plasma kallikreins I, II, and III. See text for details of experimental procedure. Asterisk (*) refers to the best preparation obtained, and parentheses ( ) to minor component.

The resulting "ceruloplasmin-containing" precipitate was chromatographed on CM Sephadex, and an increasing linear gradient in both pH and ionic strength, shown in Fig. 2, was employed. A large unabsorbed inactive peak initially emerged, followed by a second peak whose descending limb contained most of the enzyme activity. Two approaches were then used: The entire second peak (fraction A + B, Fig. 2) was pooled for subsequent chromatography on DEAE cellulose; or in other experiments, only the descending limb of the peak (fraction B, Fig. 2) was utilized for chromatography.

When the whole peak (fractions A + B) was chromatographed on DEAE cellulose with a linear gradient

TABLE I

Initial Purification of Arginine Esterase

	Total enzyme units	Protein	Specific activity	
	μmoles/ min	*mg*	*μmoles/ min per mg*	*% yield*
Frozen plasma	720	42,400	0.017	100
Cohn fraction IV_1	192	4,260	0.045	26
Saline supernatant	180	1,220	0.148	25
Crude ceruloplasmin	90	444	0.203	12.5

[16] ATMe was dissolved first in acetone and buffer was then added, to a final acetone concentration of 5%, an amount that did not appreciably inhibit enzymatic activity.

[17] These assays were performed in the laboratory of Dr. F. Bachmann who also supplied factor VIII- and IX-deficient plasma. Factor XI- and XII-deficient plasma were supplied through the kindness of Dr. L. Gaston and Dr. O. Ratnoff.

[18] These experiments were performed in the laboratory of Dr. M. E. Webster.

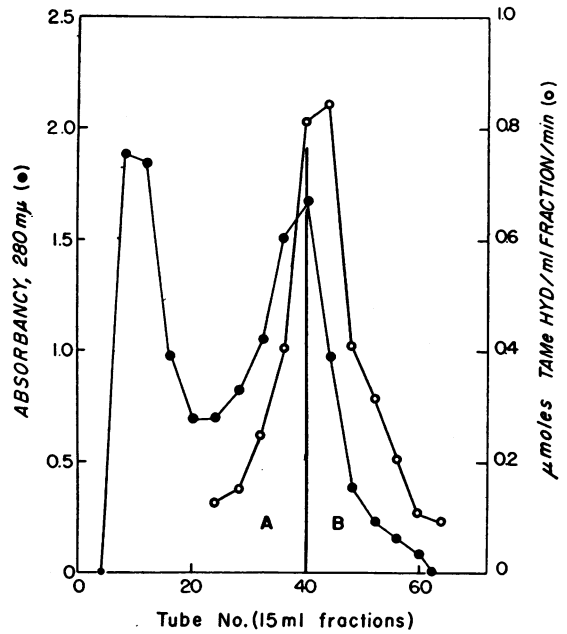

FIGURE 2 Carboxymethyl Sephadex chromatography of partially purified plasma kallikreins. Starting material was 900 mg crude ceruloplasmin (Table I). A 5 cm × 30 cm column was packed with CM-Sephadex and equilibrated with starting buffer, 0.01 M sodium phosphate, ph 6.0. After the enzyme solution was passed into the column bed, the protein eluted with a two-chambered linear gradient. The first chamber contained 1000 ml of 0.01 M sodium phosphate, pH 6.0, and the second chamber 1000 ml of 0.066 M sodium phosphate, pH 8.0, in 0.55 M NaCl. The flow rate was 105 ml/hr.

of increasing ionic strength and constant pH, as shown in Fig. 3, three peaks of enzymatic activity were obtained. Peak I was not absorbed to the DEAE cellulose and comprised 56% of the activity. Peak II emerged shortly after the application of the gradient and constituted 28% of the total activity. Peak III emerged at higher ionic strength and comprised only 16% of the arginine esterase activity. Each of these peaks contained kallikrein activity, as measured by its ability to release kinins from plasma by bioassay (Table IV) and, henceforth for convenience, the esterase activity in each of these three peaks will be referred to as kallikreins I, II, and III, respectively. Marked increase in specific activity and a substantial degree of purification were obtained by this procedure.

The top half of Table II summarizes the data obtained from column chromatography of fractions A + B, the chromatography on CM-Sephadex giving a twofold purification over the starting ceruloplasmin precipitate. The specific activity obtained for each peak on DEAE cellulose is indicated, but in calculating the estimated purification factor (last column, Table II), the assumption was made that the proportion of each peak derived from the column was the same as that one in the original starting plasma (next to last column, Table II). This assumption was also used in computing the purification factor for the preparation obtained by selective chromatography (lower half of Table II). Although this assumption is not necessarily valid, it provides a useful means for estimating the purification factor for each of the three forms of kallikrein.

Purification and characterization of kallikrein I. Kallikrein I was purified to a greater extent by selective chromatography of the last half of the CM-Sephadex peak (fraction B, Fig. 1) on DEAE cellulose, using the same conditions as above; the results are shown in Fig. 4. As shown in the lower half of Table II, an additional fourfold purification was obtained as compared to DEAE cellulose chromatography of the entire CM-Sephadex fractions A + B. There was essentially no decrease in yield since most of kallikrein I was present in fraction B alone. When compared with normal serum, disc and starch gel electrophoresis demonstrated

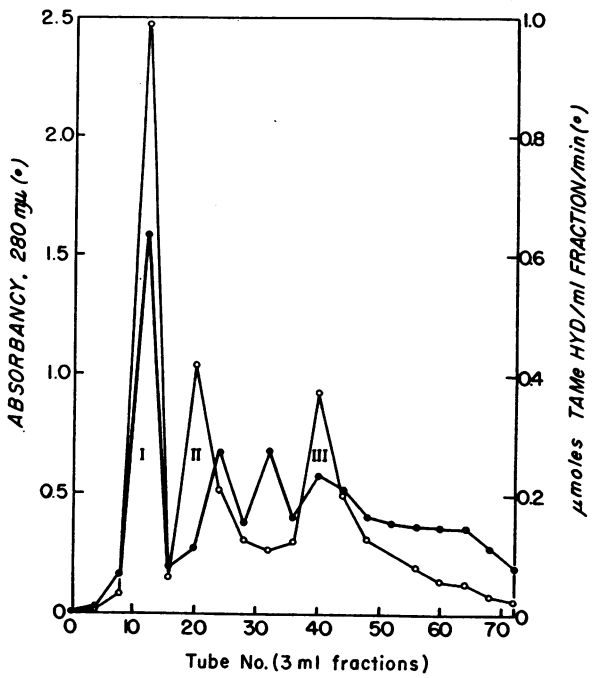

FIGURE 3 DEAE cellulose chromatography of partially purified plasma kallikreins. Starting material was 100 mg CM-Sephadex fractions A + B (Table II). A 1 cm × 24 cm column was packed with DEAE cellulose and equilibrated with the starting buffer, 0.005 M sodium phosphate, pH 8.0. After the enzyme solution was passed into the column, the protein eluted with a two-chambered linear gradient starting at tube 22. The first chamber contained 200 ml of 0.005 M sodium phosphate, pH 8.0, and the second chamber 200 ml 0.005 M sodium phosphate, pH 8.0, in 0.7 M NaCl. The flow rate was 30 ml/hr. Three peaks of esterase activity, noted as I, II, and III, were obtained.

TABLE II

Further Purification of Arginine Esterases by Column Chromatography*

Column	Specific activity	Estimated per cent total esterase activity	Estimated purification factor (activated plasma = 1)
	μM/min per mg		
CM-Sephadex fractions A+B (entire peak)	0.344	100	20.2
DEAE cellulose			
Peak I	0.600	56	62.5
Peak II	1.530	28	323.0
Peak III	0.602	16	226.0
CM-Sephadex fraction B (last half peak)	0.770	—	45.3
DEAE cellulose			
Peak I	2.400	56	250.0
Peak III	1.100	12	590.0
CM-Sephadex No. 2			
Peak I	9.300	56	970.0

three components: (*a*) a slowly moving gamma globulin; (*b*) a small amount of a faster moving gamma globulin; and (*c*) the major component, a β-globulin. Eluates from starch block electrophoresis revealed that better than 95% of the esterase activity was associated with the slowly moving gamma globulin.

Accordingly, further purification of kallikrein I was instituted, using a shallow linear gradient in increasing ionic strength and pH on CM-Sephadex (CM-Sephadex No. 2) and the results are described in Fig. 5. A large peak containing about 80% of the protein and only 20% of the enzymatic activity emerged first, as would be expected for the less positively charged β-globulin. The major peak of the enzyme activity was associated with the trailing edge of the protein peak As shown in the lower part of Table II, an additional fourfold purification was obtained; this preparation, typical of our best purification, was almost 1000-fold purified, as compared with kaolin-activated plasma, and had a specific activity of 9.3 μmoles TAMe hydrolyzed/mg per min at pH 7.6 and 37°C.

As shown in tube 1 of Fig. 6, this purified preparation could be separated into two components on polyacrylamide disc electrophoresis and, as shown in Fig. 7, the preparation also contained two components by ultracentrifugation. The major component comprised 87% of the total protein by planimetry and had a $s_{20,w}$ of 5.7 (two determinations), while the minor component comprising 13% of the protein had a $s_{20,w}$ of 8.3. With a moving boundary cell the top 5% of the sedimenting protein was shown to contain enzymatic activity similar in specific activity to the starting mixture, which indicated that the major, lighter component was kallikrein I. Analysis of sedimentation equilibrium patterns of two concentrations of protein (0.2 mg/ml, 0.4 mg/ml) revealed an average mol wt of kallikrein I of 99,800; the mol wt of the minor component was 163,000.

Despite the presence of two components on disc electrophoresis and ultracentrifugation, only a single anti-

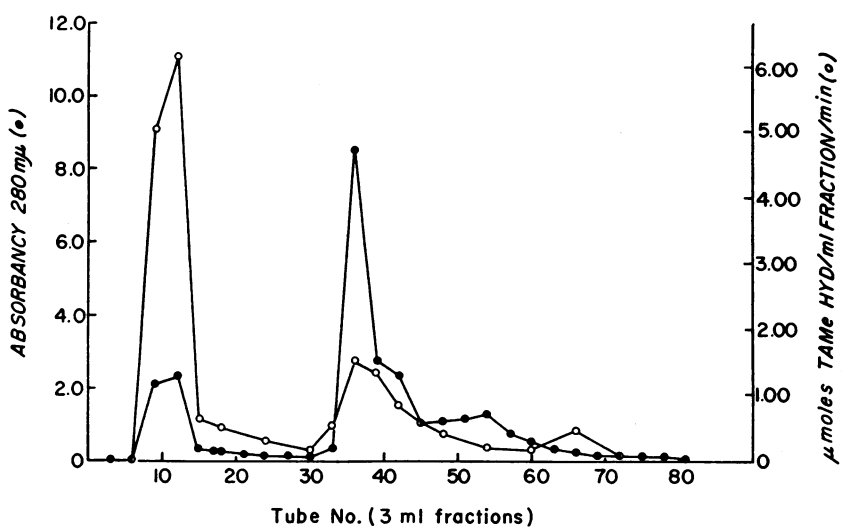

FIGURE 4 DEAE cellulose chromatography of partially purified plasma kallikreins. Starting material was CM-Sephadex fraction B, 1220 mg, (Table II). The conditions were the same as in Fig. 2, except that after the enzyme solution was passed into the column, the column was washed with 50-ml of starting buffer before beginning the linear gradient at tube 27. Three peaks of esterase activity obtained, as in Fig. 3.

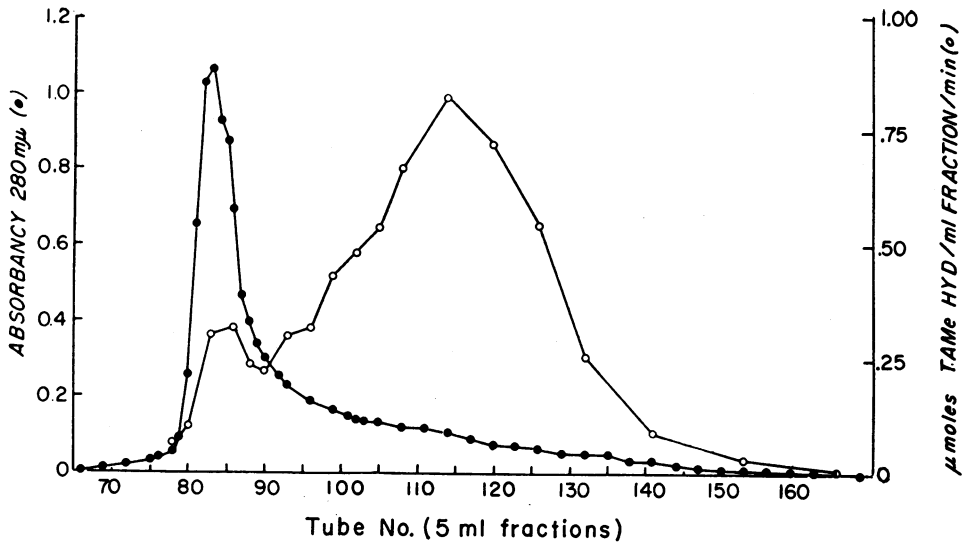

FIGURE 5 CM-Sephadex chromatography of partially purified kallikrein I. Starting material was 74.8 mg DEAE cellulose peak I (Table II). A 2.8 × 24 cm column was packed with CM-Sephadex and equilibrated with starting buffer, 0.010 M sodium phosphate, pH 6.0. After the enzyme solution was passed onto the column, the protein eluted with a two-chambered linear gradient. The first chamber contained 200 ml of 0.01 M sodium phosphate, pH 6.0, and the second chamber 200 ml of 0.016 sodium phosphate, pH 8.0, in 0.13 M NaCl. The flow rate was 60 ml/hr.

genic component was ever demonstrated on immunodiffusion and immunoelectrophoresis. The former is illustrated in Fig. 8 where well 2 contains a CM-Sephadex No. 2 preparation of kallikrein I. It is to be noted that a less purified preparation (DEAE chromatography) contained two components (well 1). A preparation of similar purity to CM-Sephadex No. 2 obtained by an alternate method (DEAE cellulose followed by gel filtration), also exhibits only one antigenic component (well 3).

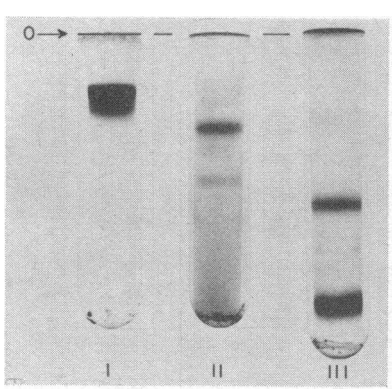

FIGURE 6 Disc polyacrylamide gel electrophoresis of preparations of kallikreins I, II, and III. Tube I: kallikrein I preparation from CM-Sephadex No. 2 chromatography; Tube II: kallikrein II preparation from DEAE cellulose chromatography; and Tube III: kallikrein III preparation from DEAE cellulose chromatography. See Table II for specific activity of these preparations. The total protein applied was 50–150 μg in 40% sucrose layered on top of gel. The buffer was 0.025 M Tris–0.19 M glycine-HCl, pH 8.9. Electrophoresis was carried out for 2 hr at 25°C, 1.5 mamp/tube.

The seemingly contrary observation that in the presence of two physically separated components, only one is observed on immunodiffusion was clarified when the minor component was identified as kallikrein II, as follows: a purified kallikrein I preparation (CM-Sephadex No. 2) was subjected to gel filtration on G-200 Sephadex, 0.016 M phosphate, pH 8.0 in 0.13 M NaCl, and two peaks were obtained with estimated molecular weights similar to the two components on ultracentrifugation. Disc electrophoresis, illustrated in Fig. 9, reveals that each of the two components migrated as a single homogeneous bond with different mobility. The major lighter component (kallikrein I, mol wt 99,800) had an electrophoretic mobility corresponding to a slow gamma globulin,[19] whereas the heavier minor component (mol wt 163,000) moved somewhat faster with a migration comparable with that of kallikrein II (*vide infra*).

[19] The mobility of kallikrein I in the slow gamma globulin region as compared with normal serum was confirmed in all the systems studied, including disc electrophoresis, immunoelectrophoresis, starch gel electrophoresis, and starch zone electrophoresis. In the last procedure, the presence of enzyme activity in the slow gamma region was confirmed by eluting the activity from an appropriate segment of the starch block.

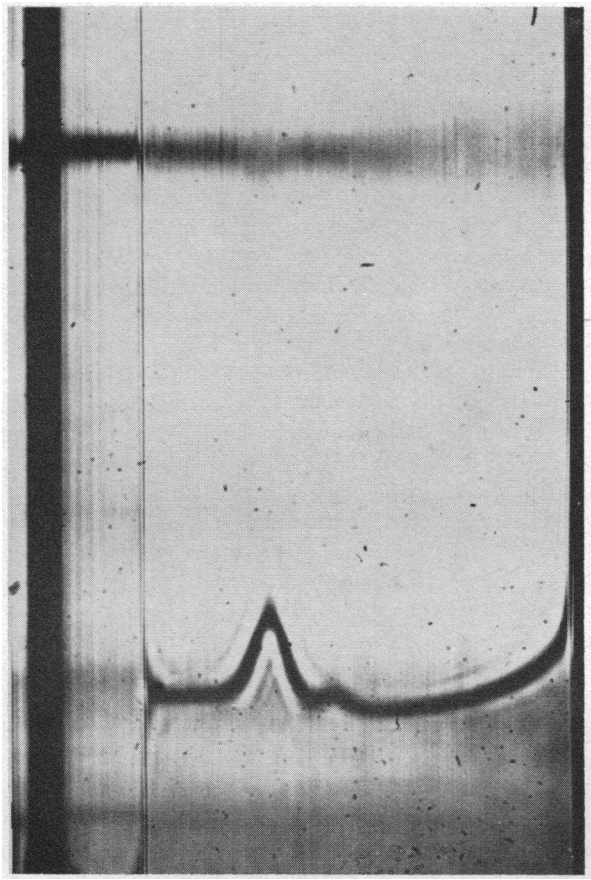

FIGURE 7 Sedimentation of plasma kallikrein I. The enzyme was in 0.015 M phosphate–0.10 M NaCl, pH 8.0, at a protein concentration of 3.0 mg/ml. Sedimentation is from left to right with a rotor speed of 59,780. The photo was taken at 51 min.

Both peaks hydrolyzed arginine esters with similar specific activity and liberated kinins, as measured by the rat uterus bioassay.

Kallikreins I and II appeared to be closely related and, perhaps, interconvertible, for not only did they exhibit a common antigenicity, but when preparations of purified kallikrein I from CM-Sephadex No. 2 (Table II, bottom line) were chromatographed on G-200 Sephadex in Tris buffer under conditions of increased ionic strength (0.3 M Tris, pH 8.0, 0.7 M NaCl), only one protein peak that was fully active enzymatically and which corresponded to a mol wt of 163,000 was obtained.

Purification and characterization of kallikrein II. Kallikrein II was best purified by chromatography of the CM-Sephadex fractions A + B (Fig. 1) on DEAE cellulose; illustrated in Fig. 3 it resulted in a preparation 320-fold–purified over activated plasma. Selective chromatography of different portions of the CM-Sephadex fractions A or B did not improve purification.

The preparation obtained showed only one component on immunoelectrophoresis and this migrated in the fast gamma region identical to the minor component of kallikrein I preparations. On immunodiffusion (Fig. 8, well 4), kallikrein II gave only one component, showing a line of identity with kallikrein I. However, as illustrated in tube II of Fig. 6, several minor components were observed on disc electrophoresis. Elution of segments of starch block, following electrophoresis, demonstrated that most of the enzymatic activity resided in the fast gamma region. The specific activity of the enzyme eluted from this region was 4.95 μmoles/min per mg with a purification factor of 1170-fold over normal plasma. Unfortunately, insufficient protein was recovered from the enzyme-rich segment to compare the physical characteristics of this peak fraction with kallikrein II derived from preparations of kallikrein I.

Purification and characterization of kallikrein III. Kallikrein III was best purified by selective chromatography of the CM-Sephadex fraction B (Fig. 1) on DEAE cellulose as depicted in Fig. 4. As shown in Table II, this resulted in a preparation 590-fold–purified, as compared with activated plasma, and 2-fold better than that

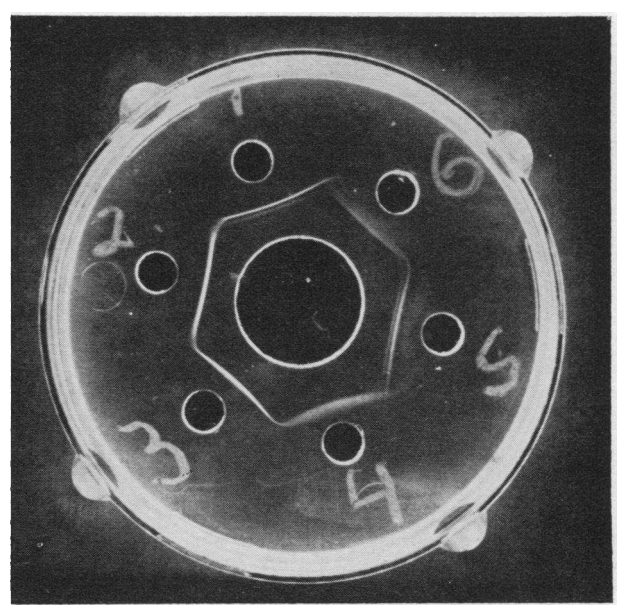

FIGURE 8 Immunodiffusion of kallikreins I, II, and III. The center well was filled with 200-μl potent polyvalent goat antihuman serum. Outside wells were filled with 20-ul of enzyme preparations containing 20–60 μg protein as follows: (*1*) kallikrein I from DEAE cellulose chromatography; (*2*) kallikrein I from CM-Sephadex No 2 chromatography; (*3*) kallikrein I from DEAE cellulose followed by G-299 Sephadex chromotography; (*4*) kallikrein II from DEAE cellulose chromatography; (*5*) kallikrein III from DEAE cellulose chromatography; and (*6*) kallikrein II from DEAE cellulose followed by G-200 Sephadex chromatography.

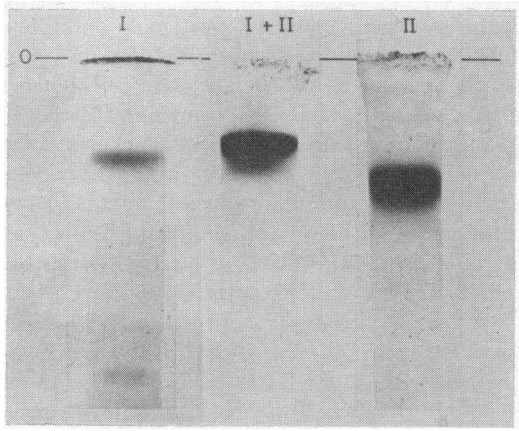

FIGURE 9 Disc polyacrylamide gel electrophoresis of a kallikrein I preparation obtained from CM-Sephadex No. 2 chromatography, which was then subjected to gel filtration on G-200 Sephadex. Tube I: second peak from Sephadex G-200 (lighter component mol wet = 99,800); Tube II: first peak from Sephadex G-200 (heavier component mol wt = 163,000. Tube I+II overlap area of two peaks I and II. The total protein was 50–150 μg in 40% sucrose layered on top of gel. The buffer was 0.025 M Tris–0.192 M glycine-HCl, pH 8.9. Electrophoresis was carried out for 2 hr at 25°C, 1.5 mamp/tube.

obtained by DEAE cellulose chromatography of fractions A + B. Unfortunately, the purification was obtained at the expense of yield, since most of kallikrein III was in the CM-Sephadex fraction A.

Polyacrylamide gel electrophoresis (tube III, Fig. 6), starch gel electrophoresis, and immunoelectrophoresis all demonstrated several components in this preparation. Eluates from starch block electrophoresis showed that the major part of the activity migrated as an alpha globulin, although this protein was still not the major component of the preparation. In an attempt to ascertain whether kallikrein III was associated with either α_1-antitrypsin or α_2-macroglobulin, two separate immunodiffusion experiments were performed with specific antiserum against these components. No precipitin lines appeared, indicating the lack of either one of these plasma proteins in the preparations of kallikrein III. On immunodiffusion against antihuman serum, one of the minor components of this preparation, the one nearest wells 5 and 6 (Fig. 8) gave a line of identity with kallikreins I and II. Gel filtration on G-200 Sephadex (0.3 M Tris, pH 8.0, 0.7 M NaCl) with known standards gave an estimated mol wt of 124,000.

Comparison of kallikreins I, II, and III. When the esterase activities of the three kallikrein preparations were compared, as shown in Table III, their substrate/TAMe ratios, employing a variety of basic amino acid esters, were quite similar; and these ratios were equivalent to those observed with the kaolin-activated plasma esterase (11). All three kallikrein preparations were completely inhibited by 10^{-4} M DFP and were 70% inhibited by diphenylcarbamyl fluoride (6.2×10^{-3} M). The three kallikrein preparations were not inhibited by the trypsin inhibitors TLCK (3.1×10^{-3} M) or ovomucoid (200 μg/ml), nor were they inhibited by 500 U heparin/ml. However, kallikrein III differed from the other two in that it was not as susceptible to polypeptide inhibitors, such as soybean trypsin inhibitor, pancreatic trypsin inhibitor, or Trasylol. Unlike kallikreins I and II, kallikrein III was not inhibited by normal human plasma. Also, although not shown in Table III, substrate inhibition at high TAMe concentrations ($5–10 \times 10^{-2}$ M) was only observed for kallikrein III.

The K_m of the kallikrein I-TAMe reaction was 7.4×10^{-3} M which compares with a human thrombin–TAMe K_m of 5.5×10^{-3} M (33). All three peaks lost 60–75% of their activity when heated at 56°C for 30 min.

Absence of other arginine esterases and related plasma proteins in the kallikrein preparations. Significant contamination of the purified kallikrein I, II, and III preparations with C'1 esterase, thrombin, plasmin, plasminogen, or clotting factors I, II, V, and VII–XII could not be demonstrated.

Identification of kallikreins I, II, and III as biologically active kallikreins. The identification of the three purified arginine esterases isolated from human plasma as kallikreins rests on experiments indicating the ability of each to release kinins in vitro and in vivo by bioaasay and in vitro by radioimmunoassay. All three purified arginine esterases were capable of forming kinin, as demonstrated by their ability to release a uterine contracting substance from human plasma when added directly to the tissue bath. For example, 7.5 μl of a moderately purified kallikrein I preparation (SA, 1.54 μmoles TAMe

TABLE III

*Effect of Ionic Strength on Arginine Esterases**

Buffer (pH 7.2)	Conductivity	Activity ratio BAMe/TAMe		Heparin (500 U/ml) % Inhibition kaolin
		No kaolin	Kaolin	
	mho			
0.050 M Phosphate	0.0078	3.71	1.63	76
0.075 M Phosphate	0.0087	2.86	1.25	48
0.100 M Phosphate	0.0116	3.37	1.18	42
0.100 M Phosphate plus 0.150 M NaCl	0.0204	No activity	0.98	19

* For the BAMe/TAMe ratio studies, the esterase activity was measured in the buffers noted in the table. For the heparin experiment, to 0.2 ml normal human siliconized plasma was added 10 mg of solid kaolin in 0.6 ml of the buffer indicated. After shaking for 1 min at 25°C, 1.4 ml of ice cold TAMe was added in buffer containing sufficient heparin to give a final concentration of 0.015 M BAMe and 0.500 U/ml of heparin. Esterase activity was assayed as indicated in Methods.

TABLE IV

Biologic and Esterolytic Activity of Various Preparations of Kallikreins I, II, and III

Enzyme preparation	Biologic* activity	Esterolytic activity (TAMe)	Purification factor (esterolytic)	Biologic / Esterolytic Ratio
	Kallikrein units/mg	*μmoles/min per mg*		
Fresh frozen ACD plasma	–	0.017	1	–
Kallikrein I	1.4	1.54	162	1.02
	5.8	5.57	586	1.04
Kallikrein II	0.71	0.324	68	2.20
	1.4	0.542	114	2.60
Kallikrein II	0.29	0.388	142	0.75
	0.33	0.506	186	0.65

* Kallikrein units are arbitrary and determined by the ability of the enzyme preparations to release kinins from human plasma, as measured by the estrus rat uterus bioassay (24).

hydrolyzed/min per mg), when mixed with a standard normal plasma, induced a contraction of 22 mm (equal to 4.4 kallikrein units/ml or 1.4 kallikrein units/mg protein). This compares with a contraction of 17.5 mm induced by 70 μl of a standard preparation (24) (0.35 kallikrein units/ml) studied under the same conditions. Similar observations were made for preparations of kallikreins II and III. The data for two preparations of each of the three kallikreins are presented in the first column of Table IV. No differences between experiments employing heated or unheated plasma were observed.

The agonist in these preparations was most likely a kinin, since incubation of enzyme and plasma outside the bath in the presence of carboxypeptidase B completely destroyed detectable biologic activity. That this action of the esterases was the result of their enzymatic activity was demonstrated by experiments utilizing SBTI; similar to its effect on the esterase activity, the addition of this inhibitor (100 μg) to the uterine bath prevented the formation of kinins by kallikrein III. Moreover, the three esterases functioned as a kallikrein, rather than a PF/dil, since they formed kinin from both unheated human plasma and human plasma heated to 61°C for 30 min. The kallikrein preparations in the absence of plasma demonstrated no kinin activity when tested directly in the rat uterus bioassay (24).

Comparison of the biologic activity (formation of kinins) with the esterolytic activity at different stages of purification, as shown in Table IV, demonstrated that these activities were directly related. It is evident that the ratios remained reasonably constant when testing preparations at different stages of purification and that the ratios of activity of the three kallikreins were of the same order of magnitude. In addition, the intradermal injection of all three esterase preparations markedly increased vascular permeability in the guinea pig as measured by the extravasation of intravenously administered dye; for example, the preparation of a moderately purified kallikrein I that contained 1.4 kallikrein units/mg (Table IV) had an activity of 310 bluing units/mg (22). This same preparation of kallikrein I when incubated with 0.6 ml of normal plasma for 3 min at 25°C, pH 8.0, released one-third (0.6 μg) of the total bradykinin in plasma, as measured by a highly specific radioimmunoassay (25).

DISCUSSION

Our primary interest in initiating this investigation was to identify the nature and source of the large amounts of Hageman factor–dependent arginine esterase activity which is released in siliconized anticoagulated plasma after kaolin activation. As will be shown in the subsequent communication (12), and as previously suspected (10), the enzyme activity is derived from the activation of plasma kallikreinogen. PF/dil activity would not be expected at the ionic strength of undiluted plasma, and in our studies (12) the conditions necessary for the elaboration of a PF/dil–like arginine esterase were also quite different.

In order to achieve our objective, it was necessary to undertake a purification of the arginine esterase activity in concert with the experiments designed to test their biological activity. It is apparent from the experiments cited in this report that the arginine esterase preparations obtained during this purification contained potent kallikrein activity: they increased vascular permeability in the guinea pig, released a kinin from heated and unheated human plasma that contracted the rat uterus and was inactivated by carboxypeptidase B, and was identified by radioimmunoassay as bradykinin and/or closely re-

lated kinins. The observation that plasma kallikrein is a known arginine esterase (8) and more specifically, that extensive purification over a range of an almost 600-fold did not dissociate the biologic-esterolytic activity ratios (Table IV), establishes the identity of the arginine esterase activity of these preparations with their kallikrein activity.

The purification procedure developed takes advantage of two facts: (*1*) Plasma kallikreinogen is present mostly in Cohn fraction IV–1, whereas prothrombin and plasminogen, the precursors of the other major arginine esterases, are removed in Cohn fractions II and III; and (*2*) The measurement of arginine esterase activity allows for the rapid screening of various fractions during the purification procedure.

The initial step in the purification also resulted in the removal of the plasma kallikrein inhibitor. However, some Hageman factor is still present and/or autocatalytic activation takes place, since progressive "spontaneous" activation was observed during the three steps that yielded the ceruloplasmin-containing precipitate. The low ionic strength isoelectric precipitation of the inactive lipoproteins of fraction IV-1 permitted subsequent chromatography, since these proteins may be difficult to handle on celulose columns. The subsequent 15% alcohol fractionation step conveniently concentrated the enzymatic activity with increasing the specific activity.

As expected, the extensive surface contact of the CM-Sephadex chromatography resulted in a preparation in which the kallikrein was in the fully active form; it was also free of Hageman factor since the latter was removed, probably in the unabsorbed peak. At this stage, and as previously suggested (9), plasma kallikrein was unstable. Large losses of enzyme activity were observed during concentration and dialysis procedures after removal from the CM-Sephadex column. Lyophilization, at this point, even from volatile buffers also resulted in marked dimunution of the total and specific activity. The instability appeared to result from the combination of high ionic strength and low protein concentration, and we were able to avoid this through the use of rapid concentration by pressure ultrafiltration followed immediately by dialysis.

The DEAE cellulose chromatography not only resulted in further purification, but allowed separation of the kallikreins into three components. Previous workers (34) have reviewed the evidence that there are two kallikreins in plasma depending on their mode of activation and method of extraction. The three entities described in this report and referred to as kallikreins I, II, and III are isoenzymes in the sense that each possesses kallikrein activity (release of kinin from heated plasma) and esterase activity with similar substrate ratios. In addition, they are all inhibited by DFP, suggesting they are all serine proteases. Furthermore, all three enzymes contain an immunologically identical component that, although demonstrated by a polyvalent antiserum in the case of kallikreins I and II, is identical to the physically homogeneous active enzyme (moving boundary cell, disc electrophoresis, and gel filtration studies).

Kallikreins I and II appear to differ slightly in charge on DEAE cellulose and electrophoresis. That they may be interconvertible is suggested by the presence, in the most purified preparation of kallikrein I, of another component of higher mol wt (163,000, as compared with 99,800 for kallikrein I) with both esterase and kallikrein activity, and with an electrophoretic mobility identical to kallikrein II. Furthermore, on gel filtration of a kallikrein I preparation under conditions of increased ionic strength and in a different buffer system (Tris instead of phosphate), all of the activity emerged as one peak with a mol wt estimated at 160,000–180,000. These findings suggest that kallikrein II may result by aggregation or dimer formation of kallikrein I.

Kallikrein III, on the other hand, differs in many ways from kallikreins I and II. It is more negatively charged, as manifested by tighter binding on DEAE cellulose and more rapid migration on disc and starch gel electrophoresis. It is not inhibited by the polypeptide inhibitors, SBTI and PTI, nor by the plasma kallikrein inhibitor which inhibits kallikreins I and II. The apparent mol wt is 124,000, larger than kallikrein I but smaller than kallikrein II. These differences may be accounted for by binding of kallikrein I to a carrier protein. Dyce et al. (35) report, in an abstract, an enzyme releasing bradykinin and hydrolyzing TAMe as associated with α_2-macroglobulin of serum. However, we were unable to detect any α_2-macroglobulin in kallikrein III preparations. Moreover, the activity is not present in the excluded volume of G-200 Sephadex, as might be expected with binding to an α_2-macroglobulin. In addition, no α_1-antitrypsin was present by immunodiffusion. Nevertheless, protein binding of this kallikrein to some other component, possibly even the plasma kallikrein C'1 esterase inhibitor, an α_2-globulin (36), still offers an explanation of these properties. That such a complex may exist in native plasma is suggested by the residual esterase activity after adding SBTI to kaolin-activated plasma (12). An alternative hypothesis is that kallikrein III represents an immunologically related entity, e.g., a product of limited proteolysis of kallikrein I or II which might conceivably affect substrate affinity. It is difficult to exclude the remote possibility that isozymes may arise as an artifact of the methods of purification employed in this investigation.

The degree of purification achieved for kallikrein I can be appreciated by considering its specific activity.

As little as 0.3 μg/ml liberated detectable bradykinin in the rat uterus assay and about 5 μg/ml gave detectable esterase activity. After further gel filtration, kallikreins I and II could be rendered homogeneous both in molecular weight and charge. The 1000-fold purification over plasma represents a purification similar to that which has been achieved with plasmin and thrombin, and allows one to calculate on the basis of the activity present in plasma, that the latter contains approximately 0.05–0.1 mg/ml of kallikreinogen, a value similar to the value estimated for plasminogen (37).

The ability to prepare highly purified preparations of plasma kallikrein should make it possible to begin an extensive investigation of the biochemical, biophysical, and physiological properties of this enzyme. Equally challenging will be the study of the molecular differences between the three kallikreins, and the isolation, purification, and characterization of plasma kallikreinogen.

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