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KININ-GENERATING AND ESTEROLYTIC ACTIVITY OF PURIFIED HUMAN URINARY KALLIKREIN (UROKALLIKREIN)*

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Abstract—Urinary kallikrein (urokallikrein), as defined by its capacity to generate kinin from heat-inactivated plasma or from purified human kininogen, was isolated from fresh concentrated male human urine and shown to be an antigenically unique urinary *p*-tosyl-L-arginine methyl ester HCl (TAMe) esterase. The isolation procedure achieved a 400- to 576-fold purification of the kinin-generating activity/mg of protein and yielded a product with albumin as the only significant contaminant at the isoelectric focusing step. The purified urokallikrein, defined by its kinin-generating activity, exhibited an isoelectric point with a range from pH 3.9 to 4.2 with charge heterogeneity, an apparent molecular weight of 25,000–40,000 on Sephadex gel filtration, and an anodal mobility on alkaline disc gels. Urokallikrein eluted from disc gels and identified by kinin-generating activity elicited monospecific antiserum in the rabbit. That purified urokallikrein is a TAMe esterase was evident from the concordance of kinin-generating activity, antigenic reactivity with a donkey antipancreas serum shown to recognize urokallikrein and esterolytic capacity as assessed after isoelectric focusing. There was suppression of the esterolytic activity of purified urokallikrein by increasing doses of TAMe or benzoyl-L-arginine methyl ester HCl (BAMe), and analysis of these data with Dixon plots indicated substrate inhibition.

Enzymes that generate kinin polypeptide activity from a plasma globulin substrate, kininogen, are termed kininogenases (EC 3.4.4.21) or kallikreins. Kallikreins have been recognized in human plasma [1], glandular tissue [2–4] and urine [4, 5] and the last, designated urokallikrein, has been considered to be a factor modulating blood pressure through the elaboration of kinins. Partial purification of urokallikrein from human [4, 6, 7] and other mammalian sources [8–10] has utilized either kinin-generating or esterolytic activity. An esterase assay, generally employing *p*-tosyl-L-arginine methyl ester HCl (TAMe),§ has been used to study the excretion of urokallikrein in hypertensive human diseases [11–14] and in experimental animal models [15–18]. However, not all urokallikrein preparations defined by kinin generation have cleaved arginine ester substrates such as TAMe [6], and human urine is known to contain esterase activity that is not attributable to urokallikrein [19].

In the present study, both esterolytic and kininogen-cleaving activities were examined and were associated at each step in the purification of urokallikrein from human urine. Nonetheless, the highly purified final preparation, when subjected to isoelectric focusing, contained some TAMe esterase activity that was not urokallikrein as assessed by immunochemical and functional criteria. Both partially and highly purified urokallikrein were markedly susceptible to inhibition by their synthetic ester substrates.

EXPERIMENTAL PROCEDURE

Materials. Synthetic bradykinin triacetate was purchased from New England Nuclear Corp. (Boston, MA); 99.9% pure methyl alcohol from Fisher Scientific Co. (Fair Lawn, NJ); human serum albumin from Schwarz/Mann Division, Becton Dickinson & Co. (Orangeburg, NY); alpha-*N*-methyl-alpha-*N*-tosyl-L-lysine-β-naphthyl ester HBr (MTLNe) and *p*-nitrophenyl-p′-guanidinobenzoate HCl (NPGB) from Nutritional Biochemical Corp. (Cleveland, OH); benzoyl-L-arginine methyl ester HCl (BAMe), *p*-tosyl-L-arginine methyl ester HCl (TAMe) and atropine sulfate from Sigma Chemical Co. (St. Louis, MO); 4,5-dihydroxy-2,7-naphthalene disulfonic acid disodium (chromotropic acid), *N*,*N*-dimethylformamide (DMF) and glacial acetic acid from Eastman Organic Chemicals (Rochester, NY); hexadimethrine bromide (Polybrene) from Aldrich Chemical Co. (Milwaukee, WI); lyophilized trypsin from Worthington Biochemical Corp. (Freehold, NJ); diethylaminoethyl (DE-52)-cellulose from Whatman Biochemicals (Clifton, NJ); Sephadex G-100 and the sizing standards: Blue Dextran 2000, ovalbumin, chymotrypsinogen A and ribonuclease A from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ); ampholine carrier ampholytes from

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§ Abbreviations used are: TAMe, *p*-tosyl-L-arginine methyl ester HCl; BAMe, benzoyl-L-arginine methyl ester HCl; MTLNe, alpha-*N*-methyl-alpha-*N*-tosyl-L-lysine-β-naphthyl ester HBr; NPGB, *p*-nitrophenyl-p′-guanidinobenzoate; CLN, carbobenzoxy-L-lysine *p*-nitrophenyl ester; DMF, *N*,*N*-dimethylformamide; Tris, Tris(hydroxymethyl)-aminomethane; DEAE, diethylaminoethyl; and TCA, trichloroacetic acid.

LKB, Inc. (Hicksville, NY); human plasminogen-free Enzodiffusion fibrin plates and streptokinase from Hyland Division, Travenol Laboratories, Inc. (Costa Mesa, CA); and rabbit anti-human albumin antiserum from Behring Diagnostics Division of Hoechst Corp. (Woodbury, NY).

Purification of urokallikrein. Two to three liters of fresh pooled normal male human urine collected over an 8-hr period was subjected to 100-fold concentration by positive pressure ultrafiltration at 4° using UM-10 Diaflo membranes (Amicon Corp., Lexington, MA) and stored at −70°. The concentrated crude urine from three collections was dialyzed for 24 hr at 4° against a 0.05 M Tris-acetate buffer, pH 7.2, 2.6 mS with two changes of dialysis buffer. The dialyzed urine concentrate was clarified by centrifugation at 900 *g* at 4°.

The entire urine concentrate was applied to DE-52 cellulose which had been packed in a 3000-ml Buchner fritted disc funnel and previously equilibrated with the dialysis buffer. After collection of 3000 ml effluent, the resin was eluted with the equilibrating buffer containing stepwise increments of 0.25, 0.50, 0.75 and 1.0 M NaCl at a rate of 300 ml/hr. Aliquots of each 300-ml fraction were screened for protein content by optical density at 280 nm and for kinin-generating and urokinase activities as described below. Fractions containing kinin-generating activity and free of urokinase were pooled and concentrated 100-fold. After a 12-hr dialysis against a 0.05 M Tris-acetate buffer, pH 6.5, 3.0 mS, this material was rechromatographed on a 5 × 100 cm column containing DE-52 cellulose equilibrated with the dialysis buffer. After collection of the effluent, a linear gradient to 1.0 M NaCl was applied. The column was run at 96 ml/hr and 16-ml fractions were collected and screened for protein content and for kinin-generating and urokinase activities.

Fractions comprising each peak of urokallikrein activity from DE-52 rechromatography were separately pooled and concentrated to 5 ml. These pooled fractions were dialyzed against a 0.05 M sodium acetate–acetic acid buffer, pH 5.0, containing 0.1 M NaCl, 12.5 mS, and applied to a 2.6 × 90 cm column of Sephadex G-100 preswollen in the same buffer. Four-ml fractions were collected at a flow rate of 24 ml/hr and screened for protein and urokallikrein activity. The peak of urokallikrein activity from Sephadex G-100 gel filtration was either concentrated *in toto* or divided into ascending and descending regions and concentrated to 5 ml.

Urokallikrein assays. The generation of kinin from kininogen by urokallikrein was bioassayed on a guinea pig terminal ileum segment suspended in Tyrode's buffer [20] made 5×10^{-7} M with atropine. Fractions were brought to pH 7.5 before assay, either by the addition of divalent cation-free Tyrode's buffer fortified with 2.5% sodium bicarbonate, or by dialysis against a 0.05 M Tris–HCl pH 7.5 buffer. One-tenth ml fractions were incubated with 0.1 ml of heat-inactivated plasma or 0.05 ml of purified kininogen for 5 min at 37° and the mixtures were immediately assayed for kinin content. The latter was quantitated by comparison with the contractile response of the ileum to a synthetic bradykinin standard. The preparation of heat-inactivated plasma for use as a kininogen source [21] was modified to utilize a 2-hr exposure at 61° in order to eliminate plasma kininase and spontaneous kinin-generating activity. Purified kininogen was prepared from fresh human plasma [22, 23] by DE-52 cellulose chromatography, Sephadex G-100 gel filtration and rechromatography on DEAE Sephadex.

The capacity of urokallikrein, neutralized by dialysis against the assay buffer, to release methanol from TAMe or BAMe was assessed with a colorometric technique [24, 25]. The enzyme–substrate interaction was carried out at 37° for 60 min in a final 0.5-ml volume of 0.05 M Tris–HCl buffer, pH 7.5. At the end of the incubation time 0.25 ml of 15% TCA was added to all samples. Controls containing substrate or enzyme alone were run simultaneously, and an amount of enzyme equivalent to that used in the experimental samples was then added to the substrate controls after the addition of TCA, to form reagent blanks. The reaction mixtures were centrifuged at 900 *g* for 20 min, and the supernatants were removed and assessed for methanol content. The net optical density at 580 nm was obtained by subtracting the optical density of the reagent blank from that of the complete reaction mixture. Net optical density was converted to methanol content using a standard curve generated with known amounts of methanol.

Urokinase assays. Urokinase, neutralized as for urokallikrein, was measured by its capacity to convert plasminogen to plasmin, which was quantitated in terms of either its fibrinolytic or esterolytic activity. In the fibrinolytic assay, 0.02-ml aliquots of column fractions were incubated with 2.9 μg of purified plasminogen [26, 27] in a final volume of 0.05 ml for 30 min at 37°, and 0.007 ml of the mixtures was applied to fibrin plates. After incubation at 37° for 24 hr, plasmin was quantitated using a standard curve developed with highly purified plasmin [27].

In the esterolytic assay, 0.05-ml aliquots of column fractions were incubated with 4.8 μg plasminogen in 0.1 ml for 30 min at 37° and 1.0 ml of 1×10^{-4} M MTLNe in 5% DMF was then added to the reaction mixture. After incubation for an additional 2 hr at the same temperature, the reaction was stopped with 0.3 ml of 1×10^{-4} M NPGB in 5% DMF and the release of beta-naphthol measured fluorometrically [28]. The plasmin generated was quantitated using a standard curve of beta-naphthol released by plasmin formed from highly purified plasminogen by streptokinase.

Analytical techniques and preparation of immunogen. Isoelectric focusing was carried out in 4 × 100 mm, 4% acrylamide gels with 2% ampholyte, pH range 3.5 to 5.0 or 3.5 to 10.0 [29]. After electrolysis to a stable minimum current, the gels were sliced into 5-mm segments and incubated for 4 hr in 0.2 ml of distilled water for pH determination. Two-tenths ml of 0.05 M Tris–HCl, pH 7.5, was then added to the slices and elution continued for 12 hr at 4°. Aliquots of 0.025 ml were removed from each eluate for albumin determination by Ouchterlony analysis. Fifty μl of the gel eluates was assessed for kinin-generating activity with either heat-inactivated plasma or purified kininogen. When both TAMe-hydrolyzing and kinin-generating activities were to be determined, gels were run in replicates of six. After slicing and pH

determination as above, elution was continued for 12 hr at 4° in 0.1 ml of 0.2 M Tris–HCl buffer, pH 8.0, containing 0.5 M NaCl. The diffusates of replicate slices were pooled according to their pH and dialyzed against 0.2 M Tris–HCl buffer with 0.5 M NaCl for 12 hr and for an additional 12 hr against the same buffer without NaCl. Two-hundredths ml of each pool was assessed for TAMe-hydrolyzing activity and 0.05 ml for kininogen cleavage. In each focusing run, one of the replicate gels was washed with 7% TCA and stained with a 0.1% solution of Coomassie blue in 20% ethanol–7% acetic acid.

Urokallikrein was identified antigenically after isoelectric focusing by two techniques, one utilizing eluates of the gel slices and the other employing the intact gel. Rheophoresis plates (Abbott Laboratories Inc., North Chicago, Ill.) were used for immunodiffusion of the eluates against a donkey anti-human pancreas antiserum absorbed with normal human plasma [19] and shown to recognize urokallikrein [30]. A focused gel sliced lengthwise was embedded in 1% agar, and a trough cut parallel to it [22] was filled with donkey anti-urokallikrein antiserum for diffusion at room temperature.

Alkaline disc gel electrophoresis was performed in a Buchler apparatus (Buchler Instruments, Fort Lee, N.J.) according to the instructions supplied by the manufacturer. Urokallikrein, purified through the Sephadex G-100 step, was electrophoresed and replicate gels were sliced into 0.2-mm segments, macerated and eluted with 0.3 ml of 0.2 M Tris–HCl, pH 8.0, for 18 hr at 4°. A replicate, unsliced gel was stained with Coomassie brilliant blue. Aliquots of 25 μl were examined for urokallikrein antigen by Ouchterlony analysis with the donkey antiserum to human pancreas. Five μl of each eluate was assessed for kinin-generating activity with heat-inactivated plasma and 50 μl was assessed for esterase activity on carbobenzoxy-L-lysine *p*-nitrophenyl ester (CLN) [31]. The remainder of the eluates from the gel segments containing kinin-generating activity was pooled, emulsified in complete Freund's adjuvant and injected into the toepads of a random-bred New Zealand rabbit.

RESULTS

Purification of urokallikrein. The chromatography of concentrated human urine on DE-52 cellulose at pH 7.2 yielded urokallikrein in the eluate range from 12.5 to 36 mS which also contained most of the protein (Fig. 1). Urokinase was predominantly present in the effluent, with a small amount appearing in the eluate between 7 and 14 mS. Urokallikrein-containing fractions eluting from 15 to 36 mS were pooled, concentrated and rechromatographed on DE-52 cellulose at pH 6.5 with a linear sodium chloride gradient (Fig. 2). Urokallikrein presented as a series of peaks in the eluate between 7 and 30 mS. Urokinase was not detectable in any column fraction.

The major functional peaks, fractions 230–280, 281–320 and 321–390, from DE-52 cellulose rechromatography were separately pooled, concentrated, dialyzed and applied to a Sephadex G-100 column. The bulk of the protein appeared in the void volumes, while kinin-generating activity from each of the three pools filtered in the molecular weight range from 25,000 to 40,000 (Fig. 3).

In a separate purification, esterase activity was assessed using 0.5-ml samples of every other column fraction obtained from G-100 gel filtration of the last and major functional peak from DE-52 cellulose rechromatography. Esterase activity utilizing 10 mM TAMe (5 μmoles in the assay mixture) was identified only in the kinin-generating peak which was comparable to the preparation depicted in Fig. 3C. The purification procedure leading to the type of preparation depicted in Fig. 3C was utilized routinely, and such preparations were further characterized by isoelectric focusing and in functional studies of urokallikrein. At this step, urokallikrein had been purified approximately 550-fold as compared with the starting urine concentrate (Table 1).

Functional and antigenic analysis of purified urokallikrein after isoelectric focusing and alkaline disc gel electrophoresis. Purified urokallikrein (Fig. 3C) was subjected to analytical isoelectric focusing in a pH range of 3.5 to 5.0. Kinin-generating activity in the eluates of a sectioned gel and urokallikrein antigen

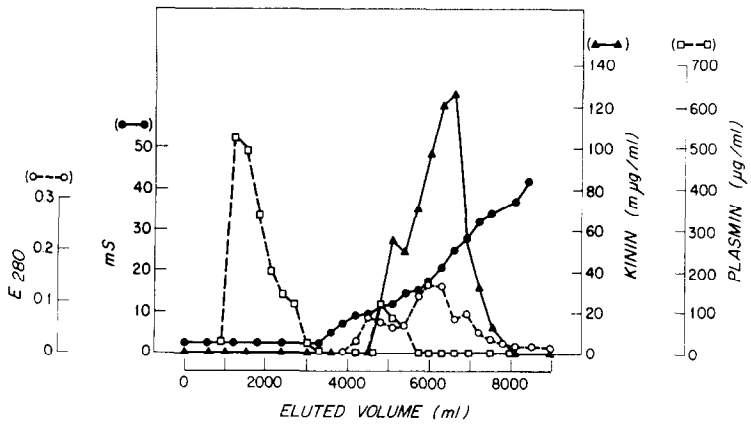

Fig. 1. Chromatography of concentrated crude urine on DE-52 cellulose using 0.05 M Tris–acetate buffer, pH 7.2, and stepwise increments of NaCl. The kinin-generating activity was measured with heat-inactivated plasma as a source of substrate. Urokinase was measured by its ability to convert plasminogen to plasmin.

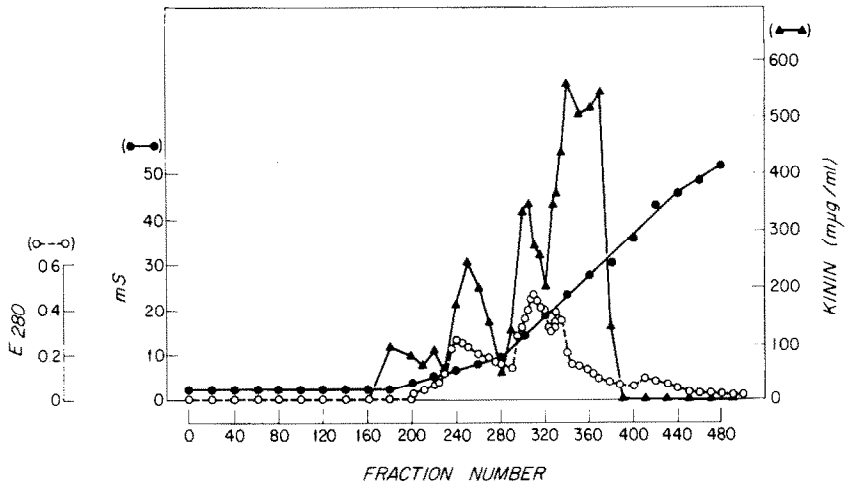

Fig. 2. Rechromatography of urokallikrein on DE-52 cellulose using a 0.05 M Tris–acetate buffer, pH 6.5, with a linear gradient to 1.0 M NaCl. The major peaks of kinin-generating activity, measured with heat-inactivated plasma, were pooled separately as indicated in the text and subjected to gel filtration.

in a replicate gel sliced longitudinally were located in the pH range of 3.9 to 4.2 (Fig. 4). The other half of the longitudinally sliced gel was stained and revealed three bands in the region corresponding to functional and antigenic urokallikrein. The apparent charge heterogeneity of purified urokallikrein was substantiated with three separate preparations which also revealed three stained bands in the region of the gel corresponding to functional and antigenic urokallikrein.

In order to determine the association between kinin-generating and TAMe esterase activities over a wider pH range, isoelectric focusing was carried out with pH 3.5 to 10 ampholytes (Fig. 5). Kinin-generating activity focused only at pH 3.9 to 4.3 and the

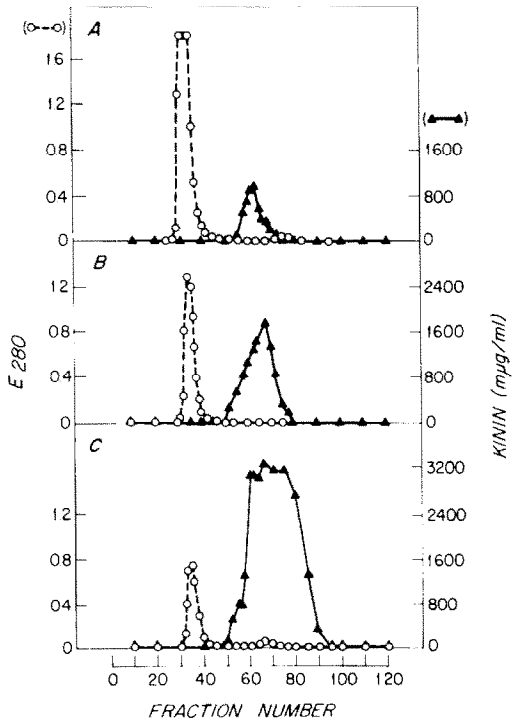

Fig. 3. Sephadex G-100 gel filtration of the major urokallikrein peaks recovered from DE-52 cellulose rechromatography in 0.05 M sodium acetate–acetic acid buffer, pH 5.0, containing 0.1 M NaCl. (A) Filtration of pooled fractions 230–280 of the experiment depicted in Fig. 2; (B) fractions 281–320; and (C) fractions 321–390. The sizing standards human serum albumin, ovalbumin, and chymotrypsinogen A eluted in fractions 50, 60 and 72, respectively. Urokallikrein was measured as for Figs. 1 and 2.

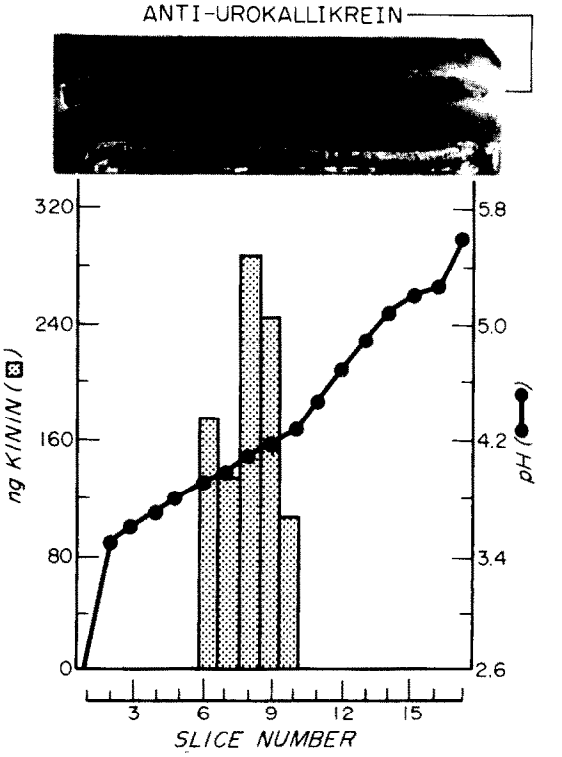

Fig. 4. Polyacrylamide gel isoelectric focusing of purified urokallikrein with pH 3.5 to 5.0 ampholytes. Kinin-generating activity of eluates from the gel slices was measured with heat-inactivated plasma. The insert depicts a replicate gel sliced longitudinally and assessed for urokallikrein antigen.

Table 1. Purification summary for human urokallikrein*

Step		Total protein recovered (mg)	Total kinin-generating activity recovered (μg kinin)	Specific activity (μg kinin generated/ mg protein)	Cumulative yield (%)	Purification factor
Starting material (crude urine concentrate)		1988	1209	0.61	100	1
DE-52 cellulose chromatography, pH 7.2		276	726	2.63	60	4.3
DE-52 cellulose chromatography, pH 6.5						
	Peaks I	84	21	0.25		0.41
	II	60	211	3.52	59	5.78
	III	43	484	11.26		18.51
G-100 Sephadex gel filtration						
	Peaks A	0.27	12	44.44		73.1
	B	0.32	106	331.25	59	544.8
	C	1.75	590	337.14		554.5

* Data are presented for one representative purification.

recovery of the applied material was 30 per cent when assayed with heat-inactivated plasma and 11.5 per cent when purified kininogen was used as substrate. The discrepancy in the recoveries of kinin-generating activity with the two substrates may be due to the limited quantities and partial denaturation of the purified kininogen used as compared to its concentration in heat-inactivated plasma. The 3.9 to 4.3 pH region where functional urokallikrein was located was associated with the only stained band in a replicate gel. Forty-two per cent of the TAMe esterase activity applied was recovered and more than half of this activity focused with the urokallikrein. The remainder of the TAME esterase activity focused in two separate peaks, neither of which exhibited kinin-generating activity or stainable protein. Antigenic analysis of eluates by rheophoresis revealed urokallikrein only in the two slices in which kinin-generating and TAMe esterase activities were located together. In a comparable experiment using a different urokallikrein preparation, only 25 per cent of the recovered esterase activity was associated with functional and antigenic urokallikrein, while the remainder again focused as two peaks in the alkaline pH range.

Disc gel electrophoresis of urokallikrein after the Sephadex G-100 gel filtration step revealed a single region of kinin-generating activity located near the anodal end of the gel with a recovery of 29 per cent. The kinin-generating activity was associated with three stained bands on a replicate gel and with the region in which the eluates formed immunoprecipitin arcs with the absorbed donkey antipancreas serum. Seventy-five per cent of the CLN-cleaving activity applied was recovered and 66 per cent of that recovered was associated with functional and antigenic urokallikrein while the remainder was present in a single region of lesser anodic mobility. The gel eluates from the region of the gel having kinin-generating activity elicited a monospecific antiserum in a rabbit which gave a line of complete identity with the

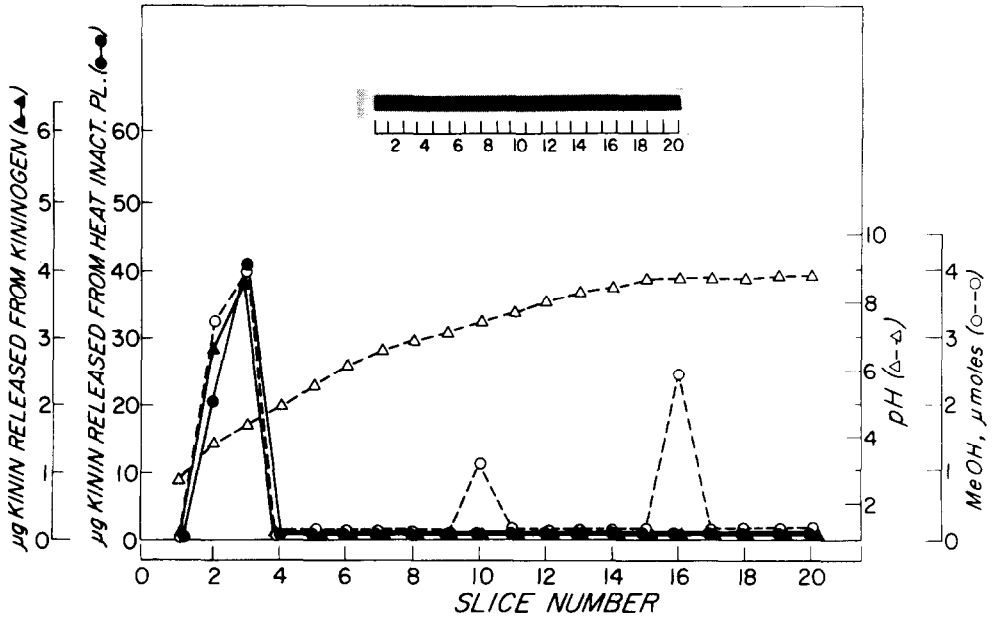

Fig. 5. Polyacrylamide gel isoelectric focusing of purified urokallikrein in pH 3.5 to 10 ampholytes. Kinin-generating activity was measured with both heat-inactivated plasma and purified kininogen and esterolytic activity was assessed with 10 mM (5 μmoles) TAMe. The insert at the top depicts a replicate gel stained with Coomassie blue.

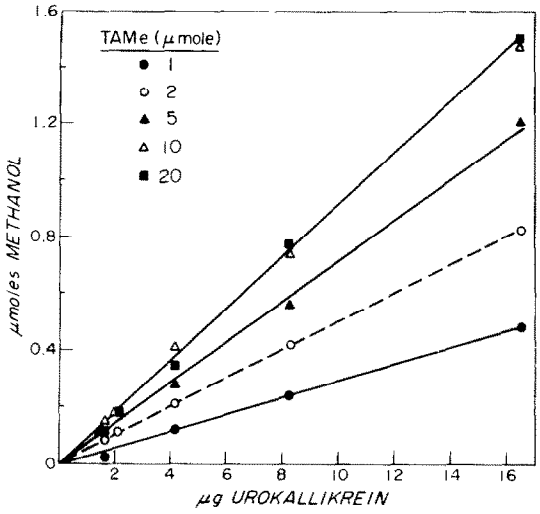

Fig. 6. Generation of methanol from 1 to 20 μmoles TAMe by increasing concentrations of purified urokallikrein.

absorbed donkey antipancreas serum when examined with either concentrated crude urine or purified urokallikrein.

Esterolytic activity of purified urokallikrein on arginine synthetic esters. Dose response and kinetic experiments were carried out with at least three different preparations of purified urokallikrein and typical experiments are presented. A 0.1-ml sample containing 16.5 μg of purified urokallikrein, capable of generating 18,000 ng kinin/ml of enzyme from heat-inactivated plasma, was diluted over a 10-fold range and incubated with 2–40 mM TAMe (1–20 μmoles in the reaction mixture) for 60 min at 37°. Methanol generation varied linearly with enzyme concentration (Fig. 6).

One-tenth ml containing 12.1 μg urokallikrein, capable of releasing 31,000 ng kinin/ml of enzyme from heat-inactivated plasma, was incubated with 10 mM TAMe (5 μmoles in the reaction mixture) for 0.5 to 90 min at 37°. Methanol release was linear over the standard 60-min incubation period (Fig. 7). Similar results were obtained with 50 mM TAMe.

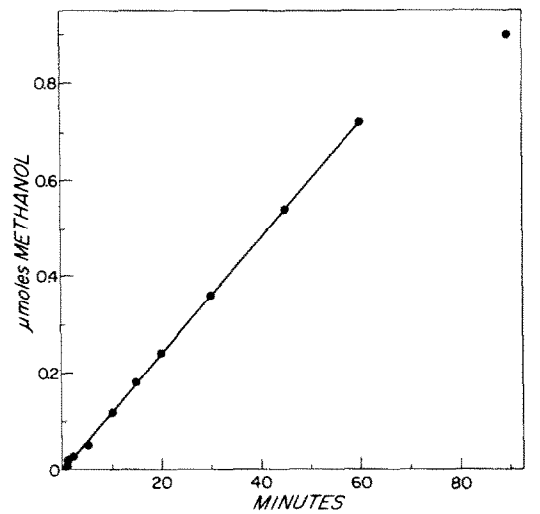

Fig. 7. Time course of release of methanol from 5 μmoles TAMe by purified urokallikrein.

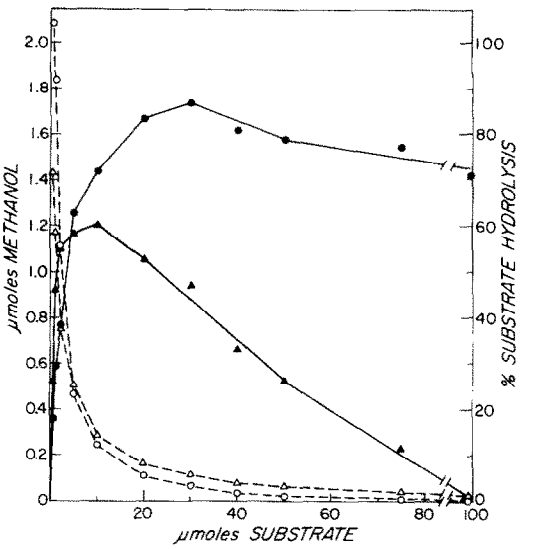

Fig. 8. Capacity of a single dose of purified urokallikrein to generate methanol from varying doses of TAMe (●) or BAMe (▲). The per cent of substrate cleavage is also indicated as a function of initial TAMe (○) or BAMe (△) concentration. The curve depicting methanol generated with 16.5 μg urokallikrein (●) is replotted in Fig. 9 as the center line (■).

When 1–200 mM TAMe or BAMe (0.5 to 100 μmoles in the reaction mixture) was incubated with 16.5 μg urokallikrein, capable of generating 18,000 ng kinin/ml of enzyme from heat-inactivated plasma, methanol release was not a linear function of substrate concentration except at very low substrate levels (Fig. 8). The per cent of substrate hydrolyzed declined significantly with more than 2.5 μmoles TAMe or BAMe. In no experiment was the pH at the end of the incubation period more than 0.1 pH

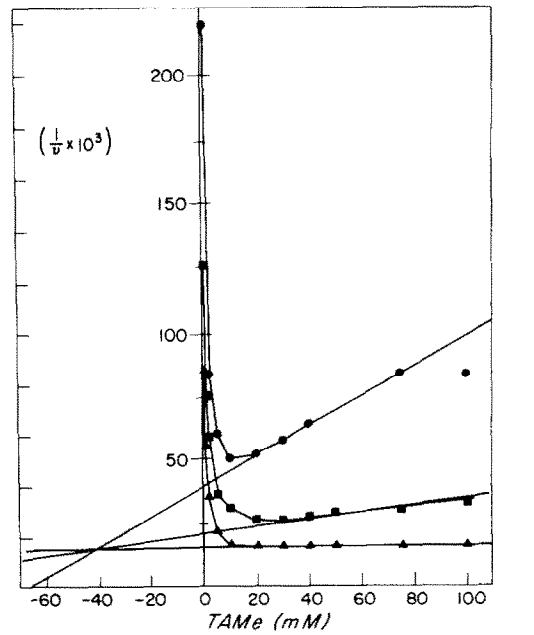

Fig. 9. Dixon analysis of the inhibition of 8.25 (●), 16.5 (■) and 33.0 (▲) μg urokallikrein by increasing initial concentrations of TAMe.

units below the starting pH of 7.5. Thus, inhibition at higher substrate levels could not be attributed to an increased release of acid during esterolysis. To examine whether this phenomenon was due to enzyme saturation or substrate inhibition, a series of curves was generated by incubation of increasing doses of TAMe with three concentrations of the urokallikrein preparation used above. At each dose of urokallikrein, methanol release reached a maximum and then showed a gradual decline as the initial TAMe concentrations were increased. When these data were replotted according to the method of Dixon and Webb [32], they yielded curves typical of substrate inhibition (Fig. 9). Lineweaver–Burk plots of the data obtained between 0 and 5 μmoles TAMe yielded a K_m of 4.35×10^{-3} M.

DISCUSSION

Urokallikrein, as defined by its capacity to generate kinin from heat-inactivated plasma or from purified kininogen, was purified from fresh, concentrated, male human urine and shown to be an antigenically unique TAMe esterase. Initial DE-52 cellulose chromatography of concentrated urine at pH 7.2 permitted almost complete recovery of urokallikrein in the eluate, with the bulk of urokinase and other proteins appearing in the effluent (Fig. 1). In three separate preparations, rechromatography at pH 6.5 of the urokallikrein fractions free of urokinase, representing 54–59 per cent of the initial product, yielded several peaks of urokallikrein in the eluate (Fig. 2). Each of the peaks was free of any detectable urokinase, and the recovery of the applied urokallikrein was almost complete when the activities of the peaks were summed. When each of the major peaks from DE-52 rechromatography was subjected to G-100 Sephadex gel filtration, the bulk of the protein appeared in the void volume while the kinin-generating activity which was entirely included exhibited an apparent molecular weight of approximately 25,000–40,000 (Fig. 3). The overall recovery at this step of the activity applied varied from 53 to 83 per cent. Because of the progressive removal of inhibitors and inactivators, the recoveries and specific activities noted in Table 1 reflect not only purification of the active principle but also the removal of factors affecting the bioassay. In each of three purifications, the bulk of urokallikrein activity appeared in the last eluate peak of DE-52 cellulose rechromatography in the range from 19 to 30 mS (Fig. 2), and Sephadex G-100 filtration of this activity (Fig. 3C) yielded a urokallikrein preparation that was purified 400- to 576-fold over the starting urine concentrate (Table 1). When analyzed by isoelectric focusing, albumin was the only significant contaminant and this product was routinely employed for further functional and immunochemical characterization. Without correcting for the albumin contamination, the kinin-generating activity varied from 305 to 830 ng/μg of enzyme when incubated with heat-inactivated plasma for 5 min at 37°. The isoelectric point of purified urokallikrein defined in terms of its kinin-generating activity was 3.9 to 4.2 when focused using ampholytes with a pH range of 3.5 to 5.0 and 3.9 to 4.3 in the pH range 3.5 to 10.0 (Figs. 4 and 5).

Published isolation procedures for esterase activity have yielded recoveries of 6–43 per cent with purification factors ranging from 25- to 650-fold [4, 7, 33]. A yield of 43 per cent with a 25-fold purification factor was reported [6] in a procedure in which kinin generation was followed. These urokallikrein preparations exhibited apparent molecular weights of 27,000 and 29,000 [33] and 43,600 [6]. Molecular heterogeneity and isoelectric points ranging from pH 3.8 to 4.2 have also been reported in previous studies in which urokallikrein was assessed by its hypotensive effect *in vivo* in the dog [33] or its esterolytic activity on N^{α}-carbobenzoxy-L-tyrosine-*p*-nitrophenyl ester [6].

In the present study, the focused urokallikrein accounted for more than half of the TAMe esterase activity in two purified preparations, with additional activities focusing in the alkaline pH range (Fig. 5). The contaminating esterolytic activities, appreciated only with the wide range ampholytes, were distinct from urokallikrein in neither being associated with kinin-generating activity nor being precipitated by specific antibody to urokallikrein. The failure of others to appreciate TAMe esterase activity in urokallikrein preparations, defined by kinin-generating activity during purification and by N^{α}-carbobenzoxy-L-tyrosine-*p*-nitrophenyl ester hydrolysis after focusing [6], could be a consequence of substrate inhibition. That highly purified urokallikrein is a TAMe esterase is evident from the isoelectric concordance of kinin-generating activity, antigenic urokallikrein and TAMe esterase activity (Figs. 4 and 5). Separation of kinin-generating and esterolytic activity from a contaminating esterase was also observed on disc gel electrophoresis using CLN as the ester substrate. When the kinin-generating fractions were used as an immunogen in a rabbit, monospecific antiserum was obtained which gave a line of identity with the absorbed donkey anti-pancreas serum when reacted with either crude urine or purified urokallikrein. This finding confirms the antigenic relationship between glandular pancreatic kallikrein and the protein in urine with kinin-generating and esterolytic activity termed urokallikrein.

The purified urokallikrein preparation known to contain some esterase activity distinct from urokallikrein was employed in further studies of TAMe and BAMe. This preparation yielded a linear dose response of substrate hydrolysis when the TAMe concentration was limited to 1–20 μmoles (Fig. 6). Cleavage of limited substrate was also linear during a 60-min interaction period (Fig. 7). On the other hand, as initial substrate concentration was increased, there was striking suppression of the per cent of hydrolysis of TAMe or BAMe (Fig. 8). When these data were analyzed according to the method of Dixon, the tangents to the curves intersected at a single point, indicative of substrate inhibition (Fig. 9). Inhibition of desialylated porcine pancreatic kallikrein by high concentrations of BAMe has been noted in experiments performed at pH 9.0 [34] and several other enzyme systems also susceptible to high substrate inhibition have been described: hydrolysis of ethyl butyrate by sheep liver carboxyesterase [35], oxidation of leucine by snake venom L-amino acid oxidase with methylene blue as hydrogen acceptor [32, 36] and the oxidation of 3β- and 17β-hydroxysteroids by

β-hydroxysteroid dehydrogenase with DPN^+ as hydrogen acceptor [37].

A sensitive assay for TAMe esterase activity has been developed using TAMe labeled with tritium in the methyl position [38] and has been applied to the measurement of excreted urokallikrein in the human [11, 39] and experimental animals [15, 40, 41]. In the experiments described herein and depicted in Figs. 4–7, 5×10^3 times as much TAMe and 5 to 20×10^3 times as much urine enzyme source were employed as in the modified method using radiolabeled TAMe [39]. Thus, the molar ratios of enzyme and substrate utilized in both assays are comparable. The K_m values of 2.6×10^{-3} M [36] and 1.14×10^{-3} M [6] previously reported for urokallikrein activity on TAMe are comparable to the value of 4.35×10^{-3} M obtained in the current studies with 0.5–2.5 mM TAMe (Fig. 8).

REFERENCES

1. E. Werle, M. Forell and L. Maier, *Naunyn-Schmiedebergs Archs exp. Path. Pharmak.* **225,** 369 (1955).
2. E. K. Frey and E. Werle, *Klin. Wschr.* **12,** 600 (1933).
3. E. Werle and P. von Roden, *Biochem. Z.* **286,** 213 (1936).
4. H. Moriya, J. V. Pierce and M. E. Webster, *Ann. N.Y. Acad. Sci.* **104,** 174 (1963).
5. E. K. Frey and H. Kraut, *Naunyn-Schmiedebergs Archs. exp. Path. Pharmak.* **133,** 1 (1928).
6. V. Hial, C. R. Diniz and M. Mares-Guia, *Biochemistry* **13,** 4311 (1974).
7. G. Porcelli, G. B. Marini-Bettolo, H. R. Croxatto and M. Di Iorio, *Ital. J. Biochem.* **23,** 1 (1974).
8. J. L. Prado, E. S. Prado, C. M. W. Brandi and A. V. Katchburian, *Ann. N.Y. Acad. Sci.* **104,** 186 (1963).
9. K. Nustad and J. W. Pierce, *Biochemistry* **13,** 2312 (1974).
10. E. Silva, C. R. Diniz and M. Mares-Guia, *Biochemistry* **13,** 4304 (1974).
11. H. S. Margolius, R. Geller, J. J. Pisano and A. Sjoerdsma, *Lancet* **2,** 1063 (1971).
12. A. Adetuyibi and I. H. Mills, *Lancet* **2,** 203 (1972).
13. H. S. Margolius, D. Horwitz, J. J. Pisano and H. R. Keiser, *Fedn Proc.* **35,** 1203 (1976).
14. J. C. McGiff and A. Nasjletti, *Fedn Proc.* **35,** 172 (1976).
15. H. S. Margolius, R. Geller, W. de Jong, J. J. Pisano and A. Sjoerdsma, *Circulation Res.* **30,** 358 (1972).
16. G. Porcelli, G. Bianchi and H. R. Croxatto, *Proc. Soc. exp. Biol. Med.* **149,** 983 (1975).
17. I. H. Mills, N. A. A. MacFarlane, P. E. Ward and L. F. O. Obika, *Fedn Proc.* **35,** 181 (1976).
18. H. R. Keiser, R. G. Geller, H. S. Margolius and J. J. Pisano, *Fedn Proc.* **35,** 199 (1976).
19. E. L. Greene, S. P. Halbert and D. Keifer, *Int. Archs Allergy appl. Immun.* **48,** 764 (1975).
20. J. H. Gaddum, in *Pharmacology*, p. 16. Oxford University Press, London (1959).
21. M. E. Webster and J. L. Prado, *Meth. Enzym.* **19,** 681 (1970).
22. J. Spragg and K. F. Austen, *J. Immun.* **107,** 1512 (1971).
23. J. Spragg and K. F. Austen, *Biochem. Pharmac.* **23,** 781 (1974).
24. A. M. Siegelman, A. S. Carlson and T. Robertson, *Archs Biochem. Biophys.* **97,** 159 (1962).
25. R. N. Colman, L. Mattler and S. Sherry, *J. clin. Invest.* **48,** 23 (1969).
26. D. G. Deutsch and E. T. Mertz, *Science, N.Y.* **170,** 1095 (1970).
27. E. H. Magoon, K. F. Austen and J. Spragg, *Clin. exp. Immun.* **17,** 345 (1974).
28. P. H. Bell, C. T. Dziobkowski and M. E. Englert, *Analyt. Biochem.* **61,** 200 (1974).
29. P. G. Righetti and J. W. Drysdale, *Biochim. biophys. Acta* **236,** 17 (1971).
30. O. ole-MoiYoi, J. Spragg and K. F. Austen, *J. Immun.* **118,** 667 (1977).
31. R. M. Silverstein, *Analyt. Biochem.* **65,** 500 (1975).
32. M. Dixon and E. C. Webb, in *Enzymes* (Eds. M. Dixon and E. C. Webb), 2nd Edn, p. 75. Academic Press, New York (1964).
33. H. Moriya, Y. Matsuda, Y. Fujimoto, Y. Hojima and C. Moriwaki, in *Kininogenases, Kallikrein First International Symposium* (Eds. G. L. Haberland and J. W. Rohen), p. 37. F. K. Schattauer Verlag, Stuttgart (1973).
34. F. Fiedler and E. Werle, *Eur. J. Biochem.* **7,** 27 (1968).
35. D. R. P. Murray, *Biochem. J.* **24,** 1890 (1930).
36. T. P. Singer and E. B. Kearney, *Archs Biochem.* **29,** 190 (1950).
37. P. I. Marcus and P. Talalay, *Proc. R. Soc. B* **144,** 116 (1955).
38. V. H. Beaven, J. V. Pierce and J. J. Pisano, *Clinica chim. Acta* **32,** 67 (1971).
39. H. S. Margolius, D. Horwitz, R. G. Geller, R. W. Alexander, J. R. Gill, Jr., J. J. Pisano and H. R. Keiser, *Circulation Res.* **35,** 812 (1974).
40. H. R. Keiser, M. J. Andrews, Jr., R. A. Guyton, H. S. Margolius and J. J. Pisano, *Proc. Soc. exp. Biol. Med.* **151,** 53 (1976).
41. I. H. Mills and P. E. Ward, *J. Physiol., Lond.* **246,** 695 (1975).