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MEASUREMENT OF HUMAN URINARY KALLIKREIN AND EVIDENCE FOR NON-KALLIKREIN URINARY TAMe ESTERASES BY DIRECT IMMUNOASSAY AND BY AFFINITY CHROMATOGRAPHY*

NORMAN G. LEVINSKY, ONESMO OLE-MOIYOI,† K. FRANK AUSTEN and JOCELYN SPRAGG‡

The Departments of Medicine, Boston University Medical Center, Boston, MA 02118, and Harvard Medical School and The Robert B. Brigham Hospital, Division of the Affiliated Hospitals Inc., Boston, MA 02115, U.S.A.

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Abstract—Kallikrein was separated from other *p*-tosyl-L-arginine methyl ester (TAMe) esterases in human urine by direct affinity chromatography of concentrated fresh pooled urine. Quantitative analysis of total TAMe esterase activity in pooled, fractionated urine indicated that less than one-third was due to urokallikrein and that the remainder was attributable to one or more esterases which lack kinin-generating activity and fail to react with a monospecific anti-urokallikrein serum. Using a radial immunodiffusion assay for human urokallikrein, recovery of purified urokallikrein added to urine was 95 per cent and the coefficient of variation in replicate analyses was 8.4 per cent. When this method was compared with a kinin-generating and a [^{3}H]TAMe esterase method for determination of kallikrein activity in urine, all three assays were well correlated in 50 urine samples from normal subjects in varying states of salt and water metabolism. However, analysis of the regression line of esterase activity on antigen concentration indicated that at least half of the urinary TAMe esterase activity was due to non-kallikrein esterases. The demonstration by direct assay and by separation techniques that at least one-half of the alkaline TAMe esterase activity of urine is not urokallikrein indicates that changes in urinary esterase activity cannot be equated solely with alterations in urokallikrein. A combination of direct immunological and kinin-generating assays should permit accurate evaluation of urokallikrein concentration and activity.

Interest in the renal kallikrein–kinin system has been stimulated by evidence that it regulates certain aspects of renal function in health and may be altered in diseases such as hypertension [1–4]. In most physiological and clinical studies, urinary esterase assays were used as an index of the activity of the renal kallikrein system. The validity of these methods depends on the assumption that human urine contains no enzymes other than kallikrein which contribute significantly to urinary *p*-tosyl-L-arginine methyl ester (TAMe) cleaving activity under assay conditions [2, 4–9]. However, recent work in our laboratory [10–12] and by others [13] indicates that more than one TAMe esterase is present in human urine. To evaluate this problem further, we have now quantified the contribution of urinary kallikrein (urokallikrein) to total urinary TAMe esterase activity and, using a newly developed immunoassay, have compared data from this method with functional measurements of kallikrein in human urine. The results demonstrate that non-kallikrein TAMe esterases contribute a large share to the TAMe esterase activity in normal human urine.

METHODS

Chromatography of urine. Fresh pooled urine from male laboratory personnel was concentrated 100-fold by ultrafiltration through an Amicon UM-10 membrane and dialyzed at 4° against 0.1 M $NaHCO_3$ with 0.5 M NaCl, adjusted to pH 7.8 with NaOH. Fifty ml of concentrated urine was applied to a 40 ml column containing the kallikrein inhibitor aprotinin (Trasylol) coupled to CH Sepharose 4B [12]. The affinity column was washed with the dialysis buffer until the $O.D._{280}$ of the effluent reached 0. The column was then eluted at 80 ml/hr with 0.1 M potassium phosphate, pH 6.5, containing 0.5 M NaCl followed by 0.1 M sodium acetate–acetic acid, pH 3.5, containing 1.0 M NaCl. Four ml fractions were collected until the $O.D._{280}$ at each elution step reached 0. In the acidic elution step, the fractions were collected in tubes to which 1.0 ml of 0.5 M potassium phosphate, pH 8.5, had been added to avoid prolonged exposure of enzymes to a pH below 6. Kinin-generating activity was tested on 2- and 100-μl samples of chromatographic fractions, using the bioassay described below. TAMe esterase activity was determined by a colorimetric method [10, 11] on 100-μl aliquots after the pH of the fraction had been adjusted to 8.5 with 0.5 M potassium phosphate. Urokallikrein antigen was examined by Ouchterlony analysis using a rabbit antiserum shown previously to be monospecific for human urinary kallikrein [10–12] and 8-μl aliquots of column fractions or serial 2-fold dilutions of each concentrated TAMe esterase pool.

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Radial immunodiffusion assay for urinary kallikrein. Urine specimens were concentrated 2–20-fold by lyophilization and subsequent reconstitution in water. Twenty μl aliquots were placed in 5.2 mm wells in 1% agar containing a 1/17 dilution of the monospecific rabbit anti-human urokallikrein serum in 0.1 M veronal, pH 8.6. After 18–24 hr of diffusion at room temperature, the agar plates were washed with 0.85% saline for 18–24 hr, and then with water for 1–2 hr. The precipitin rings were developed with 1% tannic acid for 30 min and their diameters were read to 0.1 mm with an ocular micrometer. Test specimens were assayed in duplicate and a standard curve was run with each set of specimens. Working standards were made from pooled human urine, concentrated 50–100-fold by ultrafiltration through an Amicon UM-10 membrane, or from partially purified urokallikrein. The urokallikrein concentration of the working standards was determined by immunodiffusion assay against a reference standard of human urokallikrein purified by methods described previously [12]. The protein concentration of this reference standard, which yielded a single amino terminal amino acid after dansylation [14], was determined by amino acid analysis, using the Durrum D500 analyzer.

Functional kallikrein assays. Esterase activity of unconcentrated urine specimens was determined by the radiochemical method of Margolius *et al.* [2], using [^{3}H]TAMe as a substrate and incubating for 30 min at pH 8.0 and 30°. Urokallikrein [12] in dilutions containing from 1×10^{-4} to 4×10^{-4} esterase units (E.U.) was included with each set of urine samples. The TAMe esterase activity of this purified urokallikrein standard was determined by a colorimetric method standardized with methanol [10, 11]; 1.0 E.U. = 1 μmole methanol liberated/min under the conditions of the assay.

Kinin-generating activity of unconcentrated urine was determined using 10–100 μl of heat inactivated human plasma [10] as a source of substrate and the guinea pig ileum suspended in and washed frequently with Tyrode's buffer containing 5×10^{-7} M atropine [10, 12] for the bioassay of kinin. Unknowns were bracketed with contractions caused by known concentrations of bradykinin.

Urine collection for combined functional and immunoassays. Fifty urine samples were obtained from normal volunteers of both sexes. Approximately one-third were random specimens from laboratory personnel whose salt and water intake was uncontrolled. In an attempt to increase the range of urine flows and kallikrein concentrations available for comparative assay studies, additional samples were selected from 7- to 24-hr urine collections obtained in metabolic balance experiments in normal volunteers. These studies had been performed for purposes unrelated to the present investigation and manipulations included a wide range of dietary sodium intake and infusion of saline or dextrose in water, separately or in various combinations. Since only one to three specimens from any particular state of sodium or water balance were assayed, no systematic evaluation of the effects of these manipulations on kallikrein concentration was made. All three urokallikrein assays, i.e. immunodiffusion, kinin generation, and TAMe esterase activity, were performed on each specimen. Fifteen urines were tested without prior freezing; the others had been frozen at −70° for up to several months prior to assay.

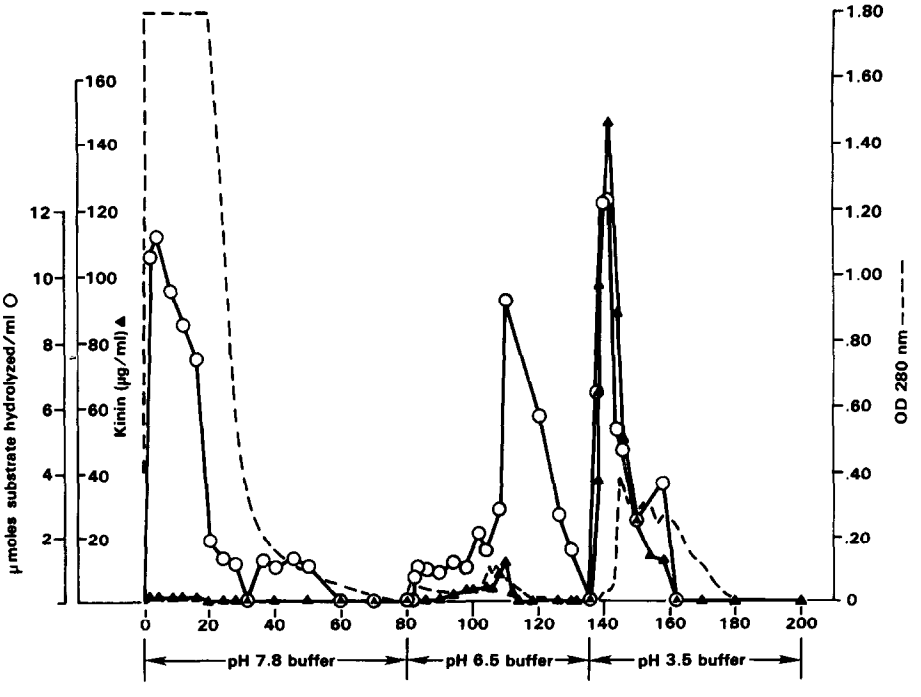

Fig. 1. Affinity chromatography of concentrated crude urine on Trasylol–Sepharose. Fractions were analyzed for protein content, alkaline TAMe esterase activity, kinin-generating activity and urokallikrein antigen. The location of urokallikrein antigen is described in Results.

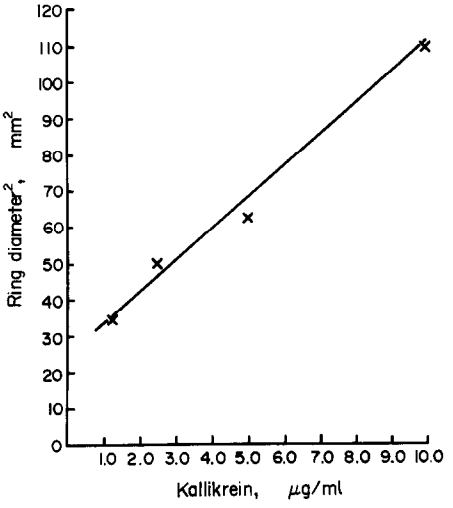

Fig. 2. Characteristic standard curve for immunodiffusion assay of urokallikrein. Concentration of purified urokallikrein is on the abscissa, and the (diameter)^2 of the precipitin ring is on the ordinate. The diameter of the sample well (5.2 mm) was not subtracted.

RESULTS

Separation of urokallikrein from other alkaline TAMe esterases in human urine. In two separate experiments, dialyzed concentrates of fresh pooled urine were chromatographed without prior purification on Trasylol–Sepharose affinity columns and the effluent and eluted fractions were assayed for kinin-generating and TAMe-hydrolyzing activities and for urokallikrein antigen. The results of one fractionation procedure are depicted in Fig. 1 and described herein; the data from the other experiment are presented in parentheses. Eighty-seven per cent (82 per cent) of the protein appeared in the effluent, which also contained 39 per cent (56 per cent) of the TAMe esterase activity but no (or 8 per cent) kinin-generating activity. No urokallikrein antigen was seen in unconcentrated fractions in either experiment. Three per cent (2 per cent) of the protein eluted at pH 6.5 and was associated with 35 per cent (18 per cent) of the esterase and 9 per cent (0 per cent) of the kinin-generating activity. A trace of urokallikrein antigen was appreciated only after 50-fold concentration of this esterase peak, and subsequent gel filtration of the concentrate on Sephadex G100 [12] yielded two esterase peaks, one completely free of kinin-generating activity and urokallikrein antigen. Ten per cent (16 per cent) of the protein eluted at pH 3.5, along with 91 per cent (92 per cent) of the kinin-generating activity and 26 per cent (26 per cent) of the TAMe esterase activity; the unconcentrated column fractions, 138–150, were positive for urokallikrein antigen by Ouchterlony testing. These data do not establish whether the non-urokallikrein TAMe esterase in the effluent and the pH 6.5 eluate are the same enzyme, but do reveal that less than one-third of the TAMe esterase activity eluted with urokallikrein defined by kinin generation and antigen.

Immunoassay of urokallikrein. A typical standard curve obtained with purified urokallikrein is shown in Fig. 2. Because recovery, tested in eleven urines to which purified urokallikrein was added prior to lyophi-

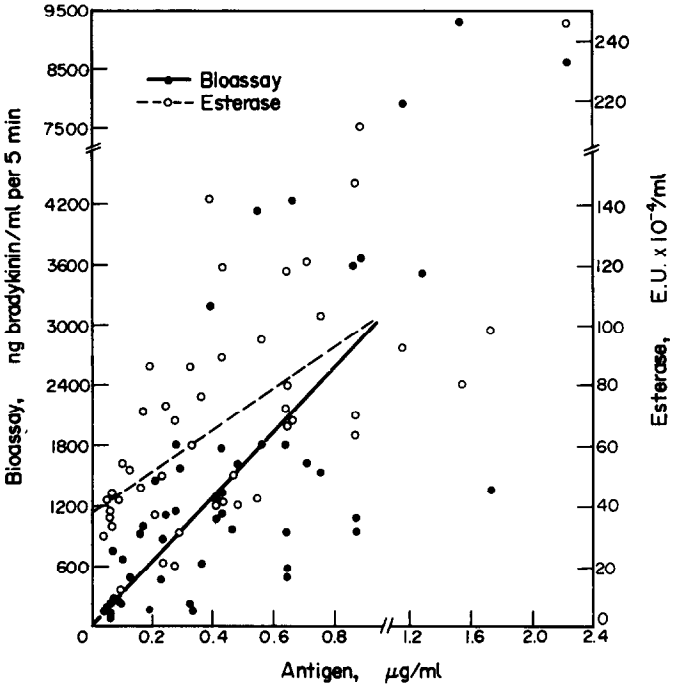

Fig. 3. Relationship of kinin generation and TAMe esterase activity to urokallikrein protein determined by immunoassay in urines from normal subjects (see Methods for conditions of collection). Regression lines are drawn by the method of least mean squares. The equation for the bioassay versus antigen line is $y = 3400x + 0.8$, $r = 0.75$, $P = <0.001$. The equation for the esterase vs antigen line is $y = 68x + 37.6$, $r = 0.70$, $P < 0.001$.

lization and assay in duplicate, was 95.0 ± 8.3 (S.D.) per cent, no correction for recovery was applied to assay results obtained in subsequent studies. The coefficient of variation was 8.4 per cent for replicate assays of twelve specimens.

Assay for 'kallikrein' in normal urine samples by three methods. Figure 3 compares the data obtained by immunological, kinin-generating and TAMe esterase assay of 50 urine samples from normal subjects and from normal individuals participating in metabolic balance studies. Results with the TAMe esterase assay and the kinin-generating assay are well correlated with urokallikrein concentration determined by immunoassay, although there is substantial variation among individual urines in the ratio of either of the functional measurements to urokallikrein antigen concentration. Values from fresh and frozen urines were comparably distributed. The y-intercept of the line describing the relation between bioassay activity and antigen concentration is not significantly different from zero. The esterase versus antigen line has a y-intercept of 38 E.U., which is significantly different ($P < 0.001$) from zero. This indicates that substantial alkaline TAMe esterase activity is present in normal urines, even when urokallikrein antigen is zero. At the mean antigen concentration of all 50 test urines, 0.48 μg/ml, 38 E.U. is equivalent to 54 per cent of the total esterase activity of 70.2 E.U. Thus, in this antigen range, over half the esterase activity is not associated with kinin-generating activity.

DISCUSSION

Direct application of fresh, concentrated pooled human urine to a Trasylol affinity column has permitted separation of alkaline TAMe esterases and has revealed that as little as one-third of the total urinary TAMe esterase activity is due to urokallikrein (Fig. 1). The remaining TAMe esterase activity is associated with one or more additional esterases which do not react with a monospecific anti-urokallikrein serum and lack kinin-generating activity. Affinity chromatography followed by gel filtration yielded a urokallikrein purified to homogeneity as determined by analytical electrophoretic and immunological criteria [12] and by detection of a single amino terminal amino acid.

Geiger *et al.* [13], who also used affinity chromatography to purify human urokallikrein, noted a sharp fall in the ratio of esterase to kinin-generating activity during purification. They concluded that there are non-urokallikrein TAMe esterases in urine which are removed during the isolation of urokallikrein. On the other hand, others have found only a single peak of esterase activity during chromatographic purification [6–9] and have observed that the ratio of esterase to kinin-generating activity is constant at each step when 'kallikrein' is purified from crude urine on the basis of esterase activity by ion exchange chromatography and gel filtration [6, 7, 9]. That urokallikrein was not separated from contaminating esterases by ion exchange chromatography and gel filtration was demonstrated when resolution was achieved after isoelectric focusing over a broad pH range [10]. The contaminating esterase(s) is more readily appreciated (Fig. 1) and separated from urokallikrein by affinity chromatography [12].

Since esterase activity cannot be assumed to be a valid measure of urokallikrein in whole urine, a direct immunoassay for urokallikrein was developed. Recovery of added urokallikrein is quantitative (95 ± 8 per cent), and interassay variability is limited (coefficient of variation, 8.3 per cent) with this simple assay (Fig. 2). Antigen concentration determined by immunoassay in normal urines obtained under varying conditions of salt and water intake correlates well with urokallikrein activity by bioassay ($r = 0.75$), although there is fairly wide variation among individual urines in the ratio of the two measurements (Fig. 3). The regression line relating urokallikrein protein by immunoassay to function by kinin generation passes through the origin, indicating that the immunoassay determines only urokallikrein. Although the [^{3}H]TAMe esterase assays also correlate well with urokallikrein antigen measurements in these same urines ($r = 0.70$), the regression line crosses the y-axis well above the origin, indicating that a large fraction of urinary alkaline TAMe esterase activity is not due to urokallikrein. Thus, when the urokallikrein antigen concentration is zero, substantial alkaline TAMe esterase activity is present in normal urine; at the mean urokallikrein antigen concentration of 0.48 μg/ml, over half the total TAMe esterase activity is not associated with kinin-generating activity. The correlation between TAMe esterase activity and urokallikrein antigen, despite the presence of non-kallikrein esterases, could indicate either that the concentrations of urokallikrein and the other esterases change proportionately in response to the altered urine produced by dietary salt and water intake in normal subjects, or that the concentration of the non-kallikrein esterase(s) does not change in response to such maneuvers.

Several other groups [2, 4, 15] have reported that TAMe esterase activity of urine correlates well with urokallikrein-related biological activities. Margolius *et al.* [2] examined ten urines from a single individual on a controlled salt diet by a kinin-generating assay, while others have measured in dogs or rats the hypotensive effect of injecting urine from both normotensive and hypertensive subjects [4, 15]. The correlations observed have been used to support the validity of the esterase assays as measurements of urokallikrein. The data of Margolius *et al.* [2] and Levy *et al.* [4] are not presented in a form which permits calculation of the y-intercept of the regression line relating TAMe esterase activity to bioassay. The data of Seino *et al.* [15] with hypertensive subjects do appear to show a regression line which passes near the origin. We found that the TAMe esterase and kinin-generating assays were well correlated in 50 urines (Fig. 3), i.e. 1 esterase unit = 0.01 ng equivalents bradykinin liberated/ml of urine + 53, $r = 0.505$, $P < 0.001$. However, the regression line reveals substantial TAMe esterase activity when the kinin-generating activity is zero.

The capacity to purify human urokallikrein to homogeneity, free of non-kallikrein esterase(s) [12], together with the development of an immunoassay for urokallikrein protein (Fig. 2), has permitted a direct assessment of this enzyme in the urine of normal subjects (Fig. 3). The cleavage of TAMe is not a valid measurement of urokallikrein due to the presence of additional TAMe

esterases. To evaluate further the possible presence of inhibitors or inactive enzyme molecules, in initial studies of disease states this immunoassay should be combined with a functional method which measures kinin liberation.

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REFERENCES

1. A. Adetuyibi and I. H. Mills, *Lancet* **2**, 203 (1972).
2. H. S. Margolius, R. G. Horwitz, R. W. Alexander, J. R. Gill, J. J. Pisano and H. R. Keiser, *Circulation Res.* **35**, 812 (1974).
3. H. S. Margolius, D. Horwitz, J. J. Pisano and H. R. Keiser, *Circulation Res.* **35**, 820 (1974).
4. S. B. Levy, J. J. Lilley, R. P. Frigon and R. A. Stone, *J. clin. Invest.* **60**, 129 (1977).
5. M. E. Webster and J. V. Pierce, *Proc. Soc. exp. Biol. Med.* **107**, 186 (1961).
6. H. Moriya, J. V. Pierce and M. E. Webster, *Ann. N.Y. Acad. Sci.* **104**, 172 (1963).
7. J. V. Pierce, in *Handbook of Experimental Pharmacology* (Ed. E. G. Erdos), p. 21. Springer, New York (1970).
8. G. Porcelli, G. B. Martini-Bettolo, H. R. Croxatto and M. Di Iorio, *Ital. J. Biochem.* **23**, 44 (1974).
9. Y. Matsuda, K. Miyazaki, H. Moriya, Y. Fujimoto, Y. Hojima and C. Moriwaki, *J. Biochem., Tokyo* **80**, 672 (1976).
10. O. ole-MoiYoi, K. F. Austen and J. Spragg, *Biochem. Pharmac.* **26**, 1893 (1977).
11. O. ole-MoiYoi, J. Spragg, S. P. Halbert and K. F. Austen, *J. Immuno.* **118**, 667 (1977).
12. O. ole-MoiYoi, J. Spragg and K. F. Austen, *J. Immun.* **121**, 66 (1978).
13. R. Geiger, K. Mann and T. Bettels, *J. clin. Chem. clin. Biochem.* **15**, 479 (1977).
14. A. M. Weiner, T. Platt and K. Weber, *J. biol. Chem.* **247**, 3242 (1972).
15. M. Seino, K. Abe, Y. Sakurai, N. Irokawa, M. Yasujima, S. Chiba, Y. Otsuka and K. Yoshinaga, *Tohoku J. exp. Med.* **121**, 111 (1977).