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ISOLATION OF RAT URINARY KALLIKREIN AND PROPERTIES OF ITS ANTIBODIES*

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Abstract—A procedure has been described for the specific and rapid isolation of rat urinary kallikrein using anion-exchange, affinity and Sephadex chromatography. Trasylol-bound Sepharose selectively adsorbed kallikrein which could be eluted between pH 5.5 and 3.5. The kallikrein was purified 694-fold by the entire procedure and 49 times in the individual step of affinity chromatography. The purification obtained by the Trasylol–Sepharose adsorption was one of the highest of any individual steps reported thus far. Antibodies generated against this kallikrein reacted with rat urine and kidney cortex homogenate, but not with bovine trypsin, dog or human urine. The activity *in vitro* and *in vivo* of kallikrein was specifically blocked by the antikallikrein serum.

The formation of hypotensive peptide kinin has been attributed to a group of serine proteinases called kallikreins (kininogenin EC 3.4.21.8). The kallikreins release kinin peptides such as bradykinin and kallidin from plasma kininogen. These enzymes have been separated into two main groups, plasma and glandular kallikreins [1]. The kallikrein found in urine has characteristics similar to glandular kallikreins. Furthermore, the urinary kallikrein, which resembles renal kallikrein [2, 3], is of special interest because of its possible involvement in the regulation of blood pressure [4] and sodium balance [5, 6].

Purification procedures of mammalian kallikreins have been reviewed [7]. Rat urinary kallikrein was isolated by Porcelli and Croxatto [8] and, more recently, by Nustad and Pierce [9] and by Silva *et al.* [10]. In the present report, we describe the use of Trasylol-bound Sepharose for isolation of pure kallikrein. In addition, antibodies have been produced against this kallikrein and examined *in vitro* and *in vivo* for specific inhibition of kallikrein activity.

MATERIALS AND METHODS

The following materials were obtained from commercial sources: DEAE-cellulose (Whatman DE-52); DEAE-Sephadex A-50, CNBr-activated Sepharose 4B, Blue Dextran and Sephadex G-100 from Pharmacia Fine Chemicals, Inc.; polyacrylamide and Coomassie Brilliant Blue R-250 from Eastman Kodak Co.; benzoyl-L-arginine ethyl ester (BAEE) from Cyclo Chemical Corp.; and crystallized human serum albumin from Nutritional Biochemicals Co. All other chemicals were of reagent grade.

Preparation of Trasylol–Sepharose (T–Sepharose)

The procedure used was similar to one described by Cuatrecasas *et al.* [11]. Binding of ligand was performed in 0.1 M sodium bicarbonate, 0.5 M sodium chloride at pH 9.5 (bicarbonate buffer). Three hundred mg Trasylol (0.15 μg equivalent to 1.0 KIU) was added to 30 g of activated Sepharose and stirred gently for 2 hr at 25° and for 18 hr at 4°. Non-covalently bound ligand was eliminated by three washing cycles; each cycle consisted of a 100-ml wash with: (1) bicarbonate buffer, (2) 0.2 M Tris–HCl, pH 7.5, and (3) 0.1 M sodium acetate, 1.0 M NaCl, pH 4.0. The product, 140 ml settled T–Sepharose, contained 284 mg of bound Trasylol as estimated by absorbance at 280 nm. Any remaining active groups of Sepharose were further reacted with 1.0 M ethanolamine at pH 8.0 for 2 hr, and the gel was re-equilibrated in bicarbonate buffer. Sodium azide (0.1%) was added as a preservative and the moist gel stored at 4° when not in use.

Purification procedure

Male Sprague–Dawley rats (200–300 g) were housed in metabolic cages with free access to normal food and water. Twelve to fifteen liters of urine was collected, free of feces, in ice-cold containers, and the proteins were precipitated with 80% saturation of ammonium sulfate. The precipitates were separated by centrifugation, redissolved in distilled water, dialyzed against the distilled water for 18 hr at 4° and lyophilized. The crude enzyme powder was stored at −20°. In the second step, the crude enzyme (10–15 g) was dissolved in 0.1 M phosphate buffer, pH 5.0, and passed through a DEAE-cellulose column (8.5 × 28 cm) equilibrated in the same buffer. The adsorbed enzyme was eluted with a linear sodium chloride gradient to 1.0 M and rechromatographed on a DEAE-Sephadex A-50 column (8.5 × 28 cm) under identical conditions.

Affinity chromatography

Major activity fractions emerging from DEAE-Sephadex A-50 chromatography were pooled, equilibrated by dialysis against the bicarbonate buffer and stirred gently with T–Sepharose for 18 hr at 4°. The gel was packed in a column (2 × 35 cm) and washed

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with 140 ml of 0.2 M disodium hydrogen phosphate at pH 9.5. The kallikrein was eluted with a pH gradient for which two chambers were used. The first contained 1 liter of 0.05 M sodium dihydrogen phosphate adjusted to pH 3.0 which was allowed to flow dropwise into a second mixing chamber which contained 100 ml of 0.2 M disodium hydrogen phosphate at pH 9.5. The second chamber was connected to the column so that the flow rate of the affinity column was proportional to the flow rate in the mixing chamber. In subsequent experiments, the contaminants were removed with 0.2 M phosphate buffer, pH 6.0, and kallikrein was eluted with 0.1 M acetic acid, pH 3.5.

The major activity emerging from the affinity column was equilibrated by dialysis with 0.1 M phosphate buffer, pH 7.2, and passed through a Sephadex G-100 (2.5 × 100 cm) column pre-equilibrated in the same buffer.

Esterase assay

BAEE was used as a substrate in a final concentration of 1.0 mM in 0.1 M phosphate buffer, pH 8.5 (phosphate buffer). The procedure of Trautschold and Werle [12] was used with the exception that the specific activity was expressed as μmoles/min/mg using a molar extinction coefficient of 2300 for the substrate. The enzyme aliquots were adjusted such that the $\Delta A^{1\,cm}_{253}$ did not exceed 0.1 in 5 min.

Estimation of kinins by bioassay

The kinin-forming activity (kininogenase) was measured using the dog bioassay procedure of Marin-Grez and Carretero [13] with some modifications. Briefly, a 0.1-ml aliquot of suitably diluted enzyme was incubated with 2000 ng of partially purified substrate (dog kininogen) in phosphate buffer. The peptidases were inhibited by incorporating 15 mM sodium EDTA and 1.5 mM *O*-phenanthroline in the assay system. The samples were incubated at 37° for 10 min and the proteolysis terminated by immersing them in a boiling water bath for an additional 10 min. The precipitates were separated by centrifugation and the supernatant was adjusted to pH 7.4 with 1 N HCl.

The samples or the standards (kallidin, Schwarz–Mann) were injected into the femoral artery through an indwelling cannula. The resulting increase in flow was recorded by an electromagnetic flowmeter and a recorder [14]. The activity of the unknown samples was bracketed between two close doses of the standard and calculated by interpolation.

Protein estimation

The protein concentration was determined by the procedure of Lowry *et al.* [15] using human serum albumin as a standard protein. An average $A^{1\,cm}_{280}$ of 1.51 was obtained for a kallikrein solution of 1 mg/ml as estimated by the Lowry procedure. This factor was used in the present preparation to estimate protein concentration.

Electrophoresis

Disc-gel electrophoresis (Canalco, Rockville, Md.) was performed in 16% polyacrylamide gel [16] using 0.025 M Tris in 0.2 M glycine at pH 9.5 according to the directions of the manufacturer using a column size of 10 cm. A current of 4 mA/gel was applied at 4° for about 4 hr when the tracking dye (Bromophenol Blue) emerged at the bottom of the column. The gel was scanned (ISCO, Lincoln, Neb.) for absorbance at 280 nm and then stained 1 hr with 0.1% Coomassie Brilliant Blue R-250 in 10% trichloroacetic acid. The destaining was performed in an aqueous solution which contained 10% of acetic acid and isopropyl alcohol. One of the unstained columns was sectioned into 0.5-cm segments, homogenized in phosphate buffer, centrifuged, and the supernatant tested for esterase and kininogenase activity.

Immunization

Four New Zealand rabbits, weighing approximately 3 kg each, were used. Two hundred μg kallikrein emulsified in 1.0 ml of complete Freund's adjuvant was injected weekly into the foot pads of rabbits for a 3-week period. Blood was drawn every week, and the booster injections were given after 4 weeks in incomplete Freund's adjuvant. The antiserum was heated at 56° for 0.5 hr and stored frozen. The development of antibody was monitored by a passive haemaglutination test.

Immunodiffusion and immunoelectrophoresis

Double diffusion in agar gel was performed according to the method of Ouchterlony [17], and immunoelectrophoresis on glass microscopic slides was employed as described by Scheidegger [18]. The antiserum was tested with the Ouchterlony technique against pure kallikrein, crude enzyme, proteins excluded by the affinity column, and homogenates of kidney and liver, all of which were obtained from the rat. For the preparation of the homogenates, rat kidney and liver were washed by the infusion of saline in the renal artery and portal vein respectively. The liver was homogenized in saline, while the kidney cortex was homogenized in 0.5% deoxycholic acid, incubated at 4° for 30 min and dialyzed against saline. In addition, human and dog urine (protein concentrate) and bovine trypsin were also tested with the antibody in the Ouchterlony technique.

Inhibition studies

The antiserum was tested *in vitro* and *in vivo* for its ability to block kallikrein activity. For this purpose, the antikallikrein and normal rabbit serum were heated at 56° for 3 hr for partial denaturation of the inhibitors [19].

In vitro. One hundred μg of crude kallikrein was mixed with 50 μl of either normal rabbit or antikallikrein serum and injected in the hind leg of the dog immediately after mixing. The blood flow response was measured by an electromagnetic flowmeter.

In vivo. The antiserum was tested in unanesthetized rats. Polyethylene catheters were implanted permanently in the femoral artery and vein and brought out from the back of the head through a small incision. The blood pressure was measured through the arterial cannula, and the test samples were injected through the venous cannula. After measuring the blood pressure response of 2 mg of crude enzyme and 1.1 μg pf pure kallikrein, 1.0 ml of normal rabbit or antikallikrein serum was injected. The animals were further tested for the blood pressure response of the same dose of kallikrein at hourly intervals.

Table 1. Purification of rat urinary kallikrein

Step	Treatment	Protein* (mg)	Sp. act. esterase (μmoles/min/mg)	Sp. act. kininogenase (μg/min/mg)	Recovery†	Purification†
1	Crude enzyme	11.250	(0.179)†	2.13	100	1
2	DEAE-52 chromatography	1.430	1.395		99	7.8
3	DEAE-Sephadex A-50 chromatography	663	2.414		79.5	13.5
4	Trasylol–Sepharose affinity chromatography	5.28	117.4		30.8	655.9
5	Sephadex G-100 chromatography	2.82	124.3	1.795	17.4	694

* Protein concentration was calculated on the basis of A_{280}^{1cm} of 1.51 for 1.0 mg/ml of kallikrein solution at 25°.
† Calculated on the basis of 66.2 per cent of the total activity as found by DEAE chromatography.

RESULTS

The analytical data, at each stage of purification, is presented in Table 1. In step 2 of the DEAE-chromatography, 'Esterase A', reportedly possessing very little kininogenase activity [9], was eliminated in the unadsorbed effluents. The esterase activity of the adsorbed enzyme was found to be 66.2 per cent of the total activity and was taken as an index to determine recovery and the degree of purification. The enzyme was eluted with a sodium chloride gradient and passed through a second column of DEAE-Sephadex A-50. In steps 2 and 3, the crude enzyme was purified 13.5-fold. The elution profile of kallikrein, in the next step of affinity chromatography, is shown in Fig. 1. Approximately 90 per cent activity emerged between pH 5.5 and 3.5, and the kallikrein was purified 49-fold in this step. In one of the other preparations, Nustad and Pierce [9] purified kallikrein 72-fold by immune precipitation of the enzyme followed by Sephadex G-100 chromatography. After the affinity chromatography, the enzyme was found to be labile and had a tendency to adhere to glass and plastic surfaces. For this reason, siliconized glassware was used. The most active fractions emerging from the affinity column were combined, equilibrated in 0.1 M phosphate buffer, pH 7.2, and passed through Sephadex G-100. The kallikrein was resolved from inactive proteins as a symmetrical peak (Fig. 2) at two times the void volume of the column. The filtration volume of the enzyme was 320 ml, while that of ovalbumin (mol. wt 45,000) was 260 ml. After the final stage of purification, the recovery of activity was 31.9 per cent for kininogenase and 17.5 per cent for esterase. The specific esterase activity of pure kallikrein was found to be 124 μmoles/min/mg, and the kininogenase activity was 1795 μg kinins/min/mg.

Figure 3 shows the results of electrophoresis in 16% polyacrylamide gel. The purified kallikrein showed a major and a minor protein band on staining. The scan of absorbance at 280 nm resulted in two peaks which coincided with the protein bands. Furthermore, the esterase and kininogenase activities, recovered in 50 per cent yield, were confined to these two components of the kallikrein.

Antiserum obtained after primary immunization of kallikrein had a titer between 1:4 to 1:64 in the passive haemaglutination test. Titers rose steadily after the booster injection, and one of the rabbits gave a titer of 1:256. An antiserum with a haemaglutination titer of 1:64 could be diluted 1:15,000 to obtain 46 per cent binding with 2300 cpm of tritiated kallikrein (unpublished results).

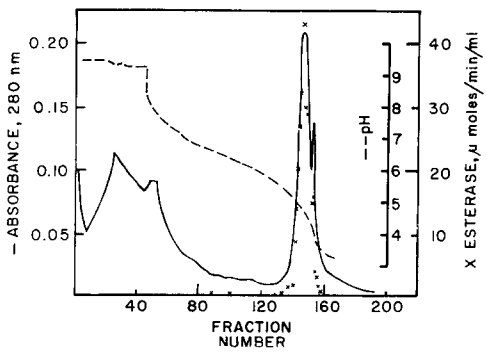

Fig. 1. Chromatography of kallikrein on a T–Sepharose column (2 × 35 cm). The enzyme was eluted in 3.0-ml fractions with a pH gradient as shown by a broken line. The absorbance at 280 nm is shown by a solid line, and the activity toward BAEE is indicated by crosses.

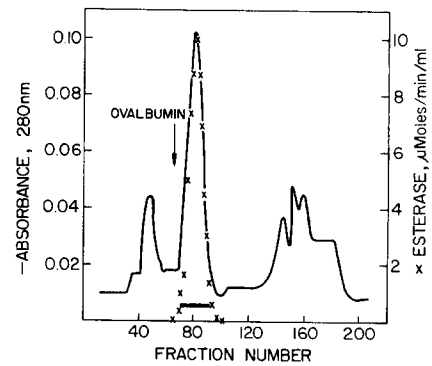

Fig. 2. Sephadex G-100 gel filtration of 5.28 mg kallikrein obtained from the previous step of affinity chromatography. Four-ml fractions were collected at the flow rate of 48 ml/hr. The solid line denotes absorbance at 280 nm, and crosses represent activity toward BAEE. The elution volume of ovalbumin is shown by the arrow. The solid bar at the bottom indicates fractions which were pooled.

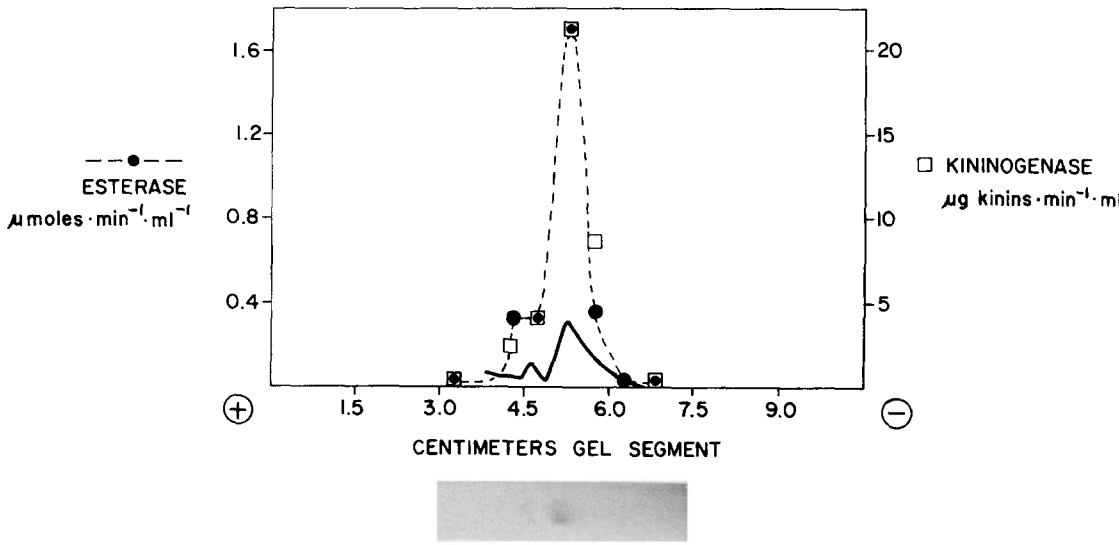

Fig. 3. Electrophoresis of 44 μg kallikrein in 16% polyacrylamide gel. The kininogenase (squares) and esterase (solid circles) activity (dashed line), recovered in 50 per cent yield, was coincident with the peaks of absorbance at 280 nm (solid line) and the protein bands were stained with Coomassie Blue as shown at the bottom of the figure.

The antikallikrein serum gave a single line of identity with kallikrein and crude enzyme (Fig. 4) but no discernible reaction with normal rat plasma, rat liver homogenate or the proteins excluded by T-Sepharose. Furthermore, the antibodies did not cross-react with bovine trypsin, dog or human urine. In immunoelectrophoresis, kallikrein and crude enzyme showed an identical anodal migration as shown in Fig. 4.

The increase in blood flow caused by 100 μg of crude kallikrein could be abolished when the enzyme was mixed *in vitro* with 50 μl of antikallikrein serum (Fig. 5). An equivalent amount of normal rabbit serum had no effect.

The effect *in vivo* of antikallikrein serum in blood pressure was examined in six rats and a typical response is presented in Fig. 6. The depressor response either of 1.1 μg of pure kallikrein or 2.0 mg of crude enzyme was abolished for a period of up to 2 hr when 1.0 ml antiserum was injected before the test. The corresponding dose of normal rabbit serum had no effect.

DISCUSSION

Several procedures [8–10] have been described for isolation of rat urinary kallikrein. Using ion-exchange and Sephadex chromatography, rat urinary kallikrein was purified 390-fold [8]. In subsequent procedures,

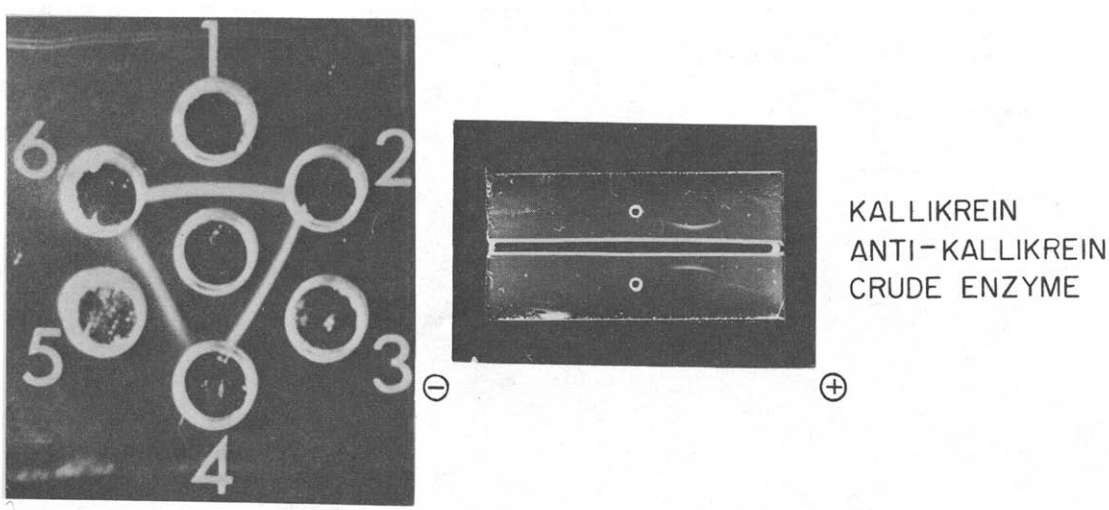

Fig. 4. On the left is shown Ouchterlony double diffusion. Center well: undiluted rabbit antikallikrein serum; peripheral wells: (1) pure kallikrein; (2) normal rat plasma; (3 and 5) 1 mg and 0.5 mg of crude kallikrein, respectively; (4) rat liver homogenate (1 mg); and (6) rat urinary proteins excluded by T-Sepharose (1 mg). On the right is shown immunoelectrophoresis of pure kallikrein (5 μg) and crude enzyme (1 mg) against antisera directed to pure kallikrein of rat urine.

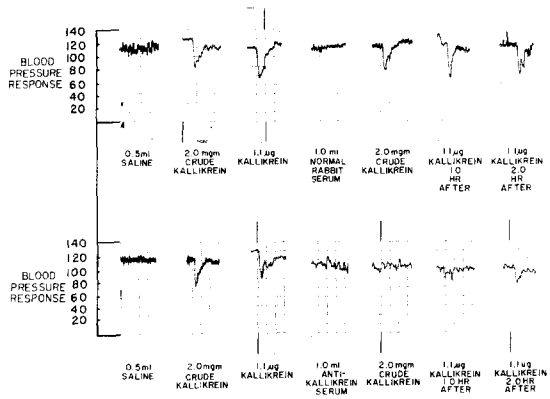

Fig. 6. The blocking activity *in vivo* of antikallikrein serum in rats. The normal rabbit serum response (upper half) was compared with the antikallikrein serum (lower half). The antikallikrein serum blocked the depressor response of kallikrein for up to 2 hr as shown in the lower half of the figure.

improved techniques have been employed such as electrofocussing [9] and affinity chromatography [10] using arginine methyl ester as the insoluble ligand. Arginine methyl ester has been reported to be a poor ligand in the isolation of trypsin [20]. Fritz and Förg-Brey [21] used guanidinated Trasylol coupled to CM-cellulose for the purification of kallikrein from the porcine pancreas, submaxillary gland and urine. The resulting enzyme had a specific activity comparable to kallikrein preparations obtained by conventional procedure. In view of this, it appears that a combination of anion-exchange, T-Sepharose and Sephadex chromatography offers a specific and rapid procedure for isolation of rat urinary kallikrein. This procedure might also have a potential use in isolation of glandular and urinary kallikrein of other species.

The specific activity of pure rat urinary kallikrein, isolated by different authors, is summarized in Table 2. Esterase activity of kallikrein has been commonly measured with BAEE and/or TAME. The specific esterase (BAEE) activity of the kallikrein preparation reported here was 124 μmoles/min/mg as compared to 51.5 μmoles/min/mg in the preparation of Porcelli and Croxatto [8]. The enzyme was assayed spectrophotometrically in both of those measurements. Using titrimetric assay, the specific activity of another preparation [9] was reported to be 193 μmoles/min/mg. In the present procedure, we used BAEE; however, we also used the colorimetric TAME assay of Roberts [24] in a subsequent preparation. The spe-

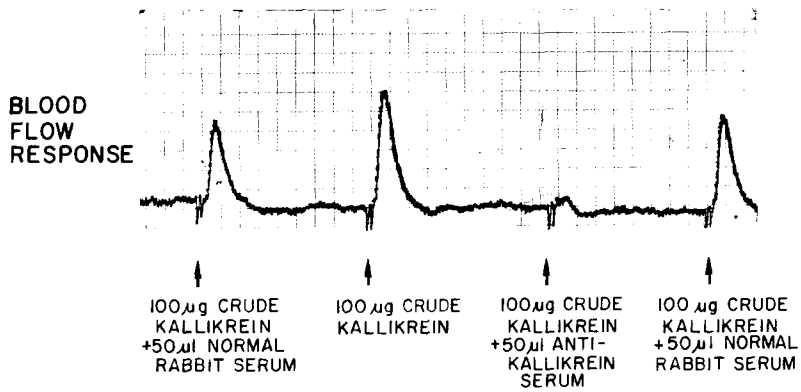

Fig. 5. Blood flow response (hind leg of the dog) to crude kallikrein in the presence and absence of antikallikrein serum. The test samples were injected as shown by the arrows

Table 2. Rat urinary kallikreins

Esterase (μmoles/min/mg)		Kininogenase (μg kinins/min/mg)	Per cent yield	Purification factor	Reference
BAEE	TAME*				
		455	18.6	390	Porcelli and Croxatto [8]
51.5†					Porcelli *et al.* [22]
	7.4	100	18.8	87	Silva *et al.* [10]
193	25	20–32	13	86‡	Nustad and Pierce [9]
	19–47	26–66§			Nustad and Pierce [23]
124		1795	17.4	694‡	Present paper

* Tosyl-arginine-methyl ester.
† Calculated from 1 esterase unit equivalent to 0.05 μmole BAEE/min.
‡ Calculated on the basis of enzyme B in rat urine.
§ Revised data on the basis of protein estimated by the Lowry procedure.

cific activity for TAME hydrolysis of another preparation was found to be 123 μmoles/min/mg. The kininogenase activity was measured with partially purified dog kininogen and found to be 1795 μg kinins/min/mg. Kininogenase activity has been measured by a variety of bioassay procedures, and the purity of kininogen is one of the important factors in the estimation. In view of this, it is difficult to compare kininogenase activity. The availability of a universal kallikrein standard should be most useful for this purpose.

The kallikrein demonstrated heterogeneity of two enzymic components when electrophoresed in 16% polyacrylamide gel. Four forms of kallikrein, with marginal differences in isoelectric points, have been reported [9]. Although the resolution obtained in the disc-gel electrophoresis and electrofocussing is not comparable, it appears that the urinary kallikrein might consist of two or more components. Nevertheless, it is not known whether the microheterogeneity is derived from a single proenzyme molecule or results from an aggregate.

The antikallikrein serum gave a single line of identity with pure kallikrein and crude enzyme. A weak reaction was also observed with kidney cortex homogenate which was perhaps due to the fact that kidney tissue contains a very small amount of kallikrein [2]. The antikallikrein serum did not show a precipitation band in immunodiffusion when reacted with the proteins excluded by the affinity column, rat liver homogenate or rat plasma. However, a possibility cannot be excluded that the antigen concentration in these preparations was below the detection limit of the Ouchterlony technique. Glandular kallikrein has been detected in lymph [25] but, as yet, not in plasma. The antikallikrein serum was found to have species-specificity, since it did not react with dog or human urinary proteins. The crude enzyme and pure kallikrein formed identical precipitin arcs in immunoelectrophoresis which confirmed the results of the Ouchterlony test. These experiments suggested that the antikallikrein serum was monospecific.

The antikallikrein serum was tested for its ability to block kallikrein activity. The increase in blood flow response (hind leg of the dog) due to kallikrein was completely blocked when the enzyme was mixed with antikallikrein serum just before the injection. The sheep antibody to rat urinary kallikrein has been reported [9] to inhibit the direct oxytocic effect of rat urinary kallikrein but not its TAME activity. Furthermore, the intravenous administration of 1.0 ml of antikallikrein serum in rats did not alter the existing blood pressure. The depressor response of crude kallikrein and pure kallikrein was blocked for up to 2 hr. Thus, urinary kallikrein appears to play no direct role in the regulation of blood pressure in normal rats, although its participation in the regulation of local blood flow cannot be excluded by these experiments.

Urinary kallikrein resembles renal kallikrein [2, 3], and is different from plasma kallikrein [1]. The physiological role of renal kallikrein is unsettled in spite of several reports relating the excretion of kallikrein with sodium and water [26–28]. These experiments could be amply validated if a specific inhibitor of kallikrein was available. The ability of the antikallikrein serum, reported here, specifically to inhibit the biological activity of kallikrein could be of importance in resolving the involvement of kallikrein in the physiological process.

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