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Specificity of the Amidase and Kininogenase Methods for the Determination of Rat Urinary Kallikrein

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Summary: The specificity of the amidase and kininogenase methods for determining rat urinary kallikrein was studied. Male and female rat urine was employed. Esterase A₁, A₂ and kallikrein were separated by DEAE-Sephadex A-50 chromatography. Esterase A₁ showed no amidase activity towards the substrate H-D-Val-Leu-Arg-*p*-nitroanilide. In contrast, esterase A₂ and kallikrein attacked the substrate, and the activity of kallikrein was especially inhibited by aprotinin, while esterase A₂ was more sensitive to soybean trypsin inhibitor. Esterase A₁ did not show kininogenase activity, whereas esterase A₂ showed this activity, but only towards the dog plasma substrate. Kallikrein possessed kininogenase activity towards both dog and rat plasma kininogen. We believe that the most specific method for measuring rat urinary kallikrein activity is the kininogenase method using partially purified rat plasma kininogen.

Introduction

The determination of rat urinary kallikrein^{1),2)} has been used as an index of activity in the renal kallikrein-kinin system. The activity of this enzyme has been measured by bioassay in the rat isolated uterus (1, 2), by its esterase activity upon synthetic arginine esters (3) and by its kininogenase activity towards heated plasma or purified fractions from bovine, dog or rat plasma (4, 5). More recently, with the availability of synthetic substrates, in which a specific amino acid sequence is coupled to a chromophore (6), the amidase method gained favour, because it was quicker and easier to apply. However, three esterase activities have been described in rat urine: esterase A₁, esterase A₂, and kallikrein (7). It has been reported that esterase A₂, a plasminogen activator, also has kininogenase activity (7, 8).

The purpose of this work was to evaluate the specificity of the amidase method using the substrate H-D-Val-Leu-Arg-*p*-nitroanilide (S-2266), and that of the kininogenase method using partially purified kininogen from dog and rat plasma.

Materials and Methods

Chemicals

The following materials were obtained from commercial sources: Lima bean trypsin inhibitor (type II-L), Soybean trypsin inhibitor (type II-S), human α_1 -proteinase inhibitor (" α_1 -antitrypsin"), aprotinin, trypsin (type XIII: TPCK¹⁾ treated) and bradykinin (Sigma); S-2266 (Kabi Vitrum, Stockholm, Sweden); DEAE-Sephadex A-50 (Pharmacia Fine Chemicals); N^ε-*p*-tosyl-arginine methyl ester (Schwarz Mann); bradykinin antibody was a gift from Dr. O. Carretero, Henry Ford Hospital, Detroit.

Experimental procedure

Collection of rat urine

Sprague-Dawley male and female rats were employed. Urine was collected under toluene over 24 h. The urine was filtered and dialysed against distilled water for 48 h, then lyophilized.

¹⁾ Abbreviations

KIU: Kallikrein inhibitor units. S-2266: H-D-Val-Leu-Arg-*p*-nitroanilide. TAME: *p*-tosyl-*L*-arginine methyl ester HCl. TPCK: *L*-1-toxylamide-2-phenylethyl chloromethyl ketone.

²⁾ Enzymes

Trypsin (EC 3.4.21.4). Tissue kallikrein (EC 3.4.21.35).

The sample of lyophilized urine was resuspended at one tenth of the initial volume in 0.01 mol/l sodium phosphate buffer pH 7.0, 0.05 mol/l NaCl.

Chromatographic separation of esterases

- A. Concentrated urine (10 ml) from male or female rats was applied to a DEAE-Sephadex A-50 column (0.6 × 20.0 cm) according to *Mc Partland* et al. (7). The eluate was collected in three pools: tubes 1 to 10, 11 to 20 and 21 to 30, corresponding to esterases A₁, A₂ and kallikrein, respectively. These pools were dialysed against 0.01 mol/l Tris HCl buffer pH 7.0 for 24 h. Esterase and amidase activities were then determined in each pool, and the recovery was calculated.
- B. Concentrated male rat urine (60 ml) was applied to a DEAE-Sephadex A-50 column (2.2 × 12.1 cm) equilibrated in 0.01 mol/l sodium phosphate buffer pH 7.0, 0.05 mol/l NaCl. The sample was applied and the column, which was then washed with 90 ml of the equilibration buffer, followed by a 500 ml gradient of 0.01 mol/l phosphate buffer pH 7.0 from 0.05 mol/l NaCl to 0.6 mol/l NaCl, with a flow rate of 35 ml/h. Three pools of eluted fractions were made: tubes 5 to 19 were considered for esterase A₁, tubes 32 to 51 for esterase A₂, and tubes 75 to 103 for kallikrein. These pools were dialysed against distilled water for 24 h before determining their enzymatic activities, which were used to calculate the recovery.

Analytical Methods

Esterase activity

The TAME¹ colorimetric assay (substrate: N^α-*p*-tosyl-arginine methyl ester) employed by *Nustad & Pierce* was applied (9). Activity was expressed as μmol of substrate consumed per minute of incubation and per liter of the eluate (U/l).

Amidase activity

The method described by *Amundsen* et al. (10) was used. Aliquots of urine or of the eluate from the column were incubated with 0.1 μmol H-*D*-Val-Leu-Arg-*p*-nitroanilide (S-2266) for 30 min in 0.2 mol/l Tris-HCl buffer pH 8.0, final volume of 1 ml, in the absence of aprotinin. The reaction was stopped with 0.1 ml 50% acetic acid and the absorbance was read at 405 nm. The activity was expressed in μmol of *p*-nitroaniline ($\epsilon_{405} = 1050 \text{ m}^2/\text{mol}$) formed in one min of incubation per liter of the eluate or urine (U/l).

Inhibition studies

Aliquots of the A₂ esterase pool, with an amidase activity of 0.58 ± 0.02 nmol/min and of the kallikrein pool with an amidase activity of 0.54 ± 0.02 nmol/min were incubated with soybean trypsin inhibitor or Lima bean trypsin inhibitor (final concentration 2.5 to 50 mg/l), aprotinin (final concentration 25 to 500 KIU/ml), or α₁-proteinase inhibitor (final concentration 0.5 to 50 mg/l) for 30 min at 37 °C in 0.2 mol/l Tris-HCl buffer pH 8.0, final volume 0.9 ml. The substrate S-2266¹ was then added and the amidase method was resumed.

Kininogenase activity

A modification of the method described by *Carretero* et al. (11) was used. One aliquot of the enzyme was incubated with kininogen (capable of generating 500 ng kinins) in 0.05 mol/l sodium phosphate buffer pH 7.4, 0.15 mol/l NaCl, 3 mmol/l EDTA, 1 mmol/l *o*-phenanthroline, 250 mg/l NaN₃, in a final

volume of 0.5 ml for 15 min. The reaction was stopped with 2 ml 960 g/l ethanol. The precipitate was washed with 1 ml 960 g/l ethanol. The supernatants containing the kinins were dried in a bath at 50 °C under a stream of air. The residue obtained was resuspended in 1 ml 0.1 mol/l Tris-HCl pH 7.4, 2 g/l gelatin, 250 mg/l NaN₃ and the amount of kinins determined by radioimmunoassay. Enzymatic activity was expressed as mg of kinins produced per minute of incubation per liter of the eluate (mg/min · l).

Kininogen preparation

The citrated plasma from rat or dog blood was heated to 56 °C–60 °C for 3 hours, followed by addition of solid ammonium sulphate (114 g/l) to 0.25 saturation. The mixture was stirred for one hour at room temperature, then centrifuged at 12100 g for 10 min; the supernatant was again precipitated with ammonium sulphate (125 g/l) at 0.43 saturation. The precipitate was resuspended and dialysed against distilled water at 4 °C for 48 h. It was then submitted to a chromatographic separation on DEAE-Sephadex A-50, previously equilibrated with 0.02 mol/l phosphate buffer pH 6.3, 0.05 mol/l NaCl. The fraction containing kininogen was eluted with a linear gradient ranging from 0.05 mol/l to 0.5 mol/l NaCl. It was finally dialysed and lyophilized. The kininogen content was estimated by incubation with an excess of trypsin or rat urinary kallikrein. The kinin content of dog kininogen per mg protein was 1400 ng with trypsin and 1152 ng with kallikrein, while the kinin content of rat kininogen was 409 ng with trypsin and 126 ng with kallikrein. Heated rat plasma and the kininogen preparations did not show kininase activity when incubated under the conditions of the kininogenase assay.

Radioimmunoassay for kinins

Briefly, the samples or standards were incubated in 0.1 mol/l Tris-HCl pH 7.4, 2 g/l gelatin, 250 mg/l sodium azide with bradykinin antiserum (1:40 000 and [¹²⁵I]bradykinin (10 000 counts/min) in a final volume of 0.6 ml at 4 °C for 20 h. One ml of dextran coated charcoal in barbital buffer pH 7.4 was then added, and after centrifugation at 4810 g for 20 min the supernatant was separated and counted in a Gamma LKB counter. The sensitivity of the RIA was 10 pg with an intraassay variability coefficient of 4.2% and an interassay variability coefficient of 6.7%.

Protein determination

Protein was measured by absorbance at 280 nm and by the method of *Lowry* et al. Bovine serum albumin was used as standard (12).

Conductivity determination

The conductivity of DEAE-Sephadex A-50 chromatography fractions was measured at 25 °C with a Radiometer conductivity meter, Copenhagen Type CDM 2e.

Results

Using DEAE-Sephadex chromatography, three peaks of TAME esterase activity were found in the urine of male rats and two peaks in the urine of female rats (fig. 1 a and 1 b). The peak that eluted at a concentration of 0.05 mol/l NaCl was present only in male rats, and did not show any amidase activity with

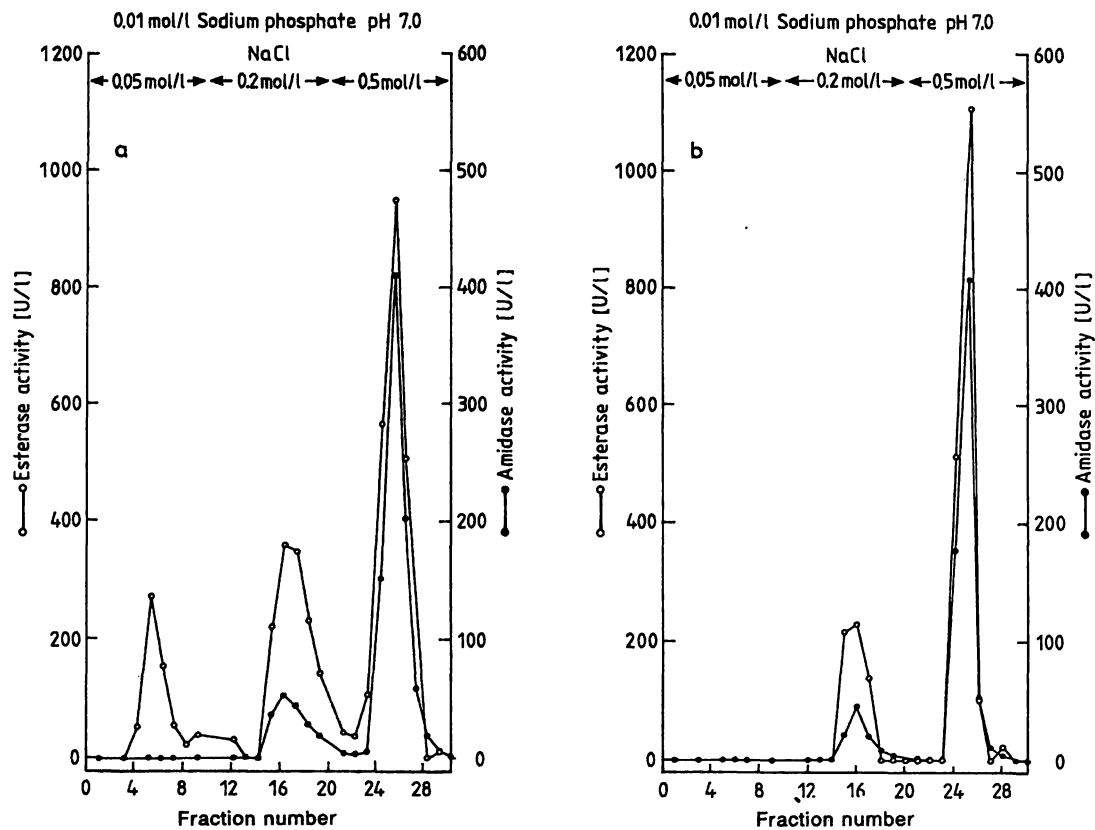


Fig. 1. DEAE-Sephadex A-50 chromatography with stepwise elution of rat urine. Elution profile of the esterase (substrate: *p*-tosyl-*L*-arginine methyl ester hydrochloride, ○—○) and amidase (substrate: *H-D*-Val-Leu-Arg-*p*-nitroanilide, ●—●) activities. (a) male rat urine; the flow rate was 24 ml/h. Fractions (1 ml) were collected, and fractions 1 to 10, 11 to 20 and 21 to 30 were pooled. (b) female rat urine; the flow rate was 9 ml/h; fractions were collected and pooled as for male rat urine.

D-Val-Leu-Arg-*p*-nitroanilide. In contrast, the peaks that eluted at concentrations of 0.2 mol/l and 0.5 mol/l NaCl, corresponding to esterase A₂ and kallikrein, did contain amidase activity from both male and female rats. The peak of esterase A₂ accounted for 16.9% of the total amidase activity present in the urine of male rats and for 14.8% of that present in female rats.

We believe that the esterase A₂ fraction is free from renal kallikrein, since the results from chromatography with stepwise elution were confirmed by a gradient elution procedure, which gave better resolution. Repetition of this procedure showed that the esterase A₂ fraction always showed kininogenase activity (see fig. 4). We submitted the esterase A₂ fraction to Western blot analysis with polyclonal antibody against rat urinary kallikrein, and the results were negative. It has been reported that highly purified esterase A₂ shows kininogenase activity towards heated dog plasma (8, 14).

The recovery of amidase activity was 92.7% for male rat urine and 72.9% for female rat urine.

The pattern of inhibition of these amidase activities was studied against some trypsin inhibitors in order to selectively inhibit one of the two enzymes. The amidase activity of esterase A₂ proved to be more sensitive than that of kallikrein to soybean trypsin inhibitor (fig. 2); on the other hand, the latter was more sensitive than esterase A₂ to aprotinin (fig. 3). However, none of these inhibitors is specific for any of these enzymes. In fact, soybean trypsin inhibitor at 50 mg/l, which inhibited 85.9% of the esterase A₂ activity, also inhibited kallikrein up to 12.3%. Likewise, aprotinin concentrations (50, 100 and 200 KIU/ml) that inhibited kallikrein up to 95.8%, 98.8% and 99.5%, respectively, also inhibited esterase A₂ up to 30%, 43.5% and 61%. Lima bean trypsin inhibitor and α₁-antitrypsin did not inhibit the amidase activity either of esterase A₂ or of kallikrein at the concentrations employed.

The study of the ability of the fractions containing esterase A₁, A₂ and kallikrein to generate kinins from kininogen from dog or rat plasma was carried out in urine from male rats. This urine was submitted to

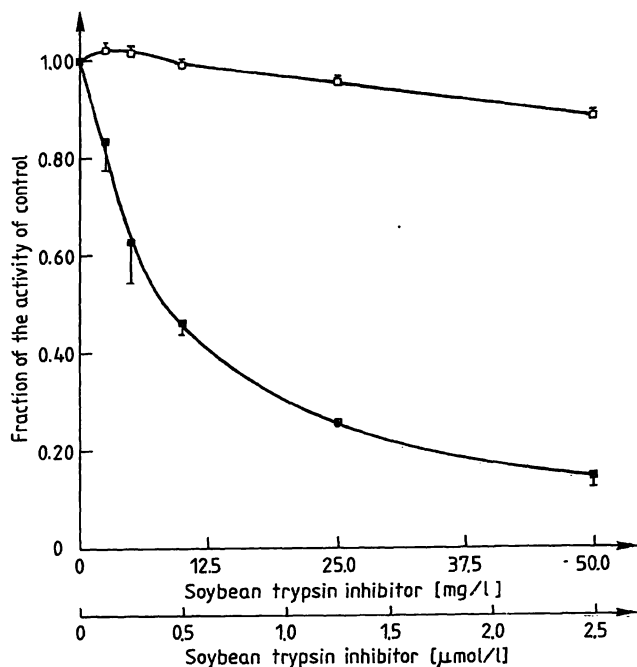


Fig. 2. Inhibition of the two amidase activities (substrate: *H-D-Val-Leu-Arg-p-nitroanilide*) present in rat urine by soybean trypsin inhibitor.

Aliquots of esterase A₂ fraction (\square) with an amidase activity of 0.58 ± 0.02 nmol/min and of kallikrein fraction (\circ) with an amidase activity of 0.54 ± 0.02 nmol/min were incubated with increasing concentration of soybean trypsin inhibitor (as described in Materials and Methods). Values are expressed as $\bar{x} \pm$ S. D. of three determinations.

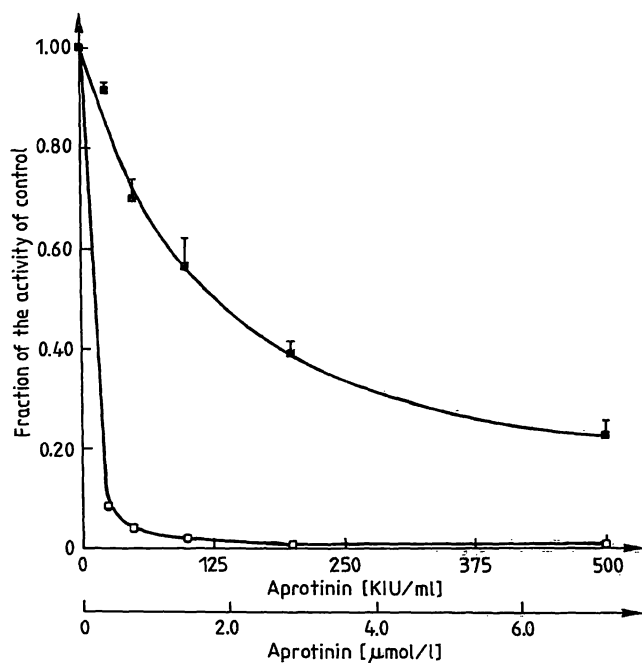


Fig. 3. Inhibition of the two amidase activities present in rat urine by aprotinin.

The conditions were the same as those described in the legend to figure 2, except that aprotinin was used instead of soybean trypsin inhibitor. (\blacksquare) esterase A₂ fraction; (\square) kallikrein fraction. Values are expressed as $\bar{x} \pm$ S. D. of three determinations.

chromatographic separation with a gradient elution in order to better resolve the peaks of enzymatic activity. It was found that the first peak of esterase activity had no kininogenase activity. However, the two peaks of esterase activities, which also possessed amidase activity, also hydrolysed dog kininogen. In contrast, rat kininogen from heated plasma, or the partially purified fraction of kininogen, was hydrolysed only by kallikrein (fig. 4). The ratios of kininogenase activity/amidase activity when using the dog

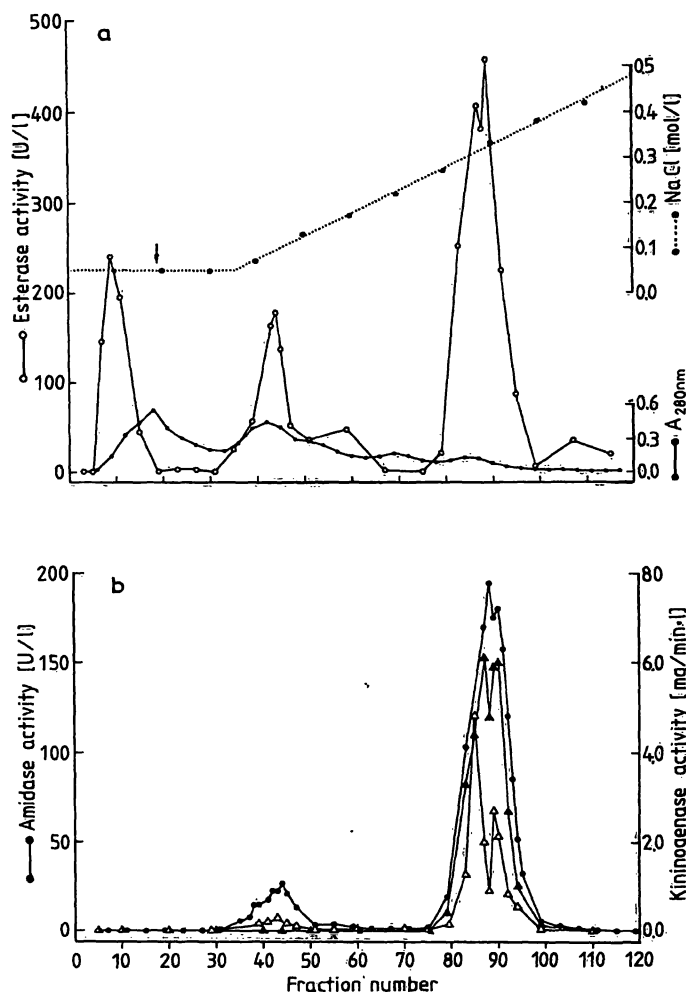


Fig. 4. Chromatography of male rat urine in DEAE-Sephadex A-50 with gradient elution.

The flow rate was 35 ml/h and 5 ml fractions were collected. The arrow indicates the beginning of the elution gradient from 0.05 mol/l NaCl to 0.5 mol/l NaCl in 0.01 mol/l sodium phosphate buffer pH 7.0.

a) Plot of A_{280} ($\bullet-\bullet$), NaCl concentrations ($\bullet\cdots\bullet$) and esterase activity (substrate: *p-tosyl-L-arginine methyl ester hydrochloride*, $\circ-\circ$). NaCl concentration in the eluate was estimated from conductivity measurements.

b) Plot of amidase activity (substrate: *H-D-Val-Leu-Arg-p-nitroanilide*, $\bullet-\bullet$) and kininogenase activity towards dog kininogen (Δ) and towards rat kininogen (\blacktriangle).

Fractions pooled were: 5-19 for esterase A₁; 32-51 for esterase A₂; and 75-103 for kallikrein.

substrate were 0.026 and 0.027 (kinin, mg/min · l)/(p-nitroaniline, $\mu\text{mol}/\text{min} \cdot \text{l}$) for esterase A₂ and kallikrein, respectively, and these values are similar to those found in whole urine (0.025). When using rat kininogen as the substrate, the ratios were 0 and 0.048, respectively. Neither esterase A₂ nor kallikrein showed kininase activity. The recovery of amidase activity was 84.3%, while that of kininogenase activity was 68.7% with dog kininogen, and 67.2% with rat kininogen.

Discussion

Three esterase activities were found in the urine of male rats: A₁, A₂ and kallikrein, whereas female rat urine had no esterase A₁ activity. According to our results, esterase A₂ showed amidase activity towards the synthetic substrate S-2266. Using this substrate, it was shown that esterase A₂ accounted for about 15% of the total amidase activity present in the urine of both male and female rats. This value was similar to that described by *Takaoka et al.* in Wistar rat urine when using the synthetic substrate Pro-Phe-Arg-4-methylcoumaryl-7-amide (13).

The study of the inhibition of the two amidase activities present in urine revealed that esterase A₂ was inhibited more by soybean trypsin inhibitor than by aprotinin, whereas kallikrein was more inhibited by aprotinin than by soybean trypsin inhibitor. Moreover, using similar initial activities of esterase A₂ and kallikrein, soybean trypsin inhibitor inhibited esterase A₂ more than kallikrein at the same concentrations of the inhibitor, whereas aprotinin inhibited the latter more than esterase A₂. These results are fully consistent with those described by *Nustad & Pierce* (9) for the esterase activity of these enzymes. Nonetheless, in contrast with our results, *McPartland et al.* found that the arginine esterase activity of esterase A₂ is more inhibited by aprotinin than by soybean trypsin inhibitor (14). On the other hand, *Morimoto et al.* found that the amidase activity of esterase A₂ with Pro-Phe-Arg-4-methylcoumaryl-7-amide is more inhibited by soybean trypsin inhibitor than that of kallikrein, which agrees with the data presented here. However, their results on aprotinin are at variance with ours, since they found that both enzymes are inhibited to a similar extent by this inhibitor (15). These discrepancies are possibly due to the different substrate used to determine enzymatic activity and to the degree of purity of the enzymatic fractions employed, since the presence of other proteins can modify the sensitivity of the enzyme to the inhibitors (14).

It must be emphasized that in our experiments, in which similar activities of esterase A₂ and kallikrein were incubated with increasing amounts of aprotinin, we observed that an aprotinin concentration of 50 KIU/ml inhibited kallikrein by 95.9% and left a remnant esterase A₂ activity of 70%. On the light of these findings; the specificity of the amidase method published by *Amundsen et al.* (10) for the determination of rat urinary kallikrein seems to be highly questionable. It is doubtful whether this method is selective for this enzyme if the sample is read against a blank in the presence of 10 KIU or 50 KIU aprotinin (10, 16, 17).

On the other hand, esterase A₂, as well as kallikrein, were able to release kinins from partially purified dog kininogen, which is in agreement with the findings of *McPartland et al.* (7, 14), *Nustad & Pierce* (9), *Chao et al.* (8) and *Morimoto et al.* (15). Both urine and the isolated fractions of esterase A₂ and kallikrein showed the same ratio kininogenase activity/amidase activity. It is then reasonable to assume a good correlation between the kininogenase method using dog substrate and the amidase method described by *Amundsen et al.* (10), an argument that has been used to validate the determination of urinary kallikrein by the amidase method (17). This implies that the kininogenase method using substrate from dog plasma is as unspecific as the amidase method for the determination of rat urinary kallikrein activity.

Obviously, the most important finding reported here is that esterase A₂ was unable to release kinins from heated rat plasma and from partially purified rat kininogen. These results agree with those of *McPartland et al.* (7) and *Nustad & Pierce* (9) who did not observe any change in the pressor test in the rat when injecting the A₂ fraction. This different behaviour of esterase A₂ towards the dog and rat substrates might be due to the specificity of the substrate. However, we cannot rule out the existence of an inhibitor in the preparation of rat kininogen that may fully inhibit fraction A₂. Studies are in progress to help elucidate this point.

The determination of rat urinary kallikrein activity may be rendered selective by using either the amidase or kininogenase assay on dog kininogen applied to urine previously subjected to a chromatographic separation (18).

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