Total and Kallikrein Arginine Esterase Activities in the Urine of Salt-Hypertensive Susceptible and Resistant Rats

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SUMMARY Urinary enzymes that hydrolyze the artificial substrate α-N-p-tosyl-L-arginine methyl ester (TAME) were studied in Dahl salt-sensitive (S) and salt-resistant (R) rats. Total urinary TAME esterase activity (kallikrein and non-kallikrein) showed a marked increase with dialysis against water, but only in hypertensive S rats with proteinuria. This phenomenon suggests the presence of dialyzable TAME esterase inhibitors in urine following renal damage, but these data do not define what urinary esterases might be affected. Partially purified urinary kallikrein exhibited a ratio of kininogenase to esterase activity which was equal for S and R rats. Thus, the marked discrepancy between kininogenase and esterase activities reported by Carretero et al. with S and R whole urine is not a function of the S and R kallikrein molecules but is probably related to interfering substances in the whole urine. Urinary kallikrein excretion was measured on individual rat samples by TAME esterase activity following dialysis and separation from non-kallikrein TAME esterase(s) using DEAE-Sephadex microcolumns. S rats had lower urinary kallikrein excretion than R when the S rats were hypertensive and showed marked proteinuria. Young S and R rats raised on low salt showed similar blood pressures and similar kallikrein excretion. High salt (8% NaCl) diet decreased kallikrein excretion in both S and R, but the decrease was greater in the S rats which became hypertensive and had increased urine protein excretion. These data suggest that the lower urinary kallikrein excretion in S rats relative to R rats is probably a consequence of hypertension and renal damage rather than a primary cause of hypertension.

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KEY WORDS • genetic hypertension • salt hypertension • urinary kallikrein • urinary arginine esterases

RATS were selectively bred by Dahl et al.1 for susceptibility (S strain) or resistance (R strain) to the hypertensive effect of a high salt (NaCl) diet. A large part of the difference in blood pressure response to salt between S and R rats appears to be caused by a genetically controlled factor(s) of renal origin.2 Urinary kallikrein is produced in the kidney where it may be involved in blood pressure regulation through the proteolytic generation of vasodilator kinin peptides from kinogen substrates.3 Carretero et al.4 have reported that there is a marked discrepancy between kallikrein measured in S and R rats by kinin generation compared to kallikrein measured by TAME esterase activity or radioimmunoassay for kallikrein protein. Their data suggest that: 1) S and R rats could have different (mutant) forms of kallikrein, with the S form being less active than the R in the kininogenase assay, or 2) substances in S urine could interfere with kinin-generating assay more than with the other assays. We present evidence herein favoring the latter hypothesis and, in addition, showing that S rats secrete less kallikrein than R only when they are hypertensive and when they have increased urinary protein excretion compared to R. This suggests that decreased kallikrein excretion in S rats is a response to hypertension rather than a cause of hypertension.

Materials and Methods

Animals and Experimental Groups

Rats from the S and R strains were originally obtained from Dr. L. K. Dahl of the Brookhaven National Laboratory (Upton, New York). At the time
of these studies, S and R rats had been inbred in our laboratory for 6 and 16 generations, respectively. Ten sublines of S and five sublines of R were maintained for breeding purposes. S and R rats for a given experiment were sampled across all sublines. Rats were maintained in a standard fashion with food and water provided ad libitum. One experimental group was composed of 5-month-old S and R rats of both sexes fed from weaning (3-weeks-old) a standard salt diet (Wayne Lab Blox, 1% NaCl). Another experimental group consisted of 2-month-old S and R male rats fed the standard salt diet for 1 week following weaning, then fed either a high salt diet (8% NaCl) or a low salt diet (0.4% NaCl). The high and low salt diets, identical except for salt content, were obtained from Brookhaven National Laboratory.

Measurement of Blood Pressure

Rat systolic blood pressures were measured by a tail cuff micropneumatic manometer method. During measurements, rats were lightly anesthetized with ether.

Collection and Processing of Urine Samples from Individual Rats

On two occasions, 4 to 7 days apart, 24-hour urine samples were collected under toluene from individual rats. During collections, rats were housed in metabolism cages and deprived of food, but allowed free access to water. The standard rat metabolism cages used effectively separated feces from urine samples. The first 24-hour urine sample from each rat was stored under toluene at 4°C until the second sample was obtained, at which time the two samples were pooled, pool volume was measured, and toluene was removed and discarded. Total TAME esterase activity of the rat urine stored under toluene showed no decrease in activity at 5°C for 8 days. A small aliquot was taken from each urine pool and stored at −20°C for later measurements of total protein and total arginine esterase activity before dialysis. Each urine pool was dialyzed at 4°C against 400 ml of distilled H₂O at 4°C. To measure the kininogen substrate concentration, the dog plasma substrate was incubated at 37°C and pH 8.6. Expression of TAME esterase activity was in esterase units (EU), with 1 EU being arbitrarily defined as the amount of esterase required to hydrolyze 1 μmole of TAME per minute. Rat urine enzyme samples often possess measurable background absorbances when run in the colorimetric assay without adding TAME. Background absorbances were determined and subtracted from corresponding assay absorbances before calculation of TAME esterase activity.

Measurement of Kallikrein Activity

Kallikrein activity was determined in fresh and dialyzed urine samples and in urinary kallikrein samples obtained from minicolumns. Assays were performed by a modified colorimetric method employing the synthetic substrate α-N-p-tosyl-L-arginine methyl ester HCl (TAME). Enzyme sample incubations were carried out at 37°C and pH 8.6. Expression of TAME esterase activity was in esterase units (EU), with 1 EU being arbitrarily defined as the amount of esterase required to hydrolyze 1 μmole of TAME per minute. Rat urinary enzyme samples often possess measurable background absorbances when run in the colorimetric assay without adding TAME. Background absorbances were determined and subtracted from corresponding assay absorbances before calculation of TAME esterase activity.

Measurement of Kallikrein Activity

Kallikrein activity was determined in some urinary kallikrein samples partially purified from DEAE minicolumns. Samples from minicolumns were dialyzed to remove KCl before kallikreinase assay. Samples were incubated at 30°C and pH 8.0 with a dog plasma kallikrein substrate preparation, and released kinins were bioassayed on an isolated rat uterus in de Jalon's solution. Synthetic bradykinin was used as a standard in the bioassay and kallikreinase activity was expressed as nanograms (ng) of bradykinin generated per minute. To prepare the substrate, citrated dog plasma was heated at 60°C for 30 minutes and then dialyzed extensively against distilled H₂O at 4°C. To measure the kallikrein substrate concentration, the dog plasma substrate was incubated with hog pancreatic kallikrein (Sigma, St. Louis) and kinins generated were bioassayed versus time. Kinin generation reached a plateau representing 2.1 μg of kinin per ml of dog plasma. When kallikrein samples were tested, 100 μl of dog plasma substrate, test sam-

DEAE-Sephadex Minicolumn Chromatography of Samples from Individual Rats

Separations of urinary kallikrein from non-kallikrein urinary arginine esterase(s) were carried out by chromatography on DEAE-Sephadex minicolumns. The chromatography procedure was based on the work of Nustad and Pierce and was performed at room temperature. A minicolumn was made by cutting off the top end of a 10 ml glass serological pipet and dropping it into a glass bead, 4 mm in diameter. The DEAE-Sephadex was equilibrated with a solution of 0.2 M KCl in chromatography buffer (0.01 M potassium phosphate pH 7.0). Each minicolumn contained a DEAE-Sephadex running bed volume of 4 ml. The sample applied to a minicolumn was a portion of the dialyzed urine pool from an individual rat. This portion was equivalent to an average 12-hour urinary volume (one-fourth of the total 48-hour volume collected) for the rat. Before application, enough KCl was added to the sample to bring its concentration to 0.2 M. Following sample application, the DEAE-Sephadex was washed extensively with equilibrating solution (5 column volumes). The unadsorbed non-kallikrein arginine esterase(s), or esterase A as it has been referred to, was removed during this wash. The adsorbed urinary kallikrein was then eluted with 0.7 M KCl in chromatography buffer. The first 1 ml of eluate was discarded and the next 7 ml of eluate, containing the urinary kallikrein, were collected as a single fraction. Experiments regarding the validity of minicolumn chromatography are given in the Results section.
Measurement of Urinary Protein

Total urinary protein was determined in urine samples by the method of Lowry et al. Bovine serum albumin was used as a protein standard.

Results

DEAE-Sephadex Minicolumn Validation Experiments

To obtain samples for testing the minicolumn technique, 24-hour urine collections were made on several occasions from adult S and R rats of both sexes as described above (see Materials and Methods). All samples from rats of a given sex and strain were pooled and toluene was removed and discarded. Each of the four group urine pools was extensively dialyzed against distilled H$_2$O at 4°C and then lyophilized.

A lyophilized sample from each of the four (S and R, male and female) group urine pools was chromatographed on a DEAE-Sephadex column at 4°C and column fractions were assayed for TAME esterase activity. The chromatography procedure was the same as that used by Nustad and Pierce. This procedure is like that described above for minicolumn chromatography, with the principal exception that the material adsorbed to the DEAE-Sephadex is eluted with a linear KCl gradient to 0.5 M, instead of with a 0.7 M KCl step. For each group urine pool the chromatography revealed two peaks of TAME esterase activity, as illustrated in figure 1 for the R male pool. These results are qualitatively similar to those obtained by Nustad and Pierce for a lyophilized female Sprague-Dawley rat urine pool. We have also obtained these two peaks with room temperature minicolumn chromatography of lyophilized and non-lyophilized urine samples from individual S and R rats. The two activity peaks shown in figure 1 are designated A and B following the nomenclature of Nustad and Pierce. These investigators demonstrated that TAME esterase peak A is composed of non-kallikrein TAME esterase(s) and that TAME esterase peak B is urinary kallikrein. We have confirmed that TAME esterase peak B from S or R rat urine pools is urinary kallikrein on the basis of the following observations: 1) it transiently lowered mean arterial blood pressure when administered intravenously to an anesthetized rat; 2) it produced a direct contraction of the isolated rat uterus, when used in an appropriately high amount, without prior incubation with kininogen substrate; 3) it generated kinins, detected in the rat uterus bioassay, from the dog plasma kininogen substrate preparation when used in an amount far below that required to cause a direct rat uterine contraction; 4) its TAME esterase activity, measured by a modified radiometric method, was not inhibited by 7.5 μM soybean trypsin inhibitor (a broad spectrum proteinase inhibitor known to have little to no effect on rat urinary kallikrein activity), but was inhibited almost 100% by 0.25 μM Trasylol (a well-known potent inhibitor of rat urinary kallikrein activity); 5) when it was further analyzed by Sephacryl S-200 gel filtration chromatography, only a single peak of TAME esterase activity was obtained.

A lyophilized sample from the R male group urine pool was chromatographed on a DEAE-Sephadex column as described above (fig. 1). The kallikrein (TAME esterase peak B) fractions were pooled, dialyzed extensively against distilled H$_2$O at 4°C and lyophilized. All of the remaining or background fractions, including those containing TAME esterase peak A, were also pooled, dialyzed and lyophilized. The kallikrein and background samples were reconstituted separately in DEAE-Sephardex equilibrating solution and then each was assayed for TAME esterase activity. Various amounts of kallikrein TAOE esterase activity were each mixed with 7.5 EU of background activity when it was further analyzed by Sephacryl S-200 gel filtration chromatography, only a single peak of TAME esterase activity was obtained.

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TAME esterase activity (originating from TAME esterase peak A) and run through the minicolumn step. The amount of background activity added corresponds approximately to the amount found in a 12 hr volume of urine from a rat. Results for the recovery of urinary kallikrein TAME esterase activity through the minicolumn step are shown in table 1. As indicated, there was essentially 100% recovery of enzyme activity within the range applied. No individual rat sample thus far run through the minicolumn step has had a urinary kallikrein TAME esterase activity outside the range of activity used in the recovery experiment and given in table 1.

In the minicolumn chromatography technique, kallikrein is eluted with 0.7 M KCl and is assayed directly for TAME esterase activity by the colorimetric method. We have found that KCl, up to 0.9 M, has no effect on S or R rat urinary kallikrein TAME esterase activity under the conditions of the colorimetric assay.

Comparisons in S and R Rats Fed a Standard Salt Diet

Presented in table 2 are means and standard errors and analysis of variance results for characteristics including total urinary TAME esterase activity and urinary kallikrein TAME esterase activity in 5-month-old S and R rats raised on a standard salt diet (1% NaCl). Systolic blood pressures in S rats eating a standard salt diet become gradually elevated and by 5 months of age the animals can be markedly hypertensive, as demonstrated by the mean S rat blood pressures given in the table. Total urinary protein was two to three times higher in S than R rats. Also, urinary protein was significantly greater in males than in females, but this is a common finding for aging rats.11,12

Total urinary TAME esterase activity, measured either before or after dialysis, was roughly twice as high in male rats as in female rats (table 2). Total TAME esterase activity was measured after dialysis, the difference between S and R rats was still significant, but much reduced. The dialysis step caused an almost twofold increase in total TAME esterase activity in S rat urine samples, but only a slight increase in R rat urine samples. We have observed that a maximal increase in total TAME esterase activity occurs during the first 24 hours of dialysis under the conditions we have employed, and thus the increases shown here can be considered maximal. The increase in total TAME esterase activity by dialysis is also illustrated in table 2 by the ratio of total TAME esterase activity after dialysis to total TAME esterase activity before dialysis. As indicated, this ratio was significantly greater for S than R urine samples. This ratio of total TAME esterase activity after dialysis to activity before dialysis was significantly and positively correlated with urinary protein in S males ($r = 0.76, p < 0.01$) and S females ($r = 0.87, p < 0.01$), but not in

![Figure 2. Relationship between kininogenase (kinin generating) activity and TAME esterase activity by the colorimetric method for 16 kallikrein samples prepared from individual rats (8S, 8R) by the mini-column technique (see Materials and Methods). The samples were dialyzed to remove the KCl used in mini-column elution prior to testing for esterase and kininogenase activity because high concentrations of K+ would interfere with the kinin bioassay on rat uterus. Regression lines calculated for S and R separately both showed slopes significantly different from zero (0.01 < p < 0.05). S and R regression lines were identical, i.e., the two slopes were not different (p > 0.5) and the two slope intercepts were not different (p > 0.5). The regression line drawn in the figure is for S and R combined. The slope of this line was significantly different from zero (p < 0.005), and the slope intercept was not significantly different from zero (p > 0.1).](http://hyper.ahajournals.org/lookup/fig/1)

<table>
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<tr>
<th>Column</th>
<th>Urinary kallikrein EU applied</th>
<th>Urinary kallikrein EU recovered</th>
<th>Percent recovery</th>
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</thead>
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<tr>
<td>1</td>
<td>0.59</td>
<td>0.59</td>
<td>100.0</td>
</tr>
<tr>
<td>2</td>
<td>2.63</td>
<td>0.62</td>
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<td>1.05</td>
<td>88.2</td>
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<td>92.0</td>
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<tr>
<td>10</td>
<td>3.91</td>
<td>4.35</td>
<td>111.3</td>
</tr>
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</table>

Average percent recovery 100.2

Abbreviation: EU = TAME esterase unit.
TABLE 2. Means and Standard Errors and Analysis of Variance Results for Characteristics Including Total Urinary TAME Esterase Activity and Urinary Kallikrein TAME Esterase Activity in 6-Month-Old S and R Rats of Both Sexes Fed a Standard Salt Diet (1% NaCl)*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Females (n = 10)</th>
<th>Males (n = 10)</th>
<th>Strain</th>
<th>Sex</th>
<th>Sex Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>219 ± 5.1</td>
<td>249 ± 2.9</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Blood pressure (mm Hg)</td>
<td>119 ± 3.7</td>
<td>184 ± 9.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary volume (ml/24 hr)</td>
<td>11.6 ± 1.2</td>
<td>15.4 ± 2.1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Urinary protein (mg/24 hr)</td>
<td>36.4 ± 1.3</td>
<td>150.3 ± 19.3</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total TAME esterase, AD (EU/24 hr)</td>
<td>10.7 ± 0.45</td>
<td>5.5 ± 2.0</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Total TAME esterase, BD (EU/24 hr)</td>
<td>11.7 ± 0.43</td>
<td>9.7 ± 0.61</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Ratio total TAME esterase, AD/total TAME esterase, BD</td>
<td>1.11 ± 0.05</td>
<td>2.1 ± 0.29</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Urinary kallikrein TAME esterase (EU/24 hr)</td>
<td>6.24 ± 0.21</td>
<td>4.42 ± 0.35</td>
<td>5.57 ± 0.24</td>
<td>3.92 ± 0.34</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*pProbabilities were obtained from standard two-way analyses of variance. Where variances were unequal, analyses were performed on log-transformed data.

Abbreviations: EU = TAME esterase unit; BD = before dialysis; AD = after dialysis; NS = not significant, i.e., p > 0.05.

R males (r = -0.27, p > 0.05) or R females (r = 0.26, p > 0.05). This suggests that the markedly decreased total urinary TAME esterase activity in S relative to R rats before dialysis was related to the proteinuria (kidney damage) in the S rats.

Urinary kallikrein TAME esterase activity, measured after removal of non-kallikrein urinary TAME esterase(s) by minicolumn chromatography, was significantly different between S and R rats, with mean values for S rats being two-thirds to three-fourths of those for R rats (table 2). Also, urinary kallikrein TAME esterase activity was slightly, but significantly, lower in males than in females. This latter finding is in contrast to the situation observed for total TAME esterase activity. As indicated above, total TAME esterase activity was substantially higher in males than in females. It thus appears that male rats possess a greater amount of non-kallikrein urinary TAME esterase activity than female rats (see Discussion).

Eight of the S and eight of the R individual rat urinary kallikrein samples obtained by minicolumn chromatography for the assay of TAME esterase activity were dialyzed extensively against distilled H₂O at 4°C and reassayed for TAME esterase activity. These same samples were then assayed for kininogenase activity. The relationship between kininogenase and TAME esterase activities for S and R urinary kallikrein samples is illustrated in figure 2. Regression lines calculated for S and R separately both showed slopes significantly different from zero (0.01 < p < 0.05). S and R regression lines were identical, i.e., the two slopes were not different (p > 0.5) and the two slope intercepts were not different (p > 0.5). The regression line in figure 2 is for S and R combined. The slope of this line was significantly different from zero (p < 0.005), and the slope intercept was not significantly different from zero (p > 0.1). The ratio of kininogenase activity to TAME esterase activity for S urinary kallikrein samples was 2.68 ± 0.30 ng (mean ± standard error) of bradykinin per minute per milliesterase unit (mEU). The same ratio for R urinary kallikrein samples was an almost identical 2.89 ± 0.32 ng bradykinin/min/mEU.

Comparisons in S and R Male Rats Fed High and Low Salt Diets

Table 3 shows the means and standard errors and analysis of variance results for characteristics including total urinary TAME esterase activity and urinary kallikrein TAME esterase activity in 2-month-old S and R male rats fed for 1 month either a high salt diet (8% NaCl) or a low salt diet (0.4% NaCl). The analysis of variance results include probabilities for both complex and simple main effects (see table footnote). As expected, S males fed the high salt diet had quite high systolic pressures in comparison to S males fed the low salt diet and R males fed either the high or the low salt diets. A similar pattern of differences was also observed for both urinary volume and urinary protein. Each of these characteristics was increased by salt-feeding in the S rats but was not increased by salt-feeding in the R rats (table 3).
Total urinary TAME esterase activity, measured either before or after dialysis, was not different between the strains, regardless of salt diet. Total TAME esterase activity, before and after dialysis, was reduced by one-third to one-half in both S and R males on the high salt diet in comparison to their counterparts on the low salt diet. The ratios of total TAME esterase activity after dialysis to total TAME esterase activity only in urine samples from S males fed the high salt diet. Correlation coefficients (r) computed between the ratios and urinary protein in S and R males were obtained by the method of Keppel. Where variances were unequal, analyses were performed on log-transformed data. Probabilities asso-

ciated with complex main effects (e.g., the effect of strain, S vs R, in rats fed the high salt diet, under column headed Strain/High) and interactions were obtained from standard two-way analyses of variance. Probabilities asso-

ciated with simple main effects (e.g., the effect of strain, S vs R, in rats fed the high salt diet, under column headed Strain/High) were obtained by the method of Keppel. Where variances were unequal, analyses were performed on log-transformed data.

**Discussion**

The measurement of kallikrein in urine is not a trivial problem. In choosing to use a partial purification step (DEAE-Sephadex minicolumn) on each individual sample prior to measurement of enzyme activity by TAME esterase and kininogenase (i.e., kinin generation) activity we considered the following factors: 1) trypsin activatable forms of kallikrein (prokallikrein) have been described in human urine and hog pancreas; 2) non-kallikrein esterases and kininogenases exist in normal rat urine; 3) in rats with marked proteinuria additional TAME esterases can be demonstrated (Sustarsic, McPartland, and Rapp, unpublished observations); and 4) kallikrein inhibitors exist in rat kidneys and rat plasma.

Our intention was to measure enzymatically active kallikrein, and so we wanted to minimize the possibility of measuring prokallikrein or kallikrein-inhibitor complexes. Both these entities would be expected to be measured by radioimmunoassay for kallikrein protein on whole urine.

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**Table 3. Means and Standard Errors and Analysis of Variance Results for Characteristics Including Total Urinary TAME Esterase Activity and Urinary Kallikrein TAME Esterase Activity in 8-Month-Old S and R Male Rats Fed Either a High Salt Diet (8% NaCl) or a Low Salt Diet (0.4% NaCl)**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>R Low (n = 7)</th>
<th>S Low (n = 8)</th>
<th>R High (n = 8)</th>
<th>S High (n = 7)</th>
<th>Probabilities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood pressure (mm Hg)</td>
<td>143 ± 6.8</td>
<td>152 ± 3.4</td>
<td>124 ± 2.6</td>
<td>190 ± 8.9</td>
<td>&lt;0.01 NS &lt;0.01 &lt;0.001 NS</td>
</tr>
<tr>
<td>Urinary volume (ml/24 hr)</td>
<td>8.8 ± 2.8</td>
<td>6.4 ± 1.1</td>
<td>8.5 ± 0.9</td>
<td>13.8 ± 0.8</td>
<td>NS &lt;0.05 NS &lt;0.01 &lt;0.001 &lt;0.05</td>
</tr>
<tr>
<td>Urinary protein (mg/24 hr)</td>
<td>18.5 ± 7.2</td>
<td>21.0 ± 3.2</td>
<td>14.0 ± 2.4</td>
<td>57.7 ± 11.9</td>
<td>&lt;0.01 &lt;0.01 &lt;0.05 &lt;0.05</td>
</tr>
<tr>
<td>Total TAME esterase, BD (EU/24 hr)</td>
<td>11.1 ± 0.53</td>
<td>11.5 ± 0.99</td>
<td>6.8 ± 0.66</td>
<td>5.8 ± 0.51</td>
<td>NS NS &lt;0.001 &lt;0.001</td>
</tr>
<tr>
<td>Total TAME esterase, AD (EU/24 hr)</td>
<td>11.2 ± 0.64</td>
<td>11.9 ± 0.73</td>
<td>6.7 ± 0.67</td>
<td>7.4 ± 0.65</td>
<td>NS NS &lt;0.001 &lt;0.001</td>
</tr>
<tr>
<td>Ratio total TAME esterase, AD/total TAME esterase, BD</td>
<td>1.00 ± 0.02</td>
<td>1.06 ± 0.06</td>
<td>0.98 ± 0.04</td>
<td>1.28 ± 0.06</td>
<td>&lt;0.001 &lt;0.001 &lt;0.05 NS</td>
</tr>
<tr>
<td>Urinary kallikrein TAME esterase (EU/24 hr)</td>
<td>5.19 ± 0.43</td>
<td>4.52 ± 0.28</td>
<td>3.04 ± 0.30</td>
<td>1.68 ± 0.19</td>
<td>&lt;0.01 &lt;0.01 &lt;0.001 &lt;0.001</td>
</tr>
</tbody>
</table>

Abbreviations: EU = TAME esterase unit; BD = before dialysis; AD = after dialysis; NS = not significant, i.e., p > 0.05.

Reduced by salt feeding was greater in the S than in the R, so that there was a significant difference between strains on high salt diet (table 3).
All the known non-kallikrein TAME esterases in rat urine are removed by the DEAE-Sephadex step. These esterases include the esterase A fraction of Nustad and Pierce. In normal rats the esterase A fraction actually contains 2 esterases, one of which is androgen dependent and is found only in sexually mature males. The effect of this androgen dependent enzyme can be seen in the present data. For example, figure 1 is from sexually mature males and shows kallikrein (peak B) to be about 25% of the total (peaks A + B). This is exactly comparable to the data for 5-month-old males in table 2 (R-male 5.57/22 = 0.253, S-males 3.92/17.4 = 0.225). (Use the after-dialysis data from table 2 for total esterase because the urine in figure 1, of course, had to be dialyzed before ion exchange chromatography and because kallikrein TAME esterase activity was obtained only after dialysis and separation on DEAE). The kallikrein percent of total esterase in females is 50% (from table 2, R-females 6.24/11.7 = 0.533, S-females 4.42/9.7 = 0.456), which is double the percent for males, mostly because females have a lower total esterase (lack of androgen dependent enzyme). In table 3, the males are 2 months old, just reaching sexual maturity and so they resemble females in the ratio of kallikrein to total esterase.

The esterases in the A fraction from DEAE do not react with antibodies to kallikrein but they do generate low amounts of kinin from dog plasma. One of these A esterases (the non-androgen dependent one) has been partially purified by us and found to generate about 1/12th as much kinin from dog plasma kininogen as kallikrein when compared on the basis of equal TAME esterase activity. It is desirable, therefore, to remove this kinin-generating activity prior to evaluating kininogenase activity of kallikrein. In addition, in old rats with marked proteinuria several other TAME esterases appear in the A fraction in addition to the two normally present (Sustarsic, McPartland and Rapp, unpublished observation). Kinin generating activity of these additional TAME esterases (possibly originating from plasma or inflammatory cells in diseased kidneys) has not been evaluated so they must be removed on the assumption that they could generate kinins.

It is quite likely that in rats with severe proteinuria, proteinase inhibitors which originate from plasma are present in urine. Most of the plasma proteins, including large molecules (albumin, globulins) are present even in normal rat urine. Two proteinase inhibitors from rat plasma which inhibit hog pancreatic kallikrein have been described. One is probably rat $\alpha_1$-macroglobulin and the identity of the other (molecular weight 73,000) is unsure but it is probably not $\alpha_1$-antitrypsin. Geiger and Mann described a low molecular weight (4,700) inhibitor of the urinary kallikrein. This inhibitor was obtained from rat kidney extracts but its presence in urine was not evaluated.

Faced with this degree of complexity (prokallikrein, proteinase inhibitors, multiple proteinases and renal damage) we feel that there is adequate reason to include a chromatographic purification step prior to assay of kallikrein. Dialysis has to precede DEAE chromatography to remove salts. This procedure probably accounts for the markedly different results obtained in comparing S and R rats by Carretero et al. and us. Carretero et al. have studied urinary kallikrein excretion in six S male and six R male rats at 4 months of age being fed a standard salt diet. They observed that total urinary TAME esterase activity, measured in whole urine samples by the colorimetric method, was in S males about one-half to one-third of that in the R males. Their measurements of kininogenase activity in whole urine samples using a dog plasma kininogen substrate revealed, however, that in the S males urinary kininogenase activity was 1/10th to 1/20th of that in the R males. Two important possibilities for this discrepancy in the S versus R comparison by Carretero et al. are: 1) S and R kallikreins could have different kinetic properties in the assays due to different mutant forms of the enzyme; 2) S rat urine could contain an inhibitor of kallikrein that affects kininogenase activity more profoundly than TAME esterase activity. Our results show an equal ratio of kininogenase activity to TAME esterase activity in S and R rats for kallikrein after the enzyme is dialyzed and separated from non-kallikrein urinary TAME esterase(s) by DEAE-Sephadex minicolum chromatography. This suggests that the partially purified S and R kallikreins have similar properties in the two assays. This is evidence against a possible kallikrein mutation. Our results showing increased total TAME esterase activity after dialysis of urine of rats with hypertension and enhanced proteinuria, support the alternate explanation of a dialyzable inhibitor in S urine occurring as a response to renal damage. We emphasize, however, that because our observations in dialysis experiments necessarily involve only total urinary TAME esterase activity, this hypothetical dialyzable inhibitor(s) in S rat urine could be affecting either non-kallikrein TAME esterase activity, urinary kallikrein TAME esterase activity, or both. It seems quite likely that the effect of dialysis would also be achieved in the Sephadex G-25 filtration step usually practiced prior to the radiometric TAME assay.

In the present studies, urinary kallikrein activity was found to be lower in S males and females than in R males and females, when the animals were 5 months old and eating a standard salt diet (1% NaCl). Urinary kallikrein activity was also observed to be lower in 2-month-old S males fed a high salt diet (8% NaCl) compared to age matched R males fed the same diet. In both of these instances where there was a strain difference in urinary kallikrein activity, there was also a marked strain difference in blood pressure and urine protein excretion. In 2-month-old S and R males fed a low salt diet (0.4% NaCl), no significant strain difference in either urinary kallikrein activity, blood pressure or urine protein was observed. These results, suggesting that in S and R rats differences in urinary kallikrein activity are only present when there are differences in blood pressure and urine protein, raise
the possibility that the differences in urinary kallikrein activity might be a response to differences in blood pressure and/or renal tubular damage. Urinary kallikrein excretion has been shown to be decreased following antiglomerular basement membrane nephritis and aminonucleoside nephrosis in the rat.**

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