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STUDIES ON THE NATIVE FORMS OF RENIN IN THE RAT KIDNEY

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Summary

This paper describes Sephadex G-100 chromatography of rat kidney extract containing various enzyme inhibitors. The high molecular weight renin (molecular weight above 50 000) constitutes about 50% of the total renin activity. Omission of the enzyme inhibitors yield solely low molecular weight renin. Upon rechromatography high molecular weight renin eluted in two peaks at lower molecular weight with a concomitant reduction of renin activity. Renin activity in the fractions from Sephadex G-100 chromatography was increased 70% by dialysis at acid as well as neutral pH through the whole molecular weight range. Cold storage of extract with low molecular weight increased renin activity about 25%. The results suggest that the fully active enzyme is not represented by the lower molecular weight forms of renin and direct connection between activation of renin and reduction of renin molecular size was not indicated.

Introduction

Renin (EC 3.4.99.19) catalyzes the formation of angiotensin I from angiotensinogen. Heterogeneity of a purified preparation of renin was first described by Haas and coworkers [1,2] who found two fractions with renin activity by free-moving-boundary electrophoresis and three forms by ultraviolet spectroscopy. Heterogeneity of renin from a variety of species has later been demonstrated by different methods [3—9].

In recent years new lines in renin research have been introduced with the finding of high molecular weight forms of renin, so-called 'big' renin and 'big

Abbreviations: MalNEt, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate.

big' renin, possessing molecular weights higher than 40 000—50 000 [10—17]. Attempts to relate these findings to the original observations of Lumbers [17] and Rubin [7] that renin activity of amniotic fluid and pig kidney extract, respectively, increased upon exposure to acids have also been made in several laboratories [10—14]. The results, however, have not been conclusive.

Differences with respect to the properties of high molecular weight form of renin and the results of acid treatment of kidney extracts might partly be due to different working conditions and partly to a complicated activation mechanism.

The present paper deals with the appearance of both high and low molecular weight forms of renin after Sephadex chromatography of kidney extracts. High molecular weight and low molecular weight renin are here defined as renin activity in fractions corresponding to molecular weights above and below 50 000, respectively.

Our purpose was to isolate as far as possible the native forms of renin in the rat kidney by rapid removal of kidneys and the use of enzyme inhibitors, and to investigate the possible relation between high molecular weight forms and acid activation.

Materials and Methods

Renin preparations

General. 6 female Wistar rats (200 g) were killed by cervical dislocation, the kidneys removed within 15 seconds and placed on ice. Cortical tissue, weighing approx. 6 g, was chopped finely with scissors. In each experiment only freshly made preparations were used.

The experiments were performed at 4°C unless stated otherwise.

Method 1. The chopped tissue was homogenized in 10 mM sodium pyrophosphate buffer/HCl/0.1 M NaCl, pH 6.5, 0°C, (1 : 5, w/v) in a Virtis blender operating at 45 000 rev./min. The homogenization medium always contained the following enzyme inhibitots: 5.4 mM EDTA, 2.3 mM *o*-phenanthroline, 1.0 mM toluene sulfonyl fluoride, 2.3 mM *p*-hydroxymercuribenzoate. In one series of experiments the homogenization medium also contained 10 mM *N*-ethylmaleimide (MalNEt).

The homogenate was frozen at —70°C and thawed (2—4°C) five times to liberate membrane-bound or intralysosomal renin. Each freezing and thawing cycle lasted for 15 min. The homogenate was centrifuged at 12 000 × *g* for 20 min in a Sorvall centrifuge type RC2-B. The supernatant was dialyzed for 4 h against 10 mM sodium pyrophosphate buffer/HCl/0.1 M NaCl, pH 6.5 (1 : 40, v/v), containing 1/20 the amount of the above mentioned enzyme inhibitors [18]. The dialyzed solution was centrifuged in the Sorvall centrifuge at 12 000 × *g* for 30 min and the supernatant chromatographed.

Method 2. The chopped tissue was homogenized in 0.45 M sucrose (1:5, w/v), 0°C in a Potter-Elvehjem glass homogenizer with a Teflon pestle (0.38 mm clearance), 500 rev./min, 5—7 strokes. The homogenate was centrifuged at 650 × *g* for 2.5 min in the Sorvall centrifuge. The supernatant (step I) was frozen and thawed as described above, and centrifuged at 6000 × *g* for 10 min in a Christ Jr. centrifuge. The supernatant (step II) was treated with enzyme

inhibitors, 5.4 mM EDTA, 2.3 mM *p*-hydroxymercuribenzoate, 2.3 mM *o*-phenanthroline by stirring for 2—3 h in the cold room and centrifuged at 12 000 × *g* for 20 min in the Sorvall centrifuge. The supernatant (step III) was dialyzed for 2—6 h against 2 l 1% glycine (pH 6.75) and centrifuged at 12 000 × *g* for 30 min in the Sorvall centrifuge. The supernatant (step IV) was dialyzed for 24 h against 2 l 10 mM sodium pyrophosphate/HCl (pH 7.0) and centrifuged at 12 000 × *g* for 20 min in the Sorvall centrifuge (step V).

2 ml each of steps I—V were kept in closed vials in the cold room and tested for renin activity every second or third day for 4 weeks to study the effect of cold storage on renin activity. The molecular weight of renin from step V was estimated by Sephadex G-100 column chromatography (see below).

Method 3. The chopped tissue was homogenized in 0.9% NaCl (1 : 2, w/v), 0°C in the Virtis blender for 1 min, frozen and thawn as described above, centrifuged at 755 × *g* for 5 min in the Sorvall centrifuge. The supernatant was used for studying acid activation and treated with 0.1% Triton X-100 (v/v). The molecular weight of renin was estimated by Sephadex G-100 column chromatography.

Column chromatography

Chromatography was performed on Sephadex G-100 fine (Pharmacia, Uppsala, Sweden) in 10 mM sodiumpyrophosphate/HCl/0.1 M NaCl (pH 6.5) with kidney extract (Method 1 and 3). Extract prepared according to Method 2 was chromatographed in a 0.1 M Tris-HCl buffer (pH 8.0) and 0.02% neomycin sulphate. Column dimensions were in all cases 100 cm × 5 cm^2, the applied volume was from 2—6 ml, flow was 20 ml/h, fractions approx. 3.5 ml.

The molecular weight of renin was estimated by the method of Andrews [19]. The following molecular weight markers were used: bovine serum albumin, 67 000; chymotrypsinogen A, 25 000; cytochrome *c*, 12 500 (all Boehringer, Mannheim, F.R.G.); egg albumin, 45 000 (Fluka, Buchs, Switzerland). Blue Dextran (Pharmacia, Uppsala, Sweden) was used in every run, and 100 µl of a saturated solution were added. Markers were run before and after each renin chromatography. The plastic vials were weighed before and after the run. The volumes of the fractions were calculated as the weight difference. The elution volumes of the renin peaks were obtained in the same way. The regression coefficients of the chromatographies with protein markers were 0.995—0.999. V_e for the markers in 21 runs differed with 2.5 ± 1.5 ml (means ± S.D.). The molecular weights of the renins were calculated from a linear plot using a least-square principle.

Protein concentration

Protein concentration was measured by determining the difference in absorbance, $E_{224} - E_{236.5}$ [20], with crystalline bovine serum albumin (BDH, Poole, Dorset, U.K.) as standard.

Acid treatment

Method A. 0.5-ml samples from every second fraction of a Sephadex G-100 chromatography of extracts prepared according to Method 1 were dialyzed against 5 l 0.08 M glycine buffer/0.08 M NaCl, pH 3, for 24 h and neutralized

by dialysis against 5 l 0.15 M phosphate buffer, pH 7.1, for another 24 h. 0.5-ml samples from the same fractions which were dialyzed for 48 h at pH 7.1 served as controls.

Method B. 0.5-ml aliquots of extracts from preparation Method 3 were diluted with 10 ml preheated 30 mM barbitone/acetate buffer, pH 3.4, 4.4, 5.4, 6.4 and 7.4, kept at 37°C for 2 h and then cooled to 0°C. The unheated preparation, kept at 0°C, served as control.

Method C. 0.5-ml samples of extract prepared by Method 3 (not centrifuged but squeezed through cheese-cloth), were dialyzed against 500 ml Sørensen glycine buffer, pH 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0. After 20 h the samples were neutralized to pH 7.4 by dialysis for another 20 h against 500 ml 0.5 M Tris-HCl/0.1 M NaCl, pH 7.4. A 0.5 ml sample, dialyzed at pH 7.4 for 40 h, was used as control. Precipitated proteins were removed by centrifugation at 8000 × *g* for 10 min.

Triton X-100 treatment

Triton X-100 (final concentration 0.1%, v/v) (Rohm and Haas, Philadelphia, PA, U.S.A.) was added to kidney extract prepared according to Method 3. The vial was shaken for 30 min and immediately tested for renin activity.

Renin and angiotensin activity

Renin and angiotensin I were measured by radioimmunoassay [21]. Standard pig renin was obtained from Medical Research Council, Mill Hill, London N.W. 7, U.K. (batch no. 69—119). Standard angiotensin I was from Bechman (lot A 0413, $\epsilon^2_{280} = 1300$, Bechman, Geneva, Switzerland), Renin substrate was prepared according to Nasjletti et al. [22]. Internal standards of angiotensin I were included in every assay, S.E.M. was 2.5% calculated from 56 consecutive radioimmunoassays.

Results

Column chromatography

When rat kidney extracts were prepared according to Method 1, in which enzyme inhibitors were present from the very first step, renin activity was eluted from a Sephadex G-100 column with 2—5 peaks. Fig. 1 shows a typical result (together with a rechromatography of renin from the same column). The molecular weights corresponding to the peak fractions found in a total of 17 extracts were: 70 000 ± 800 (S.E.) ($n = 8$); 60 000 ± 600 (S.E.) ($n = 17$); 52 000 ± 500 (S.E.) ($n = 11$); 44 000 ± 500 (S.E.) ($n = 15$) and 38 000 ± 300 (S.E.) ($n = 10$). Recovery of the applied renin activity on the column was always above 80%. The yield in peak fractions was always about 32% of total recovered activity.

The distribution of renin activity between high and low molecular weight forms of renin, in extracts prepared with and without MalNEt, showed no significant difference. In nine preparations containing 10 mM MalNEt 51 ± 3% (S.E.) appeared as high molecular weight renin, recovery 126 ± 15% (S.E.). In six preparations prepared without MalNEt the distribution between high and low molecular weight renin was identical $0.95 > P > 0.90$.

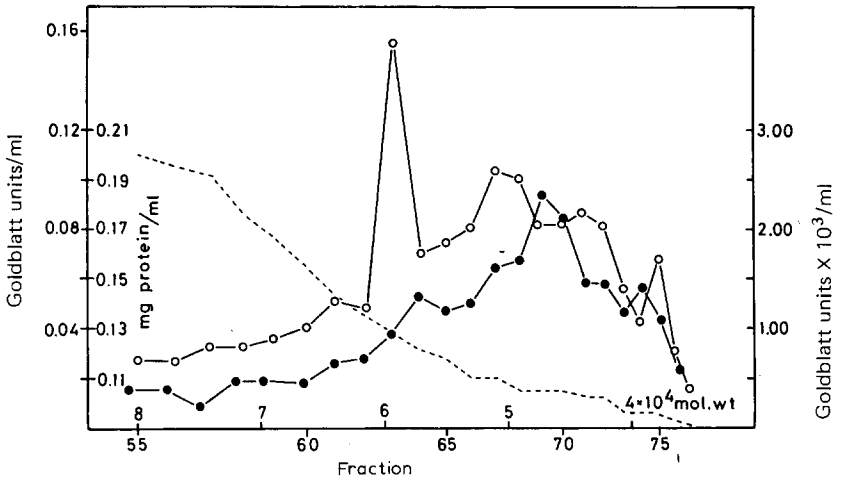

Fig. 1. Sephadex G-100 chromatography of rat kidney renin, prepared according to Method 1, and rechromatography on the same column. - - - - -, protein mg/ml; ○———○, renin activity, first chromatographic run (left ordinate); ●———●, renin activity in rechromatographed 60 000 molecular weight peak (right ordinate). In the first chromatography the load was 40 mg protein (6 Goldblatt units) in 3 ml. In the rechromatography the load was 0.8 mg protein (0.5 Goldblatt units) in 5 ml. The elution buffer was 10 mM pyrophosphate/HCl/0.1 M NaCl, pH 6.5 3.5-ml fractions.

Shoulders of renin activity, when present, were always found on the ascending limb of the chromatograph and not on the descending limb as an effect of overloading.

Rechromatography of high molecular weight renin was performed in 6 Expts., 3 from each series (Fig. 1).Renin eluted in two peaks, one at molecular weight approx. 55 000 representing 65—70% of recovered enzyme activity, and one at molecular weight approx. 45 000 representing 30—35% of the recovered activity. In one experiment from the first series, 30 mM human serum albumin was added to the pool of high molecular weight renin used for rechromatography, this prevented the conversion of high molecular weight renin (60 000), on the column. Incubation at 37°C for 2 h with albumin gave rise to a single peak at molecular weight 45 000, eliminating the protective action of albumin.

Finally, in one experiment a second rechromatography of the combined 45 000 and 55 000 molecular weight renin was carried out. In this experiment renin eluted in one peak at molecular weight 45 000, indicating either the complete removal on the column of a dissociable peptide with molecular weight of 10 000, or the complete conversion by an accompanying enzyme of high molecular weight to low molecular weight renin.

Reduction of molecular weight was never followed by increased renin activity, on the contrary, we observed a concomitant reduction of renin activity by 61 ± 14% S.E. (n = 6).

Three experiments were performed with Sprague-Dawley rats to see whether the findings were specific for Wistar rats. The results were identical and renin from these two strains are thus similar with respect to the distribution of high

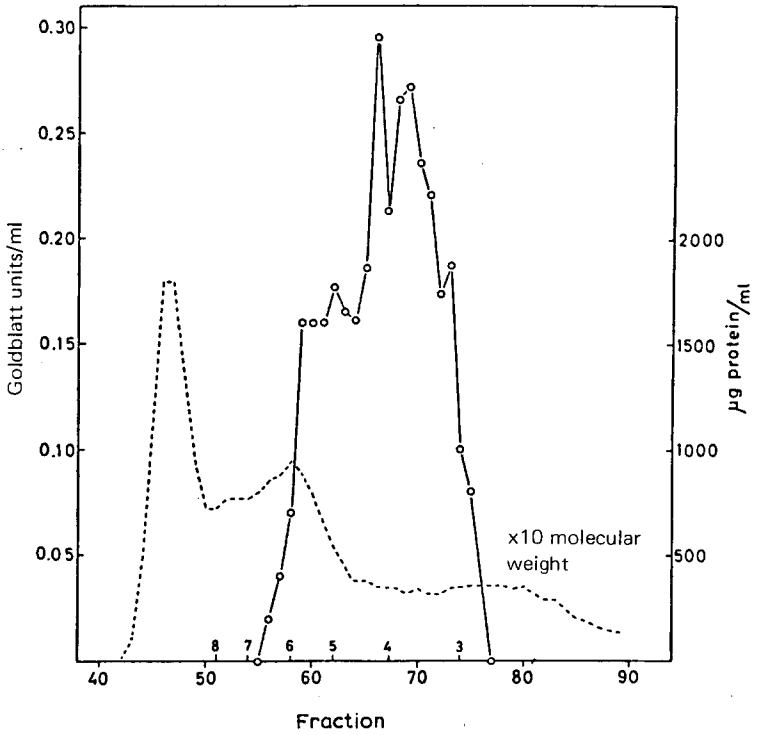

Fig. 2. Sephadex G-100 chromatography of rat kidney renin (step V) prepared according to Method 2. ------, protein mg/ml; o———o, renin activity. The load was 85 mg protein (18 Goldblatt units) in 5.5 ml. The elution buffer was 0.1 M Tris-HCl plus 0.02% Neomycin sulfate, pH 8.0.

molecular weight and low molecular weight forms of renin by Sephadex G-100 chromatography.

When enzyme inhibitors were used at a later step (Method 2), renin activity eluted in two peaks, one at molecular weight 37 000, and one at molecular weight 42 000 with a shoulder at molecular weight 52 000 (Fig. 2); the latter high molecular weight renin comprised only 18% of recovered renin activity. When enzyme inhibitors were completely omitted renin activity eluted in one peak at molecular weight 40 000 (Fig. 3).

Acid treatment

Kidney extract (Method 1) separated by Sephadex G-100 chromatography was treated with acid pH. 0.5-ml samples from every second fraction of five chromatographic runs were dialyzed at pH 3 for 24 h followed by dialysis at pH 7.1 for another 24 h. Samples from the same fractions were dialyzed at pH 7.1 for 48 h and served as controls (Fig. 4). High molecular weight renin activity increased to a mean value of 168 ± 22% (S.E.) (n = 5) and 164 ± 14% (S.E.) (n = 5). Low molecular weight renin activity increased to a mean value of 161 ± 15% (S.E.) (n = 5) and 177 ± 18% (S.E.) (n = 5) by dialysis at acid and neutral pH, respectively. The increases in renin activity are identical for the high molecular weight and low molecular weight forms by acid as well as neutral dialysis ($0.40 < P < 0.35$).

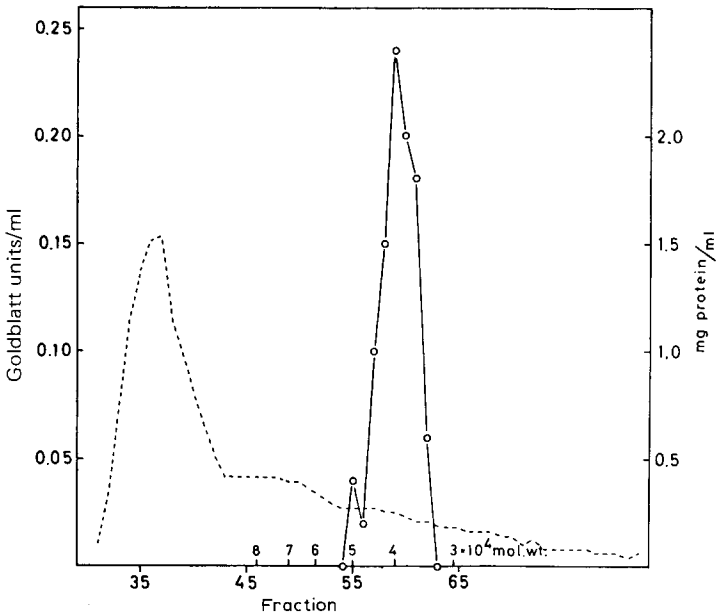

Fig. 3. Sephadex G-100 chromatography of rat kidney renin prepared according to Method 3. ------, protein mg/ml; o———o, renin activity. The load was 60 mg protein (25 Goldblatt units) in 2 ml. The elution buffer was 10 mM sodiumpyrophosphate/HCl/0.1 M NaCl, pH 6.5.

In two series of experiments the effect of acid pH on crude extracts was studied. Incubation of the extract (prepared by Method 3) at pH 3.4—7.4 (Method B) caused a decrease in renin activity. Dialysis of an extract (Method C) at pH 3 caused no increase in activity when compared to the starting material.

Cold storage

Cold storage of rat kidney extract made according to Method 2 showed significant increases of enzymatic activity in steps I, II, IV and V, but not in step III (Table I). 8 series of experiments were performed and renin activity was estimated every second or third day. In step I subcellular particles are still intact and renin activity increased to a mean value of 125% of initial activity (100%), in step II after freezing and thawing renin activity increased to a mean value of 122%. In step III after addition of enzyme inhibitors renin activity did not increase significantly. In step IV and V after dialysis against buffers of low ionic strength renin activity increased to about 120%. The increased renin activity obtained in the first week was stable during the rest of the observation period. Sephadex G-100 chromatography of step V showed two peaks and a shoulder, molecular weight 37 000, 42 000 and 52 000, respectively (Fig. 2).

To investigate whether the enhancement of renin activity was due to a process which was accellerated at 37°C 2 experiments were performed. Steps I—V were incubated at this temperature for 2.5 h in one experiment, and for 12 h in another experiment, at pH 6.50, 6.50, 6.75, 7.00 and 7.00, respec-

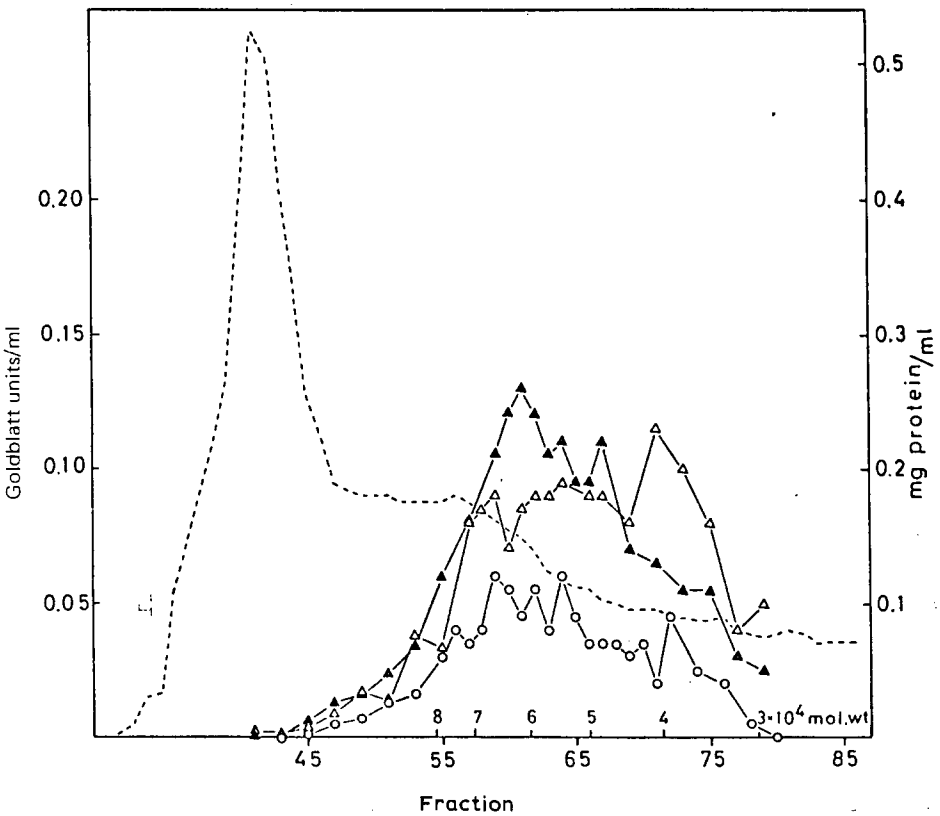

Fig. 4. Sephadex G-100 chromatography of rat kidney renin prepared according to Method 1 and acid/neutral dialysis of the fractions from the same column. ------, protein mg/ml; renin activity before (○———○) and after (▲———▲) dialysis of 0.5 ml samples from every second fraction at pH 3 and at 4°C, neutralized by dialysis for another 24 h at pH 7.1. △———△, controls dialyzed for 48 h at pH 7.1. The load was 70 mg protein (2 Goldblatt units) in 2 ml.

TABLE I

ACTIVATION OF RAT KIDNEY RENIN BY STORAGE AT 4°C.

The renin preparations were made according to Method 2 (see Materials and Methods section). The activity for each of the steps I—V was 100% on the day of preparation. Numbers in parentheses are number of experiments (χ).

Step no.	Activity of starting material (% ± S.E.M.)		
	1st week	2nd week	3rd and 4th week
I	125 ± 9 (13) **	123 ± 7 (16) *	121 ± 6 (19) *
II	122 ± 6 (13) *	121 ± 5 (16) *	119 ± 5 (17) *
III	124 ± 23 (13) ***	120 ± 14 (17) ***	117 ± 10 (17) ***
IV	121 ± 5 (13) *	118 ± 5 (17) *	118 ± 4 (19) *
V	120 ± 4 (13) *	120 ± 4 (17) *	120 ± 4 (19) *

* $P \leqslant 0.001$ or $0.01 < P < 0.005$.
** $0.025 < P < 0.020$.
*** The elevation in renin activity was not significant.

tively. Sample were withdrawn every half or second hour, respectively. The results showed a sustained or slightly decreased renin activity indicating that the 'activation' is not a simple chemical process.

Triton X-100 treatment

Extract (Method 3) was treated with the detergent Triton X-100, 0.1% (v/v) to investigate whether any membrane-bound renin activity could be released. Renin activity after treatment was 74 ± 16% (mean ± S.E., $n = 3$) as compared with the starting material (100%).

Discussion

By Sephadex G-100 chromatography renin activity eluted from the void volume to fractions corresponding to molecular weight 38 000. About 32% of the activity was recovered in 2—5 fractions which had a higher than average renin activity. These fractions corresponded to molecular weights 70 000, 60 000, 52 000, 44 000 and 38 000.

A multitude of renin forms have thus been demonstrated in rat kidney extracts when proteolytic enzyme inhibitors were present from the start of the homogenization procedure. The heterogeneity of renin seemed not to be due to non-specific interactions on the column as the protein markers eluted in single peaks. Since renin in extracts prepared without inhibitors eluted as a single peak at low molecular weight, interaction on the column specific for renin could not be responsible for the high molecular weight forms.

Renin activity in the high molecular weight range decreased when the enzyme inhibitors were added at a later step in the preparation and disappeared completely when the inhibitors were omitted. By rechromatography renin activity in the high molecular weight form shifted to low molecular weight. After a second rechromatography all renin was converted to a low molecular weight form

Inagami et al. [23] emphasized that the presence of MalNEt in the homogenization medium was critical for the preservation of high molecular weight forms of renin in the rat kidney preparation. In our case MalNEt did not exert any such influence, and we have thus no explanation for this discrepancy. Further, Inagami et al. [23] found at most only 2 peaks while we found 3—5 peaks in the rat kidney preparation. This difference is probably due to different working conditions: our preparation is frozen/thawn 5 times prior to a mild centrifugation, our preparation is not subjected to ultracentrifugation, we have used freshly prepared kidneys throughout our experiments while frozen kidneys have been the main source in Inagami's work.

The results show that homogenization and extraction leads to a gradual conversion of 'big' renin, which is only partially prevented by inhibitors. A complete inhibition of proteolytic activity could not be expected since inhibitors of acid proteases could not be included without abolishing renin activity itself. Therefore, the possibility that renin in vivo exists in forms with higher molecular weights than shown here can not be excluded. We suggest that the multiplicity of renin represents degraded products of high molecular weight renin, the many forms arising through consecutive splitting of peptide bonds by

one or several enzymes, the nature of which is unknown. The molecular weight(s) of these enzyme(s) must, however, be in the same range as renin, since the conversion takes place in the first and second rechromatography and when high molecular weight renin has been subjected to short time incubation. Degrading by autodigestion could also explain at least part of the conversion, namely the part not influenced by the addition of enzyme inhibitors.

A decrease in renin molecular weight was never accompanied by an increase in renin activity. After Sephadex G-100 chromatography, dialysis at acid and neutral pH in the cold room increased renin activity in all fractions, contradicting the prevalent view that low molecular weight forms of renin represent the fully active enzyme and again indicating that the activation is independent of renin molecular size. Cold storage alone also gave a slight (25%) but significant increase in renin activity (Table I). The increase took place during the first week, the overall increase showed a stable value which, however, the individual results fluctuated during the whole period. The half-time for loss of activity was a matter of months. This phenomenon has likewise been described for cathepsin B1 [24]. Since the increases in renin activity observed after dialysis of the fractions from Sephadex G-100 chromatography were about 70% we conclude that this increase must be due to dialysis itself and not to cold storage.

Taken together, the results show that the increase in renin activity can be related to factors, which are stable to freezing/thawing, are non-difusable and active also when the major part of renin is present as low molecular weight forms. The activation can not be accellerated by increase of temperature. The activation may be inhibited by protease inhibitors and this possible inhibition remains to be investigated.

In our hands renin activity did not increase in crude homogenates subjected to direct acidification, acidification by dialysis or Triton X-100 treatment in the initial step of our preparation.

Day and Leutscher [12,25] reported that high molecular weight renin in kidneys from patients with Wilms tumour, human plasma and human amniotic fluid, could be activated by acid without any accompanying change in molecular weight and Levine et al. [26] have reported that acid-treatment of high molecular weight renin from hog kidney led to a decrease in molecular weight to 40 000 with little (10%) if any concomitant reduction in renin activity. These results together with those presented in the present paper strongly argue against a direct connection between activation of renin and reduction of renin molecular weight.

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