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Isolation of Membrane-Bound Renal Enzymes that Metabolize Kinins and Angiotensins

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Cortex of rat kidney was homogenized and fractions enriched in plasma membrane, endoplasmic reticulum or brush border were prepared by several techniques of differential centrifugation. The identity and homogeneity of the membrane fragments were investigated by assaying marker enzymes and by transmission and scanning electron microscopy. Kallikrein was present in both plasma-membrane- and endoplasmic-reticulum-enriched fractions isolated by two fractionation procedures. Kallikrein was highly concentrated in a plasma-membrane fraction but was absent from the brush-border membrane of proximal tubular cells. Cells of transplanted renal tumours of the rat, originating from the proximal tubule, had no kallikrein activity. Kininase activity, angiotensin I-converting enzyme (kininase II) and angiotensinase were found in a plasma-membrane-enriched fraction and especially in the fraction containing isolated brush border. It is suggested that after renal kallikrein is synthesized on endoplasmic reticulum, it is subsequently reoriented to a surface membrane for activation and release. Renal kallikrein may enter the tubular filtrate distal to the proximal tubules. The brush-border membrane of proximal tubule is the major site of inactivation of kinins and angiotensin II.

It has been known for over half a century that urine contains kallikrein (EC 3.4.21.8; Frey *et al.*, 1968). Hypertensive patients excrete less of this hypotensive enzyme than normotensive subjects (Elliot & Nuzum, 1934; Margolius *et al.*, 1974). Although urinary kallikrein is believed to originate from the kidney (Frey *et al.*, 1968), some of the properties of urinary and renal kallikrein differ. For example, there appears to be more activity in the urine of the rat than can be expected from the activity found in the kidney. Carvalho & Diniz (1966) suggested that kallikrein is present in the lysosomal fraction of homogenized kidney, but Nustad (1970) found it in a microsomal fraction. In addition to kallikrein, the kininogenase that releases kinins, urine also contains the vasoactive peptides bradykinin, kallidin and Met-Lys-bradykinin (Werle & Erdös, 1954; Miwa *et al.*, 1968, 1969).

Kinins are natriuretic, diuretic, hypotensive and they release prostaglandins from the medulla of the kidney (Webster & Gilmore, 1964; Barraclough & Mills, 1965; McGiff *et al.*, 1976). These properties indicate that they may have important effects on some aspects of renal function. The kidney is very rich in kininases that terminate the action of kinins. Most of the kininase activity sediments with the microsomal fraction. Erdös & Yang (1966) described three renal kininases. One of these is similar to kininase I or carboxypeptidase N (EC 3.4.12.7) of plasma (Erdös & Sloane, 1962). A second and probably the most important one is kininase II (EC 3.4.15.1), which is identical with the angiotensin I-converting enzyme (Erdös & Yang, 1967; Yang *et al.*, 1970, 1971; Oshima *et al.*, 1974; Erdös, 1976). The ratio of activity of kininase I/kininase II is about 1:4 in the renal cortex (Erdös & Yang, 1966; Ward & Mills, 1975).

Because of the importance of angiotensin and bradykinin in renal function, we studied the cellular and subcellular localization of enzymes that metabolize them. We isolated fractions highly enriched in plasma membrane, endoplasmic reticulum or in brush border of renal proximal tubules. The identity of the fractions was confirmed by both transmission and scanning electron microscopy and by assaying marker enzymes. We found that the brush-border membrane of the proximal tubules contains kininase, angiotensin I-converting enzyme and angiotensinase, but virtually no kallikrein. Kallikrein is concentrated in a plasma-membrane-enriched and also in an endoplasmic-reticulum-enriched fraction. Transplanted renal tumours, which originated from the cells of the pars recta of proximal tubules, contained no

kallikrein activity. Some of these experiments were briefly summarized in an abstract (Ward *et al.*, 1975*a*) and in a short communication (Ward *et al.*, 1975*b*).

Experimental

Methods

Animals. Unanaesthetized male Sprague–Dawley rats (250–350g) were killed by decapitation or cervical dislocation. The aorta was clamped below the renal arteries, the vena cava opened, and the kidneys were perfused through the aorta with a solution of 0.25M-sucrose and 5mM-$NaHCO_3$, pH 7.5, or 0.9% NaCl until they were free of blood. In control studies it was ascertained that starvation for 24h before death made no difference to the results. All subsequent procedures were carried out at 4°C. For each experiment, about 10g of renal cortex dissected from three to six rats was pooled and minced.

Membrane fractionation I. Renal tissue was suspended in 9 vol. of buffer containing 0.25M-sucrose, 0.2mM-$MgSO_4$ and 10mM-Tris/HCl (pH7.4). The tissue was disrupted gently with a very loosely fitting Dounce homogenizer. The cells were completely disrupted by N_2 cavitation (Dowben *et al.*, 1968) after equilibration with N_2 at 5.6MPa (800lb/in^2) for 20min in an Artisan pressure homogenizer. EDTA was added to 1mM concentration and the homogenate centrifuged at 10000*g* for 15min. The supernatant fraction was then centrifuged at 30000*g* for 15min. The pellets, containing nuclei, mitochondria, lysosomes and cell debris, were discarded. Microsomal particles in the supernatant fraction were harvested by sedimentation at 100000*g* for 60 min in a Sorvall OTD-2 ultracentrifuge. The microsomal pellet was suspended first in 10mM-Tris (pH 8.6), sedimented at the above speed, resuspended in 1mM-Tris (pH8.6) and sedimented again. Finally, the microsomal pellet was suspended in about 30ml of 1mM-$MgSO_4$ plus 1mM-Tris (pH8.6) and dialysed against the same buffer for 2h.

Highly enriched fractions of plasma membranes and endoplasmic reticulum were obtained by a modification (Birckbichler *et al.*, 1973) of the method of Wallach & Kamat (1966). The microsomal suspension was carefully layered on 20ml of 15% (w/w) Dextran 110 in 1mM-$MgSO_4$ and 1mM-Tris/HCl (pH8.6), $\rho = 1.06$g/cm^3. The discontinuous gradients thus formed were centrifuged in a Sorvall SW-27 swinging-bucket rotor at 27000rev./min ($r = 11.5$ cm) for 15h. The plasma-membrane-rich fraction was concentrated at the interface and the endoplasmic-reticulum-rich fraction was pelleted. Both fractions were collected, washed once in 10mM-$MgSO_4$ and 10mM-Tris (pH8.6), and then suspended in 5mM-Tris (pH8.6).

Membrane fractionation II. Washed microsomal fractions from renal cortex were prepared and separated into endoplasmic-reticulum and plasma-membrane components by a procedure developed specifically for rat kidney cortex (Jakobsson, 1974). Renal cortices were homogenized in 20% (w/v) of 3mM-Tris (pH7.5) with six to eight strokes of a glass–Teflon homogenizer (Thomas, size B) at 700rev./min. The sucrose concentration was then adjusted to 0.25M and the homogenate was centrifuged at 10000*g* for 20min to remove cell debris, nuclei, mitochondria and lysosomes. Approximately 8ml of the supernatant was layered on a 3.5ml cushion of 1.6M-sucrose and centrifuged at 105000*g* for 30min with a SW-27.1 rotor in a Beckman L5-65 ultracentrifuge. The microsomal fraction at the cushion interface was removed, combined (approx. 9ml) and diluted to approx. 15ml.

Continuous linear gradients were prepared with Ficoll increasing in concentration from 0 to 8% (w/v) in 10% (w/v) sucrose. Ficoll was previously purified by dialysis against doubly distilled water for 48h at 4°C. Approximately 6ml of the concentrated microsomal fraction was then applied to the top of each of two 25ml gradients and centrifuged in a SW-25.1 swinging-bucket rotor at 50000*g* for 45–60 min. After centrifugation, 2ml fractions were pipetted sequentially from the top of each gradient, diluted with 20ml of 10mM-Tris (pH8.6) and centrifuged at 100000*g* for 45min. The pellets were suspended in 1mM-Tris (pH8.6), centrifuged for 45min and resuspended in appropriate buffer. All samples were assayed within 48h.

Brush-border preparation. Large fragments of brush-border membranes from the proximal convoluted tubular cells were prepared by the method of Wilfong & Neville (1970).

Minced cortex was homogenized in 100ml of 20mM-$NaHCO_3$ (pH8.1) with a hand-held loose glass–Teflon homogenizer (six strokes). A portion (220ml) of 10mM-$NaHCO_3$ (pH8.1) was added and the homogenate was stirred for 5min. The homogenate was poured through coarse and subsequently fine nylon screen (approx. 150μm openings). The filtrate was centrifuged at 500*g* for 20min and the pellet collected. This procedure was repeated a second time. The pellets were resuspended in approx. 28ml of 10mM-$NaHCO_3$ (pH8.1) and 60ml of 60% (w/w) sucrose was added. The final concentration of sucrose was adjusted to 40.9±0.1% (w/w) and 1ml of 40.4±0.1% sucrose was layered over the solution. After centrifugation at 90000*g* for 75min, the brown-coloured floating layer was removed and pelleted in 4mM-$NaHCO_3$ (pH8.1)/1mM-$MgCl_2$ at 3000*g* for 10min.

A linear gradient of 3–21% (w/w) sucrose in 4mM-$NaHCO_3$ (pH8.1)/1mM-$MgCl_2$ was formed over a 1.5ml cushion of 41±0.1% sucrose. The resuspended

pellet was layered on the gradient and centrifuged at 2500*g* for 15min. The brush-border fraction, which collected at the gradient–cushion interface, was diluted to 10ml in 4mM-$NaHCO_3$ (pH8.1)/1mM-$MgCl_2$ and pelleted at 20000*g* for 20min. This brush-border fraction was then assayed and embedded for transmission and scanning electron microscopy.

Tumours. Frozen Morris renal tumours MK2 and MK3, together with host animal kidneys and control kidneys, were obtained from Dr. George Weber of the Department of Pharmacology, Indiana University School of Medicine, IN, U.S.A. Transplanted Morris tumours of rat adenocarcinomas are probably derived from proximal renal tubules of the rat (Hruban *et al.*, 1973). Tumours, host kidneys and normal kidneys were thawed, weighed and suspended in 10% (w/v) 20mM-Tris (pH7.5) with an all-glass Potter–Elvehjem homogenizer or a Polytron homogenizer. The homogenate was solubilized with Triton X-100 at a final concentration of 0.1%. After 15min at 4°C and centrifugation at 10000*g* for 5min, the supernatant was dialysed against the same buffer for approx. 2h.

Enzyme assays. Renal kallikrein was assayed by incubating fractions (50–100μl) of homogenized renal tissue with kininogen substrate (100μl) in 0.1M-Tris, pH8.5 (500μl), in the presence of the kininase inhibitors, nonapeptide SQ 20881 (100μl, 1mM), EDTA (100μl, 50mM) and *o*-phenanthroline (100μl, 10mM) at 37°C (Erdös, 1971; Yang *et al.*, 1971). The amount of kinin generated was determined on an isolated rat uterus (1 unit of kallikrein activity is the amount of enzyme that forms the equivalent of 1ng of bradykinin in 1min). Incubation of synthetic bradykinin with renal extracts showed that kininases were completely inhibited. Kininogen was prepared by heating dog plasma at 61°C for 30min. The kininogen preparation had no residual kallikrein or kininase activity when incubated at 37°C. In inhibitor studies, aprotinin (12μg/ml) and soya-bean trypsin inhibitor (100μg/ml) were preincubated for 15min at 37°C before the kininogen substrate was added. In control experiments, we ascertained that the peptide released from dog plasma substrate was not angiotensin. By using specific inhibitors of kallikrein and differential bioassay on the isolated rat uterus and duodenum, we confirmed that all the material released from plasma that contracted the rat uterus was a kinin. The esterolytic activity of kallikrein was assayed with α-*N*-tosyl-L-arginine[^{3}H]methyl-ester substrate (Beaven *et al.*, 1971).

Renal kininase and angiotensinase were assayed by incubating samples of renal homogenate (20μl), with bradykinin or angiotensin II (200ng in 200μl) in 0.1M-Tris, pH7.4, 0.2M-NaCl (200μl) at 37°C then following the inactivation of the peptide on the isolated rat uterus (1 unit of kininase or angiotensinase activity is the amount of enzyme that inactivates the equivalent of 1μg of bradykinin or angiotensin II in 1min under the described conditions).

Renal angiotensin I-converting enzyme (kininase II) was assayed by incubating renal fractions (50μl), with 1mM-hippurylglycylglycine in 0.2M-Tris, pH7.4, containing 0.2M-NaCl at 37°C (Yang *et al.*, 1971). Converting enzyme activity was calculated as the amount of hippurylglycylglycine hydrolysed that could be inhibited by 0.1mM of the specific inhibitor, SQ 20881. The amount of diglycine released was assayed in a Beckman 121C amino acid analyser.

Membrane fractions were characterized by several marker enzymes. Alkaline phosphatase (EC 3.1.3.1) was assayed with *p*-nitrophenyl phosphate substrate (Linhardt & Walter, 1965). Glucose 6-phosphatase (EC 3.1.3.9) was assayed in the presence of 4mM-EDTA and 2mM-KF (Harper, 1965; Hübscher & West, 1965). $Na^+ + K^+$-activated ATPase (adenosine triphosphatase, EC 3.6.1.3) was determined by the method of Post & Sen (1967). P_i released was determined by the method of Fiske & SubbaRow (1925). Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Electron microscopy. For transmission microscopy, membrane pellets were fixed on coverslips with 3% (w/v) glutaraldehyde in 0.2M-sodium cacodylate buffer (pH7.4). The specimens were fixed overnight at 2°C, then washed and fixed secondarily with 1% (w/v) OsO_4 in a 200mM-*sym*-collidine buffer (pH 7.4) for 2h. The samples were rinsed then dehydrated stepwise in 70, 95 and 100% ethanol. The pellets were treated with propylene oxide and infiltrated overnight with Epon 812/Araldite 502, then embedded in the Epon/Araldite mixture. This was followed by polymerization at 60°C, then the coverslip was removed and the block sectioned in a Sorvall MT-2 ultramicrotome. Sections were stained with uranyl acetate and lead citrate and then examined with a JOEL 100C electron microscope.

For scanning microscopy, membrane sediment was spread thinly over a coverslip and quickly fixed with 3% (v/v) glutaraldehyde. In some cases the specimen was fixed secondarily with 1% OsO_4. This was followed by washing, dehydration and immersion in amyl acetate or acetone. They were then critical-point dried in liquid CO_2 to replace the liquid solvent. The coverslips were dried and mounted, then coated with a thin layer of palladium and gold and examined with a JOEL JSM-35 scanning electron microscope.

Materials

Dextran 110 and Ficoll (mol.wt. approx. 400000) were purchased from Pharmacia (Piscataway, NJ, U.S.A.) and α-*N*-tosyl-L-arginine [^{3}H]methyl ester from Biochemical and Nuclear Corp. (Burbank, CA

U.S.A.). Bradykinin, hippurylglycylglycine and sucrose (ultrapure) were from Schwarz–Mann (Orangeburg, NY, U.S.A.). The kallikrein inhibitor aprotinin (Trasylol) was obtained from Bayer AG, Professor G. Haberland (Wuppertal-Elberfeld), and soya-bean trypsin inhibitor from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). The synthetic converting-enzyme inhibitor SQ 20881 was obtained from Dr. Z. Horovitz of Squibb Inc. (Princeton, NJ, U.S.A.). All other reagents used were of analytical grade.

Results

Microsomal membranes

By using procedure I we found that the relative specific activities of marker enzymes of both endoplasmic reticulum and plasma membranes were elevated in the washed microsomal fraction (Table 1). The recovery and relative specific activity of kallikrein in the washed microsomal fraction was also high and similar to that reported by Nustad (1970). The extensive washing, which removes both adsorbed and intraluminal cytoplasmic contamination, removed approx. 60% of the protein of the microsomal pellet but none of the membrane markers or membrane kallikrein.

Fractions rich in either plasma membranes or in endoplasmic reticulum were prepared from washed microsomal fractions of 21 rats in six experiments, by using sedimentation in a discontinuous gradient of Dextran 110. The plasma-membrane enriched fraction, collected at the gradient interface of the Dextran 110, showed low glucose 6-phosphatase activity and high alkaline phosphatase and $Na^+ + K^+$-activated ATPase activities. The endoplasmic-reticulum-enriched fraction that pelleted had high glucose 6-phosphatase activity but lower alkaline phosphatase and $Na^+ + K^+$-activated ATPase activities (Table 1). The specific activity of glucose 6-phosphatase in the endoplasmic-reticulum-enriched fraction was nearly 8 times that of the plasma-membrane-enriched fraction, whereas the specific activities of alkaline phosphatase and $Na^+ + K^+$-activated ATPase were one-half and one-third of those of the plasma-membrane-enriched fraction.

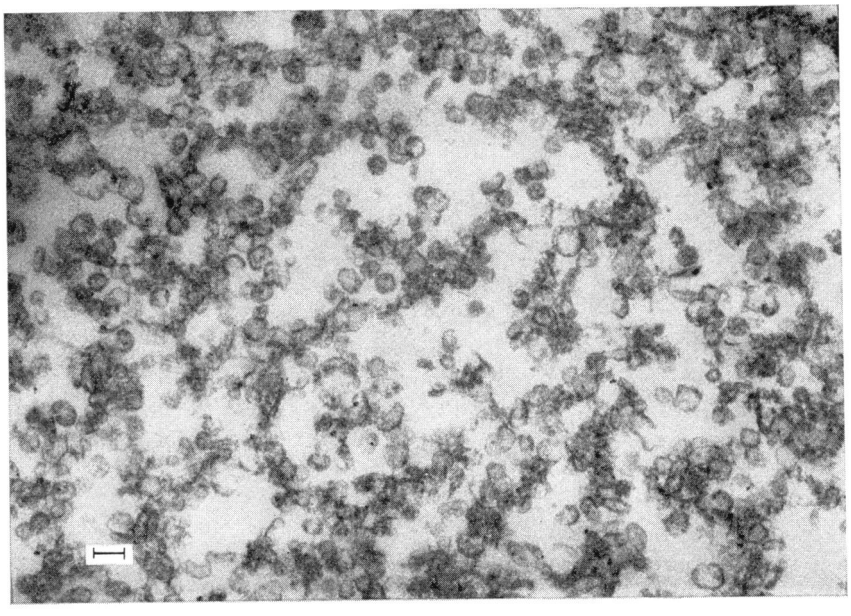
Each fraction contained approx. 10% of the total kallikrein activity of the crude homogenate. The specific activity of the plasma-membrane-enriched fraction in bioassay was 5 times that of the endoplasmic-reticulum-enriched fraction and 28 times that of the crude homogenate (Table 1). Preliminary experiments had shown that α-*N*-tosyl-L-arginine[^{3}H]-methyl esterase activity in the homogenate was not entirely due to kallikrein. However, similarly to the bioassay of kallikrein, the specific activity of the esterase in the plasma-membrane-enriched fraction

Table 1. *Distribution of enzyme activity in membrane fractions isolated from rat kidney cortex*

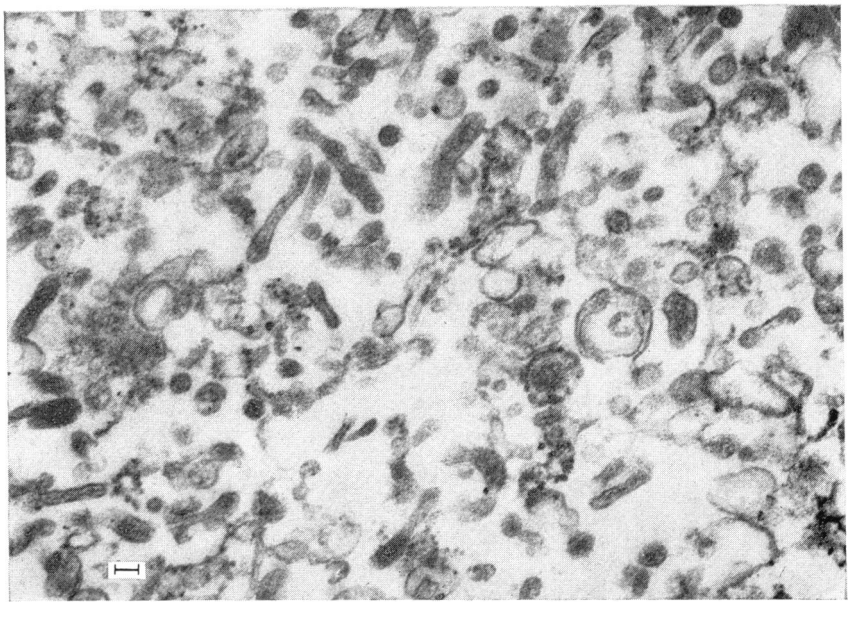
The preparation of membrane fractions and the assay of enzymes were as described under 'Methods'. All values are means ± S.E.M. Relative specific activity is (mean specific activity in the fraction)/(mean specific activity in the homogenate).

	Kallikrein		α-*N*-Tosyl-L-arginine [^{3}H]methyl ester hydrolysis		Glucose 6-phosphatase activity		Alkaline phosphatase		$Na^+ + K^+$-activated ATPase	
	Sp. activity (ng of bradykinin equiv. released/min per mg of protein)	Relative sp. activity	Sp. activity [amount of the compound (as c.p.m.) hydrolysed/min per mg of protein]	Relative sp. activity	Sp. activity (μmol of substrate hydrolysed/min per mg of protein)	Relative sp. activity	Sp. activity (μmol of substrate hydrolysed/min per mg of protein)	Relative sp. activity	Sp. activity (μmol of substrate hydrolysed/min per mg of protein)	Relative sp. activity
Homogenate	1.0±0.2	1			0.05±0.001	1	0.10±0.004	1		
Washed microsomal fraction	9.5±2.1	9.5	1290±210	1	0.33±0.03	6.5	0.52±0.11	5.3	0.14±0.12	1
Plasma-membrane fraction	28 ±5.1	28	3100±350	2.4	0.06±0.01	1.1	0.64±0.13	6.5	0.38±0.14	2.7
Endoplasmic-reticulum fraction	5.6±1.1	5.6	520±130	0.4	0.43±0.09	8.5	0.35±0.09	3.6	0.12±0.05	0.8
No. of experiments	4		2		5		3		3	

(*a*)



(*b*)

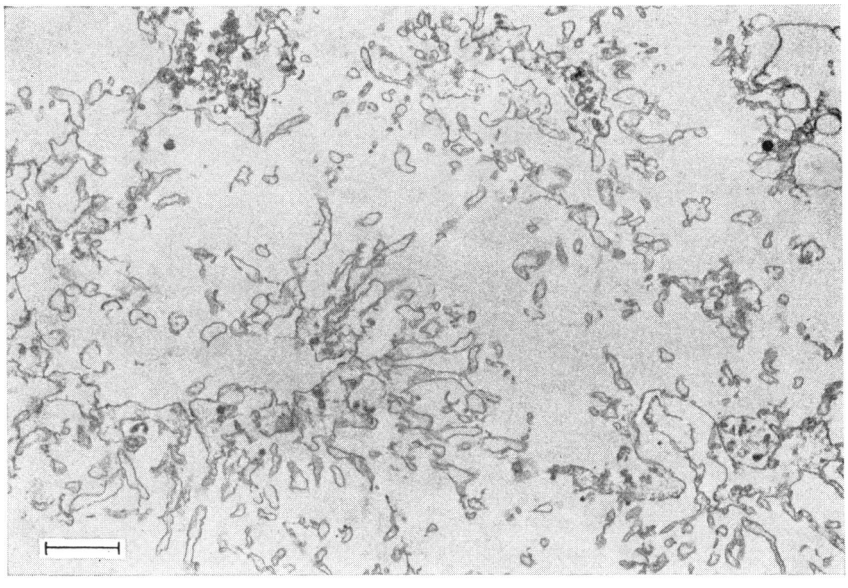


EXPLANATION OF PLATE 1

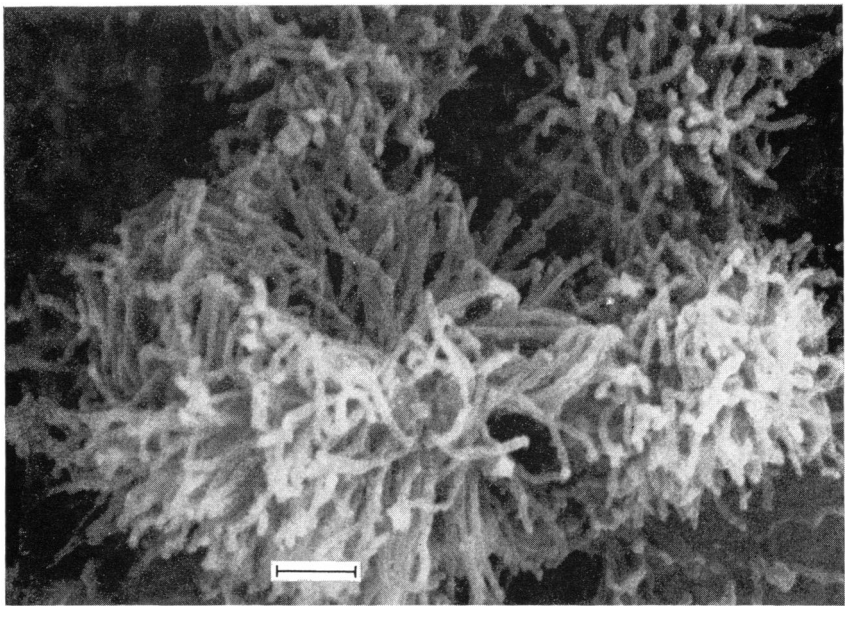
Transmission electron micrographs of subfractions of renal cortex after rate-differential centrifugation in a sucrose/Ficoll gradient

See under 'Methods' and Fig. 1. Subfractions 1–4 (*a*), from top of gradient, subfractions 9–12 (*b*) from bottom of gradient. Scale line represents 0.1 μm.

(*a*)



(*b*)



EXPLANATION OF PLATE 2

Transmission (*a*) *and scanning* (*b*) *electron micrographs of brush-border fractions prepared as described under* '*Methods*'

Scale line represents 1 μm.

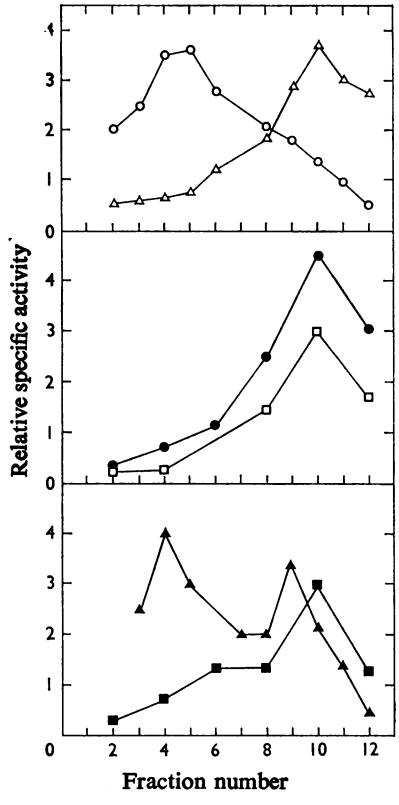


Fig. 1. *Distribution of glucose 6-phosphatase* (○), *alkaline phosphatase* (△), *kininase* (●), *angiotensin I-converting enzyme* (□), *kallikrein* (▲), *and angiotensinase* (■) *after rate-differential centrifugation of the microsomal fraction of homogenized cortex of the rat kidney in a sucrose/Ficoll gradient*

Relative specific activity is (mean specific activity of fraction)/(mean specific activity of microsomal fraction).

was approx. 3 times that of the washed microsomal fraction and 6 times that of the endoplasmic-reticulum-enriched fraction.

The kininase activities in the plasma-membrane-enriched and endoplasmic-reticulum-enriched fractions were similar. However, EDTA, a potent kininase inhibitor, was used in the preparation of these membranes and even transient exposure to this inhibitor may affect the apparent distribution and activity of renal kininase.

This procedure demonstrated that renal microsomal kallikrein was more active in the plasma-membrane-enriched fraction than in the endoplasmic-reticulum-enriched fraction. Renal cortical tissue has a variety of cell types and plasma membranes of individual renal cell types are themselves heterogeneous (Bloom & Fawcett, 1968). Therefore we isolated endoplasmic-reticulum- and plasma-membrane-enriched fractions of renal cortex by a second procedure as well.

Microsomal fractions were prepared seven times from pooled rat renal cortices by procedure II. Microsomal membranes were subfractionated into plasma-membrane- and endoplasmic-reticulum-enriched fractions by rate-differential centrifugation in a linear continuous sucrose/Ficoll gradient as described under 'Methods'. Fig. 1 shows the relative specific distribution of the endoplasmic-reticulum and the plasma-membrane marker enzymes glucose 6-phosphatase and alkaline phosphatase and of kallikrein, total kininase, angiotensin I-converting enzyme (kininase II) and angiotensinase. The distribution of the marker enzymes glucose 6-phosphatase (endoplasmic reticulum) and alkaline phosphatase (plasma membranes) demonstrates the separation of endoplasmic reticulum at the top of the gradient from plasma membranes containing brush border at the bottom. After pelleting, the structure of the membranes collected at the top and bottom of the gradient was examined by transmission electron microscopy. The top fractions contained both smooth and rough endoplasmic reticulum vesicles (Plate 1*a*). The bottom of the gradient was enriched in plasma membranes and contained brush-border membrane fragments. This separation of endoplasmic reticulum from plasma membranes is similar to that reported by Jakobsson (1974). Although other plasma-membrane marker enzymes were not determined in the present study, the distribution of all plasma-membrane-bound enzymes paralleled that of alkaline phosphatase as described by Jakobsson (1974).

Fig. 1 shows that the relative specific distribution of renal microsomal kallikrein can be resolved into two separate peaks of material paralleling both endoplasmic-reticulum and plasma-membrane fractions. Renal kininase, angiotensin I-converting enzyme (or kininase II) and angiotensinase (Fig. 1) were concentrated in the fractions that contained the plasma membrane. The relative specific distribution of these three enzymes was nearly identical with that of the marker enzyme alkaline phosphatase. Unlike procedure I, no kininase, angiotensin I-converting enzyme or angiotensinase inhibitors were used during the isolation of these membrane fractions.

Brush border

Brush borders of the proximal tubules make up a significant fraction of the plasma membrane of renal cortex. We purified intact brush borders from the proximal tubule from 27 rats in nine experiments as shown by marker enzymes and electron microscopy. Alkaline phosphatase was used as the marker enzyme of renal brush border and its relative specific activity in our purified brush-border fraction was nearly

identical with that reported (Wilfong & Neville, 1970; see Table 2). Although some contamination by endoplasmic reticulum was present, the relative specific activity of glucose 6-phosphatase remained near unity.

Electron microscopy confirmed that our preparation consisted of morphologically intact brush border (Plate 2). Transmission electron microscopy revealed thin sections of cytoplasmic fragments which represent the apical surface of the renal tubular epithelial cells. The brush borders of the cells are seen as a series of fingerlike projections measuring 0.15 μm in width and up to 1.5 μm in length. Part of the underlying cytoplasm with occasional vesicles and small amounts of endoplasmic reticulum remained attached. Scanning electron microscopy showed that the specimens consisted of spherical or irregular masses of cytoplasm with fingerlike projections representing the brush border of the tubular cells.

The relative specific activity of kallikrein in the final brush-border preparation was very low (0.2; Table 2). Unlike the marker enzyme, little or no kallikrein activity was associated with the brush border. The lack of kallikrein activity on the brush border cannot be attributed to loss of enzymic activity since the total recovery of kallikrein from all fractions was essentially 100% (not shown in Table 2). In contrast with kallikrein, kininase, angiotensin I-converting enzyme (kininase II) and angiotensinase was concentrated in the brush border. The relative specific activities of total kininase, the converting enzyme and angiotensinase in the brush border were 7.1, 10.3 and 7.2 respectively. These values were similar to those of the marker enzyme in the brush border. The angiotensinase inactivated angiotensin II at pH7.4 and was inhibited completely by 1 mM-*o*-phenanthroline.

Table 2. *Enzyme activity of the isolated brush-border preparation of rat kidney cortex*

Brush-border membranes were prepared as described by Wilfong & Neville (1970). All values given as means ± S.E.M. of five experiments. Relative specific activity is (mean specific activity in the brush border fraction)/(mean specific activity in the homogenate). Specific activities are defined in Table 1. Kininase and angiotensinase are measured as μg of bradykinin (angiotensin II) hydrolysed/min per mg of protein; and angiotensin I-converting enzyme as nmol of hippuryglycylglycine hydrolysed/min per mg of protein.

	Specific activity	Relative specific activity
Alkaline phosphatase	1.20 ± 0.22	15.0
Kallikrein	0.4 ± 0.20	0.2
Kininase	2.3 ± 0.15	7.1
Angiotensin I-converting enzyme (kininase II)	1.2 ± 0.15	10.3
Angiotensinase	0.6 ± 0.10	7.2
Glucose 6-phosphatase	0.05 ± 0.004	1.0

Tumours of proximal tubules

Homogenates of transplanted kidney tumours originating from the proximal tubules MK2 and MK3 were prepared and assayed as described under 'Methods'. No detectable activity of kallikrein was found either in the whole homogenates or in the solubilized homogenates of these tumours. Renal tissue, of both control and host animals, stored and prepared in identical fashion, had normal amounts of kallikrein. We took the results of these experiments as an additional indication that kallikrein may not be produced in normal proximal tubules.

Unlike renal kallikrein, the activity of kininase, angiotensinase and angiotensin I-converting enzyme (Hall *et al.*, 1976) in the homogenate of MK2 tumour was similar to that in renal tissue. These data provide strong evidence for the existence of kininase and angiotensinase in normal proximal tubules.

Discussion

It has been shown that a high-speed sediment, the microsomal fraction of the homogenized kidney cortex, contains enzymes that liberate or inactivate vasoactive peptides. Kininase, angiotensin I-converting enzyme (Erdös & Yang, 1966; Yang *et al.* 1971; Erdös, 1976), angiotensinase (Matsunaga *et al.*, 1968), kallikrein (Nustad, 1970; Ward *et al.*, 1975*b*) and renin (Wilson *et al.*, 1976) are among them.

By using two procedures different both in methods of homogenization and fractionation, we separated renal cortical microsomal fractions into endoplasmic-reticulum- and plasma-membrane-enriched fractions. Extensive washing procedures were used to remove adsorbed and intraluminal contamination, ensuring that the presence of the enzymes in both fractions was not due to cytoplasmic contamination. The separation of these two fractions was confirmed by the use of marker enzymes and electron microscopy. Centrifugation in Dextran 110 demonstrated that kallikrein activity was present in both fractions, but the highest specific activity was associated with the plasma-membrane fraction. Separation by rate-differential centrifugation on sucrose/Ficoll gradients also demonstrated an association of kallikrein with both fractions. In this case, however, the specific activity of kallikrein was about equally distributed between both fractions. Different isolation procedures do not necessarily produce identical membrane subpopulations. Nevertheless, both procedures do

support the concept that kallikrein is present on both endoplasmic reticulum and plasma membranes. However, a significant fraction of microsomal kallikrein may also be associated with other membranes in the microsomal fraction such as the Golgi apparatus. Renal kallikrein may be synthesized on endoplasmic reticulum and subsequently reoriented to a surface localization for activation and release.

It has been suggested that urinary kallikrein is secreted at the level of the proximal tubule (Werle & Vogel, 1960; Nustad, 1970), but brush-border membranes from proximal tubules have little or no kallikrein. In addition, renal tumour cells, ultrastructurally resembling cells of the pars recta of the proximal renal tubule (Hruban *et al.*, 1973), contained no measurable kallikrein. This lack of activity was not due to the presence of any detectable endogenous inhibitor, uninhibited kininase or lack of some exchangeable cofactor.

The absence of kallikrein from brush border of the proximal tubules, from tumours originating from proximal tubules and from the filtrate of proximal tubules (Scicli *et al.*, 1975; Carretero & Scicli, 1976) all suggests that urinary kallikrein does not originate from the proximal tubules.

In contrast with kallikrein, renal kininase, angiotensin I-converting enzyme (kininase II) and angiotensinase are present in high concentration in the brush border of proximal tubule. Electron microscopy showed the huge surface of the brush border where the enzymes are present.

The observed relationship of these enzymes with the plasma-membrane-enriched fraction, but not with the endoplasmic-reticulum-enriched fraction, can be interpreted in two ways. Either membrane-bound kininase, the converting enzyme and angiotensinase are present on plasma membranes in general (including brush border) or they are located on the brush border exclusively. The former view appears most likely. Renal converting enzyme has also been found at sites other than brush border (Hall *et al.*, 1976). However, the data do indicate that all three membrane-bound enzymes are localized on the outside membrane of renal cells, particularly concentrated in the brush border of proximal tubules. Such a localization may facilitate the cleavage of kinins and angiotensins on the cell surface.

The angiotensinase described may be identical with the aminopeptidase angiotensinase A. Similar to renal angiotensinase A (Erdös, 1971), it is a membrane-bound enzyme, active at neutral pH values and inhibited by *o*-phenanthroline. High concentration of an aminopeptidase was also found in the brush border by Quirk & Robinson (1972).

If the production of kinin and angiotensin in the kidney affects renal function, the kidney must be capable of inactivating such peptides introduced into the kidney from extrarenal sources. When a kinin (Abe, 1965; Carone *et al.*, 1975) or angiotensin II (Vane, 1969; Pullman *et al.*, 1975) is infused into the renal circulation, these peptides are nearly completely inactivated. Little or no intact kinin infused into the renal artery reaches the venous effluent (Nasjletti *et al.*, 1975) or is excreted into urine, which, however, contains kinins (Werle & Erdös, 1954; Miwa *et al.*, 1968, 1969). The low molecular weight of these peptides makes it likely that any plasma kinin or angiotensin II that escapes inactivation in the circulation is filtered at the glomerulus. Functionally oriented enzymes on the huge surface of the brush border of the proximal tubules could then inactivate both peptides. We may speculate that kallikrein or even renin bound to membranes (Wilson *et al.*, 1976), located distal to the proximal tubules, can release kinins or angiotensins that may alter ion transport and liberate prostaglandins.

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