Proteins Binding to Kallikrein and Esterase A2 in the Urine of Salt-Sensitive and Salt-Resistant Rats

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SUMMARY Iodine-labeled (\[^{125}\text{I} \]) rat urinary kallikrein and rat urinary TAME esterase A2 were used as probes to look for urinary and plasma proteins that bind to these enzymes. Such proteins are presumptive enzyme inhibitors. Complexes formed with labeled enzymes were identified by polyacrylamide gel electrophoresis followed by autoradiography. Urine from young (6 weeks old) Dahl salt-sensitive (S) rats showed no, or only traces, of protein binding to kallikrein. Concomitant with the slow development of hypertension and proteinuria in S rats fed normal rat chow, one of the six kallikrein-binding proteins demonstrable in plasma was readily found in S-rat urine. This kallikrein-binding protein was called "KBP-1." R rats showed either no or much less KBP-1 in the urine, compared to S rats up to 5 months of age. A partly purified preparation of KBP-1 was shown to inhibit the TAME esterase activity of rat urinary kallikrein in the radiometric TAME assay. Urine of proteinuric S rats also contained two TAME esterase-binding proteins, TEBP-1 and TEBP-2, detected with the \[^{125}\text{I} \]-esterase A2 probe. As S rats aged from 3 to 8 months, free KBP-1 disappeared from the urine in spite of increased and marked proteinuria and the continued presence of KBP-1 in plasma. Concomitant with this age-related loss of urinary KBP-1 there was a marked shift in S urinary proteins binding to \[^{125}\text{I} \]-esterase A2 from TEBP-1 to TEBP-2. It was speculated that KBP-1 and TEBP-1 were the same protein detectable with either labeled kallikrein or labeled esterase A2. The concomitant disappearance of free KBP-1 (TEBP-1) and the appearance of free TEBP-2 in the urine of old, hypertensive, proteinuric S rats suggests that: 1) most of the KBP-1 (TEBP-1) is bound to enzyme(s) in old rats; or 2) KBP-1 (TEBP-1) is largely converted to TEBP-2 in old rats; or 3) both are true and that binding of KBP-1 (TEBP-1) to enzymes is associated with the generation of TEBP-2. (Hypertension 4:545–555, 1982)

KEY WORDS • kallikrein • esteroprotease • hypertension • genetic hypertension • salt-sensitive rats

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den Nustad and Pierce isolated kallikrein from the urine of female rats in 1974, they also found an enzyme other than kallikrein that hydrolyzed the artificial substrate \(\alpha\)-N-p-tosyl-L-arginine methyl ester (TAME). The esterases were designated "A" and "B" in the order of elution from a DEAE ion exchange column; Nustad and Pierce showed that esterase B was rat urinary kallikrein.\(^1\) We have recently described an additional TAME esterase that is present only in the urine of male rats. This enzyme was androgen-dependent and we called it "esterase A1."\(^2\) \(^2\) Nustad and Pierce's original esterase A was renamed "esterase A2" since it eluted from DEAE ion exchange chromatography after esterase A1.\(^2\)

Urinary kallikrein is known to be decreased in human essential hypertension.\(^3\)\(^-\)\(^7\) Holland et al.\(^8\) have shown that among essential hypertensives, those patients who had slight renal insufficiency also had reduced urinary kallikrein excretion, but those patients with apparently normal renal function had normal urinary kallikrein. It has recently been reported\(^9\) that for patients with hypertension and proteinuria there is an inverse relationship between immunologically measured urinary \(\alpha\)-antitrypsin and urinary kallikrein activity following addition of exogenous kallikrein to the urine. Dahl salt-hypertension sensitive (S) rats also excrete less urinary kallikrein than Dahl salt-hypertension resistant (R) rats.\(^10\)\(^-\)\(^12\) However, we have found that there is no difference in urinary kallikrein excretion between S and R strains in young rats. Kallikrein...
excretion becomes lower in S than R concomitant with the development of elevated blood pressure and increased urinary protein excretion in the S rats. This developmental pattern suggests that low urinary kallikrein excretion is a response to hypertension rather than a cause of hypertension. Plasma is known to contain several proteinase inhibitors, among which are some that inhibit plasma kallikrein.

Our present work was designed to test the hypothesis that low urinary kallikrein in the S rat is secondary to hypertension, renal damage, and resultant leakage of plasma proteinase inhibitors into the urine. To approach the problem of proteinase inhibitors in S rat urine it was necessary to develop some unique methodology because rat urine contains several TAME esterases (noted above) and, as will be seen, there is probably more than one proteinase inhibitor in rat urine. It is well known that many proteinase inhibitors form stable complexes with the enzymes that they inhibit. It should be possible to add labeled enzyme to a sample and look for enzyme-inhibitor complexes by autoradiography following an electrophoretic separation. This approach is based on the work of Holmberg et al. who used labeled urokinase as a probe for identifying a proteinase inhibitor in the placenta, and the work of Ganrot and Gauthier et al. who studied interactions of various labeled proteinases with plasma inhibitors.

Materials and Methods

The animals used were the salt-sensitive (S) and salt-resistant (R) rats developed by Dahl. Stocks were originally obtained from Dr. L. K. Dahl of Brookhaven National Laboratory (Upton, New York) and were inbred by brother-sister matings in our laboratory for 25 generations in the R and 15 generations in the S rats. Blood pressures were taken by the tail-cuff micromanometer method with the rats under ether anesthesia. Blood was collected from the abdominal aorta of rats under urethane anesthesia. The rats were given 500 units of heparin intravenously (i.v.) prior to collection. When disodium (ethylenedinitrilo)-tetracetic acid (EDTA) was added to the blood following collection, the amount used was 2 mg/ml of blood. Plasma was obtained following centrifugation to remove cells. The plasma was carefully separated from the cellular layer following centrifugation and recentrifugation to remove all white cells, to avoid contamination with proteinases.

During isolation of the rat urinary esterases, chromatographic column effluents were monitored by hydrolysis of the artificial substrate α-N-p-tosyl-L-arginine methyl ester HCl (TAME). The assay is based on the method of Roberts as modified by Nustad and Pierre. One esterase unit (EU) was defined as the amount of enzyme required to hydrolyze 1 μmole of TAME per minute at pH 8.6, 37°C. TAME esterase was also measured using the radiometric assay in which [3H]-TAME (213 mCi/mmol, Amersham, Arlington Heights, Illinois) was the substrate. Radiactive TAME was purified before use, and the enzymatic assay was that of Beaver et al. as modified by Margolius.

Rat urinary esterases A2 and B (esterase B is rat urinary kallikrein) were isolated as follows. Urine was collected into toluene from rats in metabolism cages; during collection, rats were given water but no food. Urine from R female rats under 6 months of age was collected and stored under toluene at 4°C. Pools of urine 2 to 3 liters in size were filtered and dialyzed at 4°C against four changes of distilled water. Potassium phosphate buffer 0.1 M pH 7.0 was added to yield a final concentration of 0.01 M phosphate. Potassium chloride (KCl) was added to a final concentration of 0.05 M. The prepared urine was applied directly to a 5 × 30 cm diethylamino ethyl (DEAE) Sephadex column made by boiling 25 g of DEAE-Sephadex A50-120 (Pharmacia) in 0.05 M KCl, 0.01 M phosphate buffer at pH 7.0. After the urine had flowed onto the column, the column was washed with 2 liters of 0.05 M KCl, 0.01 M phosphate buffer pH 7.0 and the wash was discarded. Esterase A2 was eluted with 2 liters of 0.2 M KCl in 0.01 M phosphate buffer pH 7.0 (20 ml fractions), and esterase B (rat urinary kallikrein) was eluted in a linear gradient (20 ml fractions) using 1 liter of 0.2 M KCl and 1 liter of 1.0 M KCl both in 0.01 M phosphate buffer pH 7.0. Fractions were assayed for TAME esterase activity, and the appropriate esterase-containing fractions were pooled, dialyzed against distilled water, and lyophilized.

The lyophilized esterase A2 was further purified by dissolving it in 4 ml of 0.2 M Tris-HCl buffer pH 8.0 and applying it to a 25 ml column of aprotinin-agarose (Sigma) containing 0.2 M Tris-Cl, pH 8.0. The column was washed with 120 ml of Tris buffer before changing to a 0.05 M phosphate buffer, pH 8.0, containing 1 M NaCl. After collecting 10 6-ml fractions, esterase A2 was eluted with 0.05 M citrate-phosphate buffer pH 3.0 containing 1 M NaCl. Aliquots of each 6 ml fraction were assayed for TAME esterase activity. The active fractions were pooled and adjusted to pH 7 with 0.75 M Tris-Cl at pH 8.6, and dialyzed for 3 days against distilled water. The sample was then lyophilized in silanized lyophilization bottles. This product was chromatographed on a molecular sieve column (Sephacryl S-200, Pharmacia) dialyzed, lyophilized, and reconstituted in water. The final preparation had a specific activity of 68 EU/mg and was used for iodination. Figure 1 shows this urinary esterase A2 preparation run in polyacrylamide gel electrophoresis and autoradiography following an electrophoretic separation.
Figure 1. Polyacrylamide gel electrophoretogram of purified rat urinary kallikrein (K) and purified rat urinary TAME esterase A2. The gel was stained for protein. Gel concentration was 13.5%.

The kallikrein fraction from the DEAE column was dialyzed, lyophlilized, and run over Sephacryl S-200. This final preparation was dialyzed, lyophlilized, and reconstituted in water. This preparation had a specific activity of 225 EU/mg and was used for iodination. Figure 1 shows this kallikrein preparation run in polyacrylamide gel electrophoresis and stained for protein. The heterogeneity seen is well known for urinary kallikrein and arises from variable change in the carbohydrate side chain. Moreover, like esterase A2, all the kallikrein bands in figure 1 did stain for esterase activity using the substrate N-acetyl-L-methionine α-naphthyl ester.

Both esterase A2 and kallikrein were iodinated by the chloramine T procedure of Greenwood et al., which is given in detail for kallikrein by Shimamoto et al. Two µg of either enzyme was reacted with 125I (Amersham) and the iodinated enzyme was separated from iodide on a Sephacryl S-200 column, 0.9 x 60 cm in 0.01 M phosphate buffer containing 0.14 M NaCl and 0.1% lysozyme. The iodinated enzymes were frozen until used and were usable for up to 2 months. Lysozyme serves as a carrier protein but has the desirable property of moving in the opposite direction from plasma proteins on polyacrylamide gel electrophoresis at pH 8.6 because of its high isoelectric point (pI = 11). It does not, therefore, enter the gel.

When urine from individual rats was studied, 24-hour urine samples were collected into toluene. Rats were given water but no food during collection. The toluene was removed and the urine was filtered to remove debris. Urine protein was measured by the method of Lowry et al. using bovine albumin as standard. Further processing consisted of dialysis against three changes of distilled water at 4°C, and lyophilization followed by reconstitution in water to yield an eightfold concentration. Samples were cleared of any precipitate by centrifuging at 1000 g for 10 minutes at 4°C.

Binding of [123I]-labelled TAME esterases to plasma or urinary proteins was performed as follows. We added 20 µl of [123I]-labelled enzyme (100,000 cpm) in phosphate-buffered saline, pH 7, containing 0.1% lysozyme, to 20 µl of concentrated urine (or other sample) and 10 µl deionized water. The mixture was incubated at 37°C for 30 minutes. At the end of the incubation, the mixture was chilled in ice, and 20 µl of a 50% sucrose solution containing 0.02% bromphenol blue tracking dye was added. Then 20 µl of this final mixture was applied to each sample well in a polyacrylamide gel, and the gel was subjected to electrophoresis and autoradiography.
cm above the bottom of the gel. Gels were stained for
protein in 0.04% Coomassie Brilliant Blue G 250 in
perchloric acid and destained in 7.5% acetic acid.
Autoradiography of [125I]-labeled proteins was car-
ried out by bringing the gel in contact with Kodak X-
omat R film sandwiched between two calcium tung-
state X-ray intensifying screens at —80° for 2 to 3
hours. The films were developed in a Kodak X-
omat film processor.

Results

Table 1 gives the time course of development of
proteinuria and blood pressure in S and R male and
female rats maintained up to 12 months of age on
commercial laboratory rat chow (Wayne Lab Blox),
which contains a normal amount of salt (1% NaCl). It
is obvious that with increasing age S rats slowly de-
velop hypertension even without excess salt feeding
and that concomitant with this a pronounced pro-
teinuria occurs. Very old R male rats also eventually be-
come hypertensive and markedly proteinuric. It is im-
portant to note that, at every age studied, male rats
excreted more urinary protein than females.

Plasma is well known to contain a variety of protein-
ase inhibitors. A simple way to demonstrate binding
of kallikrein to plasma proteins (presumptive inhibi-
tors) is to incubate [125I]-kallikrein with plasma and
look for displacement of the labeled kallikrein into new
bands with polyacrylamide gel electrophoresis and au-
торadiography. Figure 2 shows such an autoradio-
graph. If plasma is collected with heparin, six radioac-
tive bands (besides kallikrein) are seen. If EDTA is
added to heparinized plasma, one band disappears and
only five radioactive bands appear (fig. 2). The effect
of EDTA may be the result of removal of a divalent
cation, possibly Zn2+, which is thought to bind to
inter-α-trypsin inhibitor of human plasma.

We wanted to determine if any of the plasma kalli-
krein-binding proteins were present in urine of S and R
rats. Figure 3 shows an autoradiograph of a gel electro-
phoretogram using eightfold concentrated urine incu-
bated with [125I]-kallikrein. Urine from 3- and 5-
month-old female R rats showed no kallikrein-binding
proteins. Three-month-old S rats, however, showed a
pronounced band of complexed [125I]-kallikrein having
a mobility identical to the fastest running [123I]-kalli-
krein protein complex found in plasma (fig. 3). The
urinary and plasma complexes had identical electro-
phoretic mobilities, with polyacrylamide gel concen-
trations between 6% and 15%, suggesting identity of
the complexes formed from the two sources. This

<table>
<thead>
<tr>
<th>Table 1. Urinary Protein Excretion and Blood Pressure at Various Ages for Salt-Sensitive (S) and Salt-Resistant (R) Rats Fed 1% NaCl Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat age</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>1 5 months old:</td>
</tr>
<tr>
<td>Urine protein (mg/24 hrs)</td>
</tr>
<tr>
<td>Blood pressure (mm Hg)</td>
</tr>
<tr>
<td>No. of rats</td>
</tr>
<tr>
<td>3 months old:</td>
</tr>
<tr>
<td>Urine protein (mg/24 hrs)</td>
</tr>
<tr>
<td>Blood pressure (mm Hg)</td>
</tr>
<tr>
<td>No. of rats</td>
</tr>
<tr>
<td>6 months old:</td>
</tr>
<tr>
<td>Urine protein (mg/24 hrs)</td>
</tr>
<tr>
<td>Blood pressure (mm Hg)</td>
</tr>
<tr>
<td>No. of rats</td>
</tr>
<tr>
<td>12 months old:</td>
</tr>
<tr>
<td>Urine protein (mg/24 hrs)</td>
</tr>
<tr>
<td>Blood pressure (mm Hg)</td>
</tr>
<tr>
<td>No. of rats</td>
</tr>
</tbody>
</table>

Values are means ± se. NS = not significant, i.e., p > 0.1. A probability of 0.05-0.1 was considered to be of questionable significance. Analysis was by a 2 x 2 factorial analysis of variance, except at the 12-month point where a t test was appropriate.
binding protein is named "kallikrein-binding protein number 1," abbreviated as "KBP-1." The band seen on the gel autoradiograph is of course a complex between $[^{125}I]\text{-kallikrein}$ and KBP-1. This complex is abbreviated as "K*KBP-1." As the female S rats grow older, there is a progressive loss of the formation of K*KBP-1 complex. This is obvious by comparing the 3-, 5-, and 8-month-old S females in figure 3.

Figure 4 shows similar data for S and R male rats. Urine from 3-month-old male S rats shows a pronounced binding of $[^{125}I]\text{-kallikrein}$ to form the K*KBP-1 complex. Some of the 5-month-old S males show the formation of the K*KBP-1 complex and some do not. By 8-months of age, male S rat urine usually fails to form the K*KBP-1 complex. The pattern for male R rat urine is somewhat different. The younger 3-month-old R males show no K*KBP-1 complex, or only traces of complex formation, but K*KBP-1 complex-forming activity increases gradually with age in R males. K*KBP-1 does not become undetectable in R males at 8 months of age, as it did in S males. The results for urinary KBP-1 for all the S and R male and female rats studied are summarized in table 2.

The binding of $[^{125}I]\text{-esterase A2}$ by urine from S and R rats has also been studied. Figure 5 shows representative results from S and R male rats. Urine from 3-month-old male R rats shows the formation of a complex with esterase A2. The protein forming this complex is called "TAME esterase binding protein number 1" (TEBP-1) and so the complex with A2 is labeled A2*TEBP-1 in figure 5. With increasing age, a second faster migrating complex appears in R male urine. The protein forming this complex is called "TAME-esterase binding protein number 2" (TEBP-2), and the complex with A2 is abbreviated A2*TEBP-2 (figure 5). Urine of male S rats shows the same complexes, A2*TEBP-1 and A2*TEBP-2, as did that of the R rat, but the intensity of the bands on the autoradiographs is darker. In urine from 3- and 5-month-old S rats, some rats showed formation of both A2*TEBP-1 and A2*TEBP-2 complexes, and some showed only A2*TEBP-1. By 8 months of age, however, only the A2*TEBP-2 complex was seen (fig. 5). Thus, in both S and R rats there appears to be a shift with age from the
formation of A2×TEBP-1 to A2×TEBP-2. These results are summarized in Table 3 for all S and R male and female rats studied for binding of urinary proteins to esterase A2.

Obviously, there are marked shifts in the patterns of binding kallikrein and esterase A2 by the urine of S rats with age. It was found that these age-related shifts for the two enzymes are correlated. Table 4 shows that for S rats 3 to 8 months of age, when an S rat urine was positive for the presence of KBP-1 then it was usually positive for the presence of TEBP-1 with the [125I]-

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**Table 2. Summary of Results for Detection of KBP-1 in the Urine of Salt-Sensitive (S) and Salt-Resistant (R) Rats by Gel Electrophoresis and Autoradiography**

<table>
<thead>
<tr>
<th>Rat strain</th>
<th>Sex</th>
<th>Age (mos)</th>
<th>KBP-1 negative</th>
<th>KBP-1 trace</th>
<th>KBP-1 weak positive</th>
<th>KBP-1 strong</th>
</tr>
</thead>
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<td>1.5</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R</td>
<td>female</td>
<td>3</td>
<td>*</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R</td>
<td>female</td>
<td>5</td>
<td>*</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>R</td>
<td>female</td>
<td>8</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>S</td>
<td>female</td>
<td>1.5</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>S</td>
<td>female</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>8</td>
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<td>1</td>
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<td>S</td>
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<td>8</td>
<td>6</td>
<td>0</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>R</td>
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<tr>
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<td>5</td>
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<td>1</td>
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</tr>
<tr>
<td>R</td>
<td>male</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>S</td>
<td>male</td>
<td>1.5</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>0</td>
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<tr>
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<td>0</td>
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</tr>
<tr>
<td>S</td>
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<td>1</td>
<td>0</td>
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<td>2</td>
</tr>
<tr>
<td>S</td>
<td>male</td>
<td>8</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

The entries in the table are the numbers of rats seen in each category.

*No data were available for 1.5-month-old S and R females and 8-month-old R females.

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**Table 3. Summary of Results for Detection of TEBP-1 and TEBP-2 in the Urine of Salt-Sensitive (S) and Salt-Resistant (R) Rats by Gel Electrophoresis and Autoradiography**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>Age (mos)</th>
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<th>Only TEBP-1</th>
<th>Only TEBP-2</th>
</tr>
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<tbody>
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<td>female</td>
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<td>4</td>
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</tr>
<tr>
<td>R</td>
<td>female</td>
<td>5</td>
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<td>1</td>
</tr>
<tr>
<td>R</td>
<td>female</td>
<td>8</td>
<td>*</td>
<td>*</td>
<td>*</td>
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<tr>
<td>S</td>
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<td>9</td>
</tr>
<tr>
<td>S</td>
<td>female</td>
<td>5</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>S</td>
<td>female</td>
<td>8</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>R</td>
<td>male</td>
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<tr>
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</tr>
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<td>8</td>
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<td>0</td>
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</tr>
<tr>
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<td>7</td>
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<tr>
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<tr>
<td>S</td>
<td>male</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

The entries in the table are numbers of rats seen in each category. TEBP-1 and TEBP-2 are TAME esterase binding protein number 1 and number 2.

*No data were available for 8-month-old R females.
FIGURE 5. Autoradiograph of polyacrylamide (8.5%) gel electrophoretogram. The left-most lane contained [125I]-kallikrein. The next 12 lanes contained concentrated urine samples from S and R male rats of various ages as indicated, and incubated with [125I]-esterase A2. TEBP = TAME esterase binding protein; A2-TEBP = a complex between esterase A2 and a TAME esterase-binding protein. There are two complexes formed with esterase A2, and these are labeled A2-TEBP-1 and A2-TEBP-2.

FIGURE 6. Autoradiograph of polyacrylamide (8.5%) gel electrophoretogram. Urine samples from individual rats were selected on the basis of the presence or absence of KBP-1. Aliquots of these urine samples were incubated with either [125I]-kallikrein (right side of the gel) or [125I]-esterase A2 (left side of the gel). Thus, counting lanes left to right, the same urine samples were used in Lanes 1 and 10, 2 and 11, etc. The right-most lane was [125I]-kallikrein incubated with the dialyzed supernatant of a 60% ammonium sulfate precipitation of rat plasma. Note that when a urine sample was positive for KBP-1, then it contained TEBP-1 or a mixture of TEBP-1 and TEBP-2. When a urine sample was negative for KBP-1, then it contained only TEBP-2.
TABLE 4  Contingency Table for Binding of Labeled Esterase A2 to TAME-Esterase-Binding Proteins (TEBP) and for Binding of Labeled Kallikrein to Kallikrein-Binding Protein (KBP-1) in the Urine of Proteinuric S Male and Female Rats 3 to 8 Months Old

<table>
<thead>
<tr>
<th>Presence of KBP-1</th>
<th>Presence of [125I]-Esterase A2 mixture of TEBP-1 and TEBP-2</th>
<th>TEBP detected with [125I]-Esterase A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detected with [125I]-kallikrein</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>Positive</td>
<td>0</td>
<td>6*</td>
</tr>
<tr>
<td>Weak positive</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
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</tbody>
</table>

The entries in the table are the numbers of rats seen in each category. A chi-square test showed that there was a significant ($p < 0.005$) association between the kind of complex formed with esterase A2 and the presence or absence of formation of a complex between kallikrein and urinary kallikrein binding protein. The total numbers of rats in this table differ slightly from those in table 2 because data on TEBP were not obtained on a few rats.

*Includes one 5-month-old S female graded as trace for KBP-1.

KBP-1 in urine were tested and all were found to still contain readily detectable amounts of KBP-1 in plasma (gel not shown).

KBP-1 has been partially purified from plasma by ammonium sulfate fractionation and column chromatography on Affi-Gel Blue (Biorad). KBP-1 is soluble in 60% saturated ammonium sulfate (see right side of fig. 6) whereas the other proteins that bind to kallikrein (i.e., those that form kallikrein complexes that run near the top of the gel in fig. 2) are precipitated. The 60% ammonium sulfate supernatant was dialyzed, lyophilized, and applied to a 2.5 × 20 cm Affi-Gel Blue (Bio Rad) column in 0.05 M Tris-Cl, 0.05 M NaCl, 0.02% NaN₃, pH 8.0. KBP-1 binds to Affi-Gel Blue and was eluted with 1.4 M NaCl in the same buffer. Some albumin elutes with the 1.4 M NaCl wash also, but most of it remains bound to the column, being eluted only with 1 M KSCN. The 1.4 M NaCl Affi-Gel Blue fraction was dialyzed, lyophilized, and reconstituted in water. The KBP-1 preparation was free of the other TAME esterase-binding proteins that form complexes with kallikrein as judged by autoradiographic analysis following incubation with labeled kallikrein. This preparation was able to inhibit the TAME esterase activity of kallikrein using the radiometric assay (table 5). The KBP-1 preparation by itself had slight TAME esterase activity (490 cpm released above blank value), probably due to slight contamination with a plasma TAME esterase that is not inhibited by KBP-1.

The binding of [125I]-esterase A2 by rat plasma is demonstrated in figure 7. With whole plasma, A2 forms a single complex migrating much slower than the urinary A2-TEBP complexes. (It is probably bound to a macroglobulin.) Plasma, however, does contain a protein that forms a complex with esterase A2 and migrates in electrophoresis similarly to the complexes formed with urine. This is demonstrated in figure 7 by fractionating plasma with ammonium sulfate to remove high-molecular-weight proteinase inhibitors. This plasma fraction (60% ammonium sulfate supernatant) is the same one that contains KBP-1, as shown on the right side of figure 6.
Discussion

The data presented show that rat urine contains proteins that form stable complexes with the TAME esterase enzymes normally present in rat urine. Moreover, there are pronounced temporal differences in the detectability of these proteins in the urine of S and R rats.

The importance of these alterations centers around the likely possibility that these urinary TAME esterase-binding proteins are inhibitors of the enzymes to which they bind. Inhibition of kallikrein could reduce intrarenal kinin formation, which theoretically may exacerbate hypertension via reduced renal blood flow. Recently we have found that purified urinary esterase A2 has kinin generating ability equal to one-half that of rat urinary kallikrein. Previous estimates of kinin generation by esterase A2 were erroneously low due to contaminating kininase in the impure preparations used. Although the discussion will include the possibility that the binding proteins described are enzyme inhibitors, it is emphasized that definitive data showing the binding proteins to be proteinase inhibitors are lacking. A partially purified preparation of plasma KBP-1, which was devoid of other kallikrein binding proteins, did inhibit the TAME esterase activity of rat urinary kallikrein. Another fact suggesting that KBP-1 is an inhibitor is that the K*KBP-1 complex was stable to boiling in mercaptoethanol and sodium dodecyl sulfate (unpublished data). The complex between trypsin and α1-antitrypsin, for example, was also stable to this treatment.50 These data suggest, but do not prove, that KBP-1 is a kallikrein inhibitor. It is likely on the basis of our unpublished experience with KBP-1 on molecular sieve and ion exchange columns that KBP-1 is identical to the smaller of two inhibitors of glandular kallikreins described by Hojima et al.46 in rat plasma. This inhibitor was partly purified by Hojima et al. and shown to inhibit kallikrein esterase activity and also to inhibit kallikrein activity in the dog vasodilator assay.

It is also conceivable that an enzyme-binding protein could be a substrate for the enzyme rather than an inhibitor. Plasma kallikrein and plasma prekallikrein are known to form complexes with kininogens.7-9

There are no data concerning inhibitory properties of the TEBP-1 and TEBP-2 described here using labeled esterase A2. It is possible, however, that TEBP-1 is identical to KBP-1. Old S rats in which no urinary KBP-1 could be detected almost always (13 out of 14, table 4) failed to yield detectable TEBP-1. The reverse was usually true, that is, if no TEBP-1 were detected, KBP-1 was also usually absent (13 out of 16, table 4). Thus, KBP-1 and TEBP-1 disappear concomitantly, suggesting that they may be identical. The few exceptions to this could easily arise by misclassification of a rat as negative for KBP-1 or TEBP-1 when in fact it was positive. Autoradiography is subject to this kind of error because a longer exposure time may sometimes reveal a band not seen with shorter exposures. Further evidence that KBP-1 and TEBP-1 are identical is that 60% ammonium-sulfate fractionated plasma contained KBP-1 (fig. 6, right-most lane) and a TEBP (fig. 7, center lane).

The impetus behind looking for kallikrein-binding proteins in rat urine was to try to explain the decrease in urinary, DEAE extractable kallikrein in S compared to R rats.12,13 Since this decrease in S urinary kallikrein excretion occurred concomitantly with the development of hypertension and subsequent renal damage and proteinuria,15 it makes sense that plasma proteinase inhibitor(s) enters the urine, complexes with kallikrein, and thus lowers the free DEAE extractable kallikrein excretion. Renal lesions in S rats40,41 and their correlation with blood pressure41,42 have been described.

It is clear that a possible origin of urinary KBP-1 is plasma since the K*KBP-1 complex was formed with both whole plasma and urine, as defined by the identical electrophoretic properties of the complexes from both sources. In the case of the binding of [125I]-esterase A2 to whole plasma, one major slowly migrating complex was seen (fig. 7). This was probably a complex with a macroglobulin. Macroglobulins are, however, easily precipitated with ammonium sulfate leaving smaller molecules in the supernatant. The 60% ammonium sulfate supernatant, did show marked formation of a complex with esterase A2 that had similar mobility to complexes formed in urine with esterase A2. Obviously, in whole plasma the affinity of one of the macroglobulin proteinase inhibitors for A2 must be greater than the affinity of TEBP for esterase A2, and in whole plasma this macroglobulin takes up all the available labeled esterase A2. Similar competition between plasma inhibitors using trypsin or chymotrypsin in whole plasma is well documented.18

In most of the 8-month-old S rats studied, KBP-1 was completely undetectable in urine. It was, however, still present in plasma and the rats were still markedly proteinuric. Obviously, the methods used were able to detect only the free moiety of the binding proteins. It is possible, therefore, that the apparent absence of KBP-1 from urine of old S rats means that all the available KBP-1 was bound to endogenous unlabeled kallikrein and/or esterase A2. Another possible explanation for the disappearance of KBP-1 (TEBP-1) from urine in old S rats is that it may be converted to TEBP-2. The only evidence for this speculation is that the disappearance of KBP-1 (TEBP-1) is associated in these rats with the appearance of TEBP-2. In this case one would expect DEAE-extractable urinary kallikrein to increase in old rats because of the replacement of a protein with strong kallikrein-binding ability by one with weak or no kallikrein-binding ability. This was not observed, however, in a study of S and R rats up to 6 months of age.13 An additional speculation is that the binding of KBP-1 (TEBP-1) to kallikrein or esterase A2 results in the proteolytic generation of a fragment that has affinity for esterase A2 but not for kallikrein. This is possible as many proteinase inhibitors have multiple inhibitory domains.10

A further possibility for the temporal changes in urinary KBP-1 and TEBP-2 is that these changes might
reflect activation and inactivation of different genes. The reciprocal relationship between KBP-1 and TEBP-2 might arise from the concomitant deactivation of a gene coding for KBP-1 and the activation of a gene coding for TEBP-2 in kidney tissue. The stimulus for such hypothetical gene activation is obscure. On the basis of the information available so far we favor the idea that KBP-1 is metabolized to TEBP-2 over the gene-switching theory. This bias arises from the result that plasma is the apparent source of KBP-1, and because the plasma KBP-1 is still present when urinary KBP-1 has disappeared. It is also obvious, however, that immunologic data are needed to establish the identity of KBP-1 from plasma and urine.

Human urine is known to contain acidic, acid-stable trypsin inhibitor(s) in the molecular weight range 15,000–45,000,44,45 and these are immunologically related to human plasma inter-α-trypsin inhibitor,46,47 which is a much larger molecule (molecular weight is 180,000). In most of the work on human urinary trypsin inhibitors, acid treatment was part of the isolation scheme. If acid treatment is avoided, a larger inhibitor (molecular weight 68,000–70,000) can be isolated.48 Recently, Muramatu et al.49 showed that this larger (molecular weight = 68,000) urinary trypsin inhibitor from human urine is degraded at low pH by acid-activated uropoepsin to smaller active trypsin inhibitors. It is also possible to cleave inter-α-trypsin inhibitor with a variety of serine proteases to form lower molecular-weight active trypsin-inhibitors.50 In addition to these serine protease inhibitors in human urine, which are apparently derived from plasma inhibitors, human urine also contains inhibitors of thiol proteinases, which are probably degradation products of thiol proteinase inhibitors from human plasma.51 An inhibitor (m.w. = 4700) of rat urinary kallikrein has been isolated from rat kidney by Geiger and Mann.52 Whether this inhibitor is present in rat urine is unknown. Clearly it is not identical to the binding proteins described here because it is much smaller than these molecules (unpublished gel filtration data).

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*Hypertension.* 1982;4:545-555
doi: 10.1161/01.HYP.4.4.545

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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