A release of renin from kidney cortex slices in vitro was first reported by the present authors (1). Recently, the renin release from kidney slices of rats, pretreated with deoxycorticosterone acetate, clipping of renal artery, bleeding or decapitation, was shown by Jong (2) and Bočović et al. (3). In their experiments, the fundamental process of renin release has not been made clear. In our previous paper, it was pointed out that renin is released from dog kidney slices into an incubation medium depending on the temperature and pH of the medium and about 10% of renin contained in the original slices is released within 60 minutes of incubation.

Presently, the effects of metal ions in the incubation medium and atmosphere on renin release were studied. From the present findings, it is assumed that renin is released, partially included in the renin granule, into the incubation medium. Further study was made on the release of several enzymes found in other cell particles to compare the releasing pattern of renin from slices.

MATERIALS AND METHODS

Preparation of the kidney cortex slices

Mongrel dogs weighing 10 to 20 kg were used. Their kidneys were removed under pentobarbital anesthesia and immediately washed by gentle infusion of cold Krebs' bicarbonate solution through the renal artery until the solution coming out from the renal vein became colourless. After removal of the capsule, the kidney was cut longitudinally through the hilus, and cortex slices, 0.4 mm thick, were prepared with a razor blade under low temperature.

Preparation of renin substrate

Male mongrel dogs weighing 10 to 20 kg, were anesthetized with pentobarbital. Following heparinization (100 U/kg, i.v.) both kidneys were removed to reduce circulating renin (4) and to increase renin substrate in plasma (5, 6). About 4 hours later the blood was slowly drawn from the carotid artery. The plasma was added by solid ammonium sulfate at a final concentration of 30% saturation and then pH was adjusted

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An outline of the present experiments was reported at the seminar on "control of renin secretion", sponsored by Japan-U.S. Cooperative Science Program in Kyoto, October, 1969.
to 4.0 with HCl. After removing the precipitate by centrifugation, the supernatant was treated by the addition of solid ammonium sulfate at a final concentration of 50% saturation. The precipitate was dissolved in distilled water and the solution was dialyzed against distilled water until sulfate-free, then against 0.1 M EDTA-Na₂ solution to inhibit angiotensinase activity, and further against 0.005 M phosphate buffer (pH 7.4). The dialysis sac contents were centrifuged, and a total volume of the supernatant was adjusted to one tenth of the original plasma volume with 0.005 M phosphate buffer (pH 7.4). This solution was added by sodium chloride at a final concentration of 0.9%, and used as substrate solution (Fig. 1). Neither renin activity nor angiotensinase activity was observed in the substrate solution. More than 400 μg of angiotensin was liberated from 1 ml of the substrate solution when incubated with excess of dog renin.

<table>
<thead>
<tr>
<th>Dog plasma</th>
<th>Ammonium sulfate 30% saturation</th>
<th>adjusted pH to 4.0</th>
<th>centrifuged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>Ammonium sulfate 50% saturation</td>
<td>centrifuged</td>
<td></td>
</tr>
<tr>
<td>Precipitate</td>
<td>dissolved in distilled water</td>
<td>dialyzed against distilled water,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 M EDTA-Na₂ solution, then 0.005 M phosphate buffer (pH 7.4)</td>
<td>centrifuged</td>
<td></td>
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<tr>
<td>Clear sup</td>
<td></td>
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</tbody>
</table>

**Fig. 1. Preparation of renin substrate.**

**Incubation medium**

Krebs' bicarbonate solution (pH 7.4) was used as standard incubation medium, which contained (mM) NaCl 118, KCl 4.74, CaCl₂ 2.50, KH₂PO₄ 1.19, MgSO₄ 1.18, and NaHCO₃ 12.2. Variations of the components will be described under the chapter of results. When the components were changed, osmolarity was adjusted by addition of choline chloride.

**Incubation of kidney cortex slices**

Kidney cortex slices (200 or 300 mg) were incubated at 25°C in Krebs' bicarbonate solution (10 ml), containing 0.02% neomycin. Aliquots were taken out from the incubated medium at various intervals (3, 10, 60 and 120 minutes) and centrifuged at 2,000 rpm for 10 minutes to eliminate slices and cell debris. The supernatant was used for renin assay.

In some experiment, succinic dehydrogenase (succinic-DH), acid phosphatase (acid P-ase) and glucose-6-phosphatase (G6P-ase) released into an incubation medium were determined as marker enzymes of cell particles, i.e., succinic-DH for mitochondria, acid P-ase for lysosome and G6P-ase for microsome. Enzyme activities were comparatively studied in reference to released protein and renin activity. In these experiments, 20
ml of a standard medium containing 0.02% neomycin was prewarmed to 25°C and at zero time 4 g of slices was added. Incubation was carried out for 120 minutes. The supernatant was used for enzyme assay.

**Enzyme assays**

**Renin**

One tenth ml of the supernatant of the incubated medium was mixed with 1 ml of phosphate saline buffer (0.155 M, pH 7.4) and 0.1 ml of 0.1 M EDTA-Na₂ solution, and incubated at 37°C for 10 minutes to inhibit angiotensinase activity. Angiotensin formation was started by addition of 0.8 ml of the renin substrate. Incubation was continued at 37°C for 2 hours. Then the mixture was heated at 100°C for 10 minutes and centrifuged to remove the heat-denatured protein precipitate.

The pressor activity of the supernatant was measured on rat blood pressure in comparison with that of synthetic angiotensin II (Hypertensin, CIBA) and was expressed in terms of angiotensin equivalents (7). A pressor substance produced following incubation was confirmed to be angiotensin on the basis of: 1) thermostable and dialysable, 2) being destroyed by a treatment with trypsin, and 3) not influenced by pretreatment with either phenoxybenzamine or pyrethiazine in rats. Renin in the original slices was quantitatively measured following homogenization of slices.

**Succinic-DH**

Succinic-DH was determined by measuring a reduction of ferricyanide in the presence of succinate at 20°C, following the method of Slater and Bonner (8). The activity is expressed in arbitrary units as a decrease of absorbancy per milliliter of an incubated medium per hour.

**Acid P-ase and G6P-ase**

Three tenths ml of incubated medium separated from slices and cell debris were preincubated with 2.0 ml of 0.2 M tris-acetic acid buffer (pH 5.0 for acid P-ase, pH 6.5 for G6P-ase) and 0.2 ml of distilled water at 37°C for 10 minutes. The reaction was started with the addition of 0.5 ml of substrate solution (50 mM β-glycerophosphate for acid P-ase, 10 mM glucose-6-phosphate for G6P-ase), and carried out for 60 minutes at 37°C, and terminated by the addition of 0.75 ml of ice-cold 60% perchloric acid. After the precipitated protein was discarded by centrifugation, the released inorganic phosphate was determined. Simultaneously, the non-enzymatic inorganic phosphate in each sample and substrate solution was determined and subtracted from the value obtained by incubation. Acid P-ase and G6P-ase activities were expressed in μmoles of released inorganic phosphate per milliliter of an incubated medium per hour.

**Assay of inorganic phosphate**

The inorganic phosphate was determined by a modified method of Fiske and Subbarow (9). Half ml of the supernatant was mixed with 3.0 ml of distilled water, 0.75 ml of 60% perchloric acid and 0.5 ml of 4.5% ammonium molybdate, and then added with 0.25 ml of aminonaphtholsulfonate solution (Daiichi Pure Chemical Co. LTD, Tokyo, Japan) as reducing reagent. An optical density at 750 μm was read in the spec-
trophotometer. The produced molybdenum blue was stable for 8–20 minutes and its optical density was linear with the inorganic phosphate concentration in a range of 1–5 μg per ml. Accordingly, measuring the optical density was carried out exactly 15 minutes after the addition of the reducing reagent.

**Protein assay**

The protein content of the supernatnat was determined by the method of Lowry et al. (10), using crystalline bovine albumin as standard.

**RESULTS**

*Effects of atmosphere on renin release*

The previous report (1) showed that renin release from the kidney cortex slices depended upon the temperature and pH of an incubation medium and that about 10% of renin contained in the original slices was released into the medium.

A study was made of effects of atmosphere on renin release. The slices were incubated in the standard medium (pH 7.4) at 25°C under gentle bubbling with varying gas; air, oxygen containing 5% carbon dioxide or nitrogen containing 5% carbon dioxide. At intervals of 30 minutes the incubation medium was taken out to determine its renin content. No significant differences in renin release were observed between incubation of slices with various atmospheres for 30 or 60 minutes (Fig. 2).

*Effects of metal ions on renin release*

Since it has been reported by many workers that sodium depletion in animals and man produces a greater increase in renin secretion and higher response to stimuli than
in controls, the effects of sodium concentrations in incubation media on renin release were examined. Slices were incubated in Krebs' bicarbonate buffer solution containing low sodium (25 mM or 85 mM) and renin release was examined up to 120 minutes. For 30 minutes no significant differences were observed between low sodium and standard sodium (144 mM) media, while for 60 minutes renin release was slightly greater in a 25 mM sodium medium than in a 85 mM or 144 mM sodium medium.

Effects of calcium and magnesium on renin release were also examined. The renin release was smaller at 1.25 than 2.50 mM calcium, and further decrease was observed in a calcium-free medium with or without magnesium. However, in the presence of calcium (2.5 mM), a decrease of the concentration of magnesium from 1.18 to 0.59 mM or omission of the cation from the incubation medium did not result in significant changes in renin release (Fig. 3).

Effects of freezing and thawing
The standard incubated medium was centrifuged at 15,000 g for 30 minutes. The renin activity was consistently lower in the supernatant than in the uncentrifuged medium taken before centrifugation and 12.5-21.4% of renin activity was noted in the sediment. Therefore, the effects of freezing and thawing on renin activity in the incubated medium were examined. Aliquots were taken out of the incubated medium at various time intervals (30, 60 and 120 minutes), and centrifuged at 2,000 rpm for 10 minutes to remove slices and cell debris. Part of this supernatant was immediately frozen in dry-ice aceton and thawed. This procedure was repeated ten times. The renin activity of the treated supernatant was significantly higher than that of the nontreated supernatant (Fig. 4). These findings suggest that renin, included in cellular particles, may be partially released during incubation.
Fig. 4. Effects of freezing and thawing on renin activity in the incubated medium.

Fig. 5. Relationship between acid P-ase and G6P-ase activities and incubation time. A range 0.15-0.90 ml (-O-, 0.15 ml : -x-, 0.30 ml : -△-, 0.60 ml : -●-, 0.90 ml) of the kidney cortex homogenate (10%) was mixed with 6 ml of 0.2 M tris-acetic acid buffer (pH 5.0 for acid P-ase, pH 6.5 for G6P-ase), and a total volume of the mixture was adjusted to 7.5 ml with distilled water. The mixture was preincubated for 10 minutes at 37°C and the reaction was started with the addition of 1.5 ml of substrate solution (50 mM β-glycerophosphate for acid P-ase, 10 mM glucose-6-phosphate for G6P-ase). Enzyme activities were expressed as liberated Pi (μM/ml).

A release of various enzymes from the kidney cortex slices

A possibility that the renin granule is released into the medium from the cell led the present authors to test the release of several enzymes, found in other cell particles, into the incubation medium. The activity and stability of G6P-ase (a microsomal marker),
acid P-ase (a lysosomal marker) or succinic-DH (a mitochondrial marker) in the homogenate of kidney cortex were determined. Activities of G6P-ase and acid P-ase was linear relation with incubation time of 30–120 minutes and with enzyme concentrations in a range 0.15–0.90 ml of the homogenate (Fig. 5). When the homogenate was stored at 0°C, enzyme activities (G6P-ase and acid P-ase) remained unchanged until the following day. In assay of succinic-DH activity, a reduction of ferricyanide was also found to be linear with the amounts of the homogenate, but a marked decrease in enzyme activity was noted after the storage at 0°C over night.

The time course of activities of G6P-ase, acid P-ase and succinic-DH in the medium were studied in comparison with that of renin and the protein amounts in the medium were simultaneously determined. Table 1 shows the results obtained in eight experiments. All enzyme activities in the incubated medium were almost parallel in their increasing patterns with incubation time. The specific activities of these enzymes, however, tended to decrease up to 30 minutes after incubation and showed no significant changes thereafter, revealing a similar release pattern (Fig. 6).

**TABLE 1. Release of renin, G6P-ase, acid P-ase and succinic-DH from dog kidney cortex slices.**

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Incubation time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Renin*</td>
<td>1.32±0.0642</td>
</tr>
<tr>
<td>G6P-ase**</td>
<td>0.800±0.0623</td>
</tr>
<tr>
<td>Acid P-ase**</td>
<td>0.589±0.0530</td>
</tr>
<tr>
<td>Succinic-DH***</td>
<td>0.398±0.0523</td>
</tr>
<tr>
<td>Protein****</td>
<td>1.03±0.0811</td>
</tr>
</tbody>
</table>

Figures are given as mean±S.E.
* : Angiotensin equivalents µg/ml of incubated medium/hr.
** : Pi µM/ml of incubated medium/hr.
*** : µA/ml of incubated medium/hr.
**** : mg/ml of incubated medium/hr.
DISCUSSION

Although a physiological regulation of renin secretion from the kidney has been discussed by a great number of investigators, the basic mechanism of renin secretion remains unclarified. It seems that an in vitro experiment would be useful in elucidating the nature of storage and secretion of renin in vivo. Robertson et al. (11, 12) and Sz. Szalay et al. (13) demonstrated a production and release of renin in an organ culture of the mammalian kidney and Michelakis (14) reported that renin was produced in a dog renal cell suspension. Recently, Božović et al. (3) presented a hypothesis of the active mechanism about renin release by finding that renin release from the rat kidney cortex was glucose-dependent in some experimental conditions. However, there is a possibility that incubation with metabolic substrate results in the production of renin during incubation, so that it does not seem reasonable to quantify a net renin release.

In the present experiments, an analysis of release was done without addition of an exogenous metabolic substrate. When slices were incubated with Krebs' bicarbonate buffer (pH 7.4), renin was gradually released at 25°C. During incubation in an atmosphere of oxygen, air or nitrogen, no significant differences were observed in renin release. Sodium balance is an important factor for a control of renin secretion in vivo; sodium depletion, caused by low sodium diets or by natriuretic agents, increases plasma renin levels in dogs and man. In the present experiments, when the slices were incubated in a medium with low sodium (25 mm), there was a slight increase in renin release. Conversely, reduction of calcium content in the incubation medium markedly decreased a release of renin into the medium but not in the case of magnesium. Evidence that calcium ion is essential for a release of the contents of secretory granules has been observed in many tissues, such as the submaxillary gland (15), neurohypophysis (16) and pancreas (17). Accordingly, it is assumed that calcium may play a role in mediating a renin release in vitro as well as other secretory tissues.

As pointed out in the previous paper (1), a significant higher activity of renin was obtained during incubation with an acidic medium than with the standard medium (pH 7.4), suggesting a similarity between renin granule and lysosomal granule which is most labile at pH 4–5 (18). The work of Cook and Pickering (19) suggested that the renin of rabbit kidney cortex may be present in lysosomes, or some very similar particles. Fisher (20) has indicated that juxtaglomerular granules are identified as lysosome on a combined histochemical and electronmicroscopic technique. Ogino et al. (21) have suggested that renin may be located in the lysosome on the basis of a marked pressor response to the kidney lysosomal fraction. In the present experiments, renin activity was observed in the sediment obtained by centrifuging (15,000 g, 30 minutes) the incubated medium with slices. This suggests that renin-containing granules, at least in part, might be released into the medium during incubation. Furthermore, this possibility would be supported by evidence that the renin activity in the incubated medium was augmented by repeated freezing and thawing. Since renin appears to be in a granule of the lyso-
somal fraction, the activity of acid P-ase, a marker enzyme of lysosome, in the incubated medium was comparatively measured, showing no difference in releasing patterns between two enzymes. On the other hand, a similar pattern was also observed in a release of marker enzymes (G6P-ase and succinic-DH) of other cell particles. Thus it is unlikely that, of these enzymes tested, renin is released through a specific process under this experimental condition and rather a common process as a leakage phenomenon is probably implicated to such release. Two different processes may be involved about the renin release into the incubation medium; 1) release of intact renin granule into the medium, and subsequent release of renin from the granule, 2) intracellular release of renin from the granule in the cytoplasma, followed by a leakage through the cell membrane. Further study is in progress to elucidate whether an active mechanism participates in renin release in vitro.

**SUMMARY**

1. When dog kidney cortex slices were incubated with Krebs' bicarbonate buffer, renin was released into the medium and this release was not influenced under either aerobic or anaerobic condition.

2. An intense lowering of sodium concentration in the medium resulted in a slight increase in renin release. When calcium was omitted from the medium, renin release was markedly suppressed.

3. The renin activity of the incubated medium was augmented by repeated freezing and thawing. After centrifuging the incubated medium for 30 minutes at 15,000 g, a large amount of released renin activity was found in the supernatant, and a small amount in sediments.

4. Specific activities of succinic-DH, acid P-ase and G6P-ase in the incubated medium were comparatively studied in reference to released renin activity, revealing a similar release pattern.

5. A possible mechanism of renin release is discussed.

**Acknowledgement:** This work was supported by a grant from Japan Ministry of Education.

**REFERENCES**

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