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METABOLISM OF KININS AND ANGIOTENSINS IN THE ISOLATED GLOMERULU--ETC(U)
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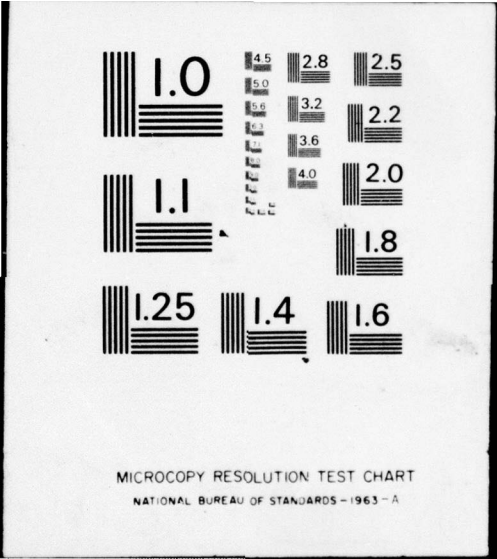
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TECHNICAL REPORT NO. 6

METABOLISM OF KININS AND ANGIOTENSINS
IN THE ISOLATED GLOMERULUS AND BRUSH BORDER OF RAT KIDNEY

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15 October 1976

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
Technical Report No. 6		
4. TITLE (and Subtitle)	5. TYPE OF REPORT & PERIOD COVERED	
6 METABOLISM OF KININS AND ANGIOTENSINS IN THE ISOLATED GLOMERULUS AND BRUSH BORDER OF RAT KIDNEY.	Technical 3/1/75 - 5/31/77	
7. AUTHOR(s)		6. PERFORMING ORG. REPORT NUMBER
10 Patrick E. Ward, Werner Schultz, Rolland C. Reynolds and Ervin G. Erdős		
9. PERFORMING ORGANIZATION NAME AND ADDRESS		B. CONTRACT OR GRANT NUMBER(s)
Univ. Texas Health Science Center @ Dallas Dept. Pharmacology 5323 Harry Hines Blvd., Dallas, TX 75325		15 N00014-75-C-0807 New
11. CONTROLLING OFFICE NAME AND ADDRESS		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
Office of Naval Research Biological & Medical Sciences Division Medical and Dental Sciences Program, Code 444 Arlington, Virginia		16 NR-105-785
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		12. REPORT DATE
11 15 Oct 76 12 29 p.		10/15/76
		13. NUMBER OF PAGES
		24
		15. SECURITY CLASS. (of this report)
16. DISTRIBUTION STATEMENT (of this Report)		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
9 Distribution of this document is unlimited		
14 TR-6		7 Technical rept. 1 May 75 - 31 May 77
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number)		
Peptidases, glomerulus, renal brush border, ultrastructure, freeze etching, kallikrein, kinins, electron microscopy.		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number)		
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Isolated glomeruli contained little or no kallikrein. In addition, compared to renal brush border, renal glomeruli contained relatively low concentrations of kininase, angiotensin I converting enzyme and angiotensinase. The results of these experiments support the idea that the brush border of the proximal tubule is the major site of inactivation of kinins and angiotensins and that renal kallikrein enters the tubular filtrate distal to the glomeruli and proximal tubule. ↗

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METABOLISM OF KININS AND ANGIOTENSINS
IN THE ISOLATED GLOMERULUS AND BRUSH BORDER OF RAT KIDNEY

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Running Title: Renal metabolism of peptides

ABSTRACT

In order to localize the activities of kallikrein, kininase, angiotensin I converting enzyme and angiotensinase in the kidney, rat kidneys were homogenized and glomeruli and brush border were isolated. The yield and purity of glomerular preparations were high. The similarity of the structure of the isolated glomeruli to glomeruli in situ was established by scanning and transmission electron microscopy and freeze fracture. The morphology of isolated brush border of proximal tubules was compared to brush border in situ. Isolated brush border, devoid of core material, retained its converting enzyme, kininase and angiotensinase activity confirming our previous findings that these enzymes are bound to plasma membrane.

Isolated glomeruli contained little or no kallikrein. In addition, compared to renal brush border, renal glomeruli contained relatively low concentration of kininase, angiotensin I converting enzyme and angiotensinase. The results of these experiments support the idea that the brush border of the proximal tubule is the major site of inactivation of kinins and angiotensins and that renal kallikrein enters the tubular filtrate distal to the glomeruli and proximal tubule.

Kinins are active polypeptides released by kallikreins (E.C. 3.4.21.8) from plasma alpha-2-globulin. Kallikrein in the urine originates from the kidney (10, 27) and isozymes of urinary kallikrein have been isolated in highly purified form (26). Kallikrein has been reported to be present in renal glomeruli (17), although Scicli *et al.* (30) found little kallikrein in isolated glomeruli.

The kidney is rich in kininases that inactivate kinins (7,34) and most of this activity sediments with the microsomal fraction of the homogenized kidney. Erdős and Yang (7) described three of these kininases. One of them, kininase II (peptidyl dipeptide hydrolase, E.C. 3.4.15.1) is identical with the angiotensin I converting enzyme (8,28,39,40).

Changes in kallikrein excretion can coincide with changes in sodium excretion and occur in certain hypertensive conditions (6, 18, 19, 22). Kinins are natriuretic, diuretic, hypotensive (25, 35) and they release other vasoactive materials such as prostaglandins (20). Kinins are present in urine (34), but they do not originate from plasma (1). These properties suggest that kinin generation by renal kallikrein has physiological significance.

Recently we found that kallikrein is present in both plasma membrane and endoplasmic reticulum of the homogenized rat kidney cortex (33,35). Brush border of proximal tubules contained high concentrations of kininase, angiotensinase and angiotensin I converting enzyme, but little or no kallikrein.

The importance of enzymes that release or inactivate kinins and angiotensins within the kidney, the controversial reports on the localization of renal kallikrein and the availability of techniques to isolate and study pure renal organelles induced us to continue our investigations of the enzyme content of fractions of the kidney. In the present study we compared the structures of the isolated glomeruli and brush border with those of the

intact kidney. In addition, we determined the content of enzymes in the fractions that metabolize kinins and angiotensins.

MATERIALS AND METHODS

Preparation of Glomeruli and Brush Border

Male Sprague-Dawley rats (250-350 g) were anesthetized with sodium pentobarbital (50 mg/kg) and 100 units of heparin were injected via the femoral vein. The abdomen was exposed via a midline incision and a polyethylene catheter was introduced into the aorta beside the renal arteries. Circulation above and below the kidneys was stopped by clamping and both renal veins were cut. The kidneys were perfused at physiological pressure (100 mm Hg) with Earle's balanced salt solution buffered at pH 7.2 with 20 mM hydroxyethyl piperazine-N-2-ethanesulfonic acid (HEPES). Perfusion was carried out until the kidneys had completely blanched. Iron oxide (2.5 mg/ml) or tantalum powder (25 mg/ml) diluted in the above medium, was then perfused into the kidneys until a uniform grey-black color was attained (21). Perfusion pressure was not allowed to exceed 150 mm Hg and was recorded with a Statham pressure transducer connected to a Grass polygraph. In control studies heparin was excluded and higher perfusion pressures (>200 mm Hg) were used.

After removal of the medulla, the cortex was minced with scissors and lightly homogenized in 30 ml of the same buffer with five up and down strokes of a hand held glass Teflon homogenizer (Thomas, size B). The homogenate was then poured over a 211 micron nylon sieve and washed through with buffer by applying gentle pressure. The material that passed through the 211 micron sieve was washed through a 153 micron sieve. Both sieves served to remove large pieces of broken tissues. The effluent of the 153 micron sieve was then passed through a 63 micron sieve. Material remaining on the 63 micron sieve was washed thoroughly and collected in a 15 ml plastic conical centrifuge tube. This material was suspended in buffer and subsequently allowed to

settle by gravity when tantalum powder was used or by a permanent magnet when iron oxide was used. The buffer and cell debris above the fraction were immediately removed by pipetting. This procedure was repeated ten times. The resulting sediment was estimated to be a 98-100% pure preparation of glomeruli, as observed by light microscopy.

Magnetic iron oxide was prepared as described by Cook and Pickering (5). Before use, tantalum powder was washed with 6 N HCl to remove any traces of heavy metal.

Brush border of proximal tubule was prepared according to our previous publications by differential centrifugation of homogenized rat kidney cortex (35).

Enzyme Content

The enzyme content of isolated glomeruli was determined after disruption of the extracts either by sonication for one minute or by homogenization in a Potter-Elvehjem homogenizer or by freezing and thawing five times. The techniques selected for disruption of the glomeruli had little effect on the relative activities of the enzymes. No activity was lost during the preparation of the glomeruli as compared to the activity in the crude homogenate of the kidney cortex.

Initially in control experiments, only one kidney was perfused with iron oxide or tantalum powder. Then cortices of both kidneys were homogenized separately and their enzyme activities determined. Neither iron oxide or tantalum had any effect on total kallikrein, kininase, converting enzyme or angiotensinase activities.

Renal kallikrein, total kininase activity and angiotensinase were determined by bioassay on the isolated rat uterus as previously described (33,35). The activity of kallikrein was estimated from the amount of kinin it released from heated dog kininogen. The kininase and angiotensinase activities were determined by following the disappearance of the activity of synthetic bradykinin

or angiotensin II when incubated with renal extracts. The esterolytic activity of kallikrein was assayed with alpha-N-tosyl-L-arginine ^3H methyl ester as substrate (^3H -Tame;3). Renal converting enzyme was assayed with hippuryl-glycylglycine as substrate (8,35). The activity of angiotensin I converting enzyme (kininase II) was calculated as the amount of hippuryl-glycylglycine hydrolyzed that could be inhibited by the specific inhibitor SQ 20881. The amount of glycylglycine released by the enzyme was determined by a programmed Beckman 121C amino acid analyzer.

Alkaline phosphatase (E.C. 3.1.3.1) was assayed with p-nitrophenyl phosphate as substrate (15). Glucose-6-phosphatase (E.C. 3.1.3.9) was assayed in the presence of 4 mM EDTA and 2 mM KF (12,13). Lactic dehydrogenase (LDH, E.C. 1.1.2.3) was assayed according to Wroblewski and LaDue (38). Renin (E.C. 3.4.99.19) was determined by radioimmunoassay (37). The activities of the enzymes were expressed as the amount of substrate cleaved in one minute per mg protein. The relative specific activity is calculated by comparing it to whole kidney which is taken as one. Inorganic phosphate was determined by the method of Fiske and SubbaRow (9) and proteins with bovine serum albumin as standard (16).

Electron Microscopy

For transmission electron microscopy (TEM), specimens were fixed with 3% glutaraldehyde in .2 M sodium cacodylate buffer (pH 7.4). After fixing overnight at 2° , the specimens were washed and fixed secondarily with 1% osmium in a 200 mM-sym-collidine buffer for two hours. After treatment with propylene oxide and overnight infiltration with Epon 812/araldite 502, the sediments were embedded in the Epon mixture. The epon was polymerized at 60° , and the blocks were sectioned with a Sorvall MT-2 ultramicrotome. The sections were stained with uranyl acetate and lead citrate.

Specimens which had been previously fixed in 3% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) or fresh, unfixed tissues were prepared

fer or fixative. Following a minimum exposure to glycerol for two hours the specimens were placed in hinged gold opposed specimen holders and quick frozen by immersion in liquid Freon 22 at approximately -150° . Fracture, etching, and replication were carried out in the Denton freeze etch apparatus DFE-3 using an electron gun of our own design. In general, the specimens were fractured at -115° followed by shadowing within one minute. Thus, virtually no etching was allowed at this temperature. Replicas were cleaned routinely for 24 hours in a 1:1 mixture of saturated sodium chloride and commercial bleach (5.25% sodium hypochlorite).

For scanning electron microscopy (SEM), cell fractions including brush border and glomeruli were spread thinly over a coverslip and quickly fixed with 3% glutaraldehyde. In some cases the specimen was fixed secondarily with osmium tetroxide. This was followed by washing and dehydration in graded alcohols. They were then critical point dried from absolute ethanol using liquid CO_2 . After drying, the coverslips were mounted, coated with a thin layer of palladium and gold and examined with a JEOL JSM-35 scanning electron microscope.

Solid tissues for SEM were fixed initially in 3% glutaraldehyde and 0.2 M sodium cacodylate buffer. After fixation the tissue blocks were dehydrated in graded acetone or alcohol. Some specimens were then critically point dried and coated with gold and palladium. Other specimens were subjected to snap-freezing in liquid nitrogen either in absolute alcohol or after being critically point dried. After freezing the specimens were cracked with the sharp edge of a razor blade while still immersed in liquid nitrogen. The specimens in absolute alcohol were thawed and critically point dried. Specimens which had previously been critically point dried were allowed to thaw to room temperature. The specimens were then mounted on stubs so that the fractured surfaces could be examined after coating with palladium and gold.

Ultrathin sections and replicas were examined and photographed with a JEOL 100C electron microscope operated at 80 kV.

Materials

Tantalum powder of 1.0 micron particle size was obtained from Fansteel, Inc. (Chicago, Ill.). The sieves used in the isolation of the glomeruli were prepared from Nitex Nylon monofilament blotting cloth obtained in the designated mesh sizes from Tetko Inc. (Houston, Texas).

Alpha-N-tosyl-L-arginine(³H)methyl ester was obtained from Biochemical and Nuclear Corp. (Burbank, Calif.). Bradykinin, angiotensin II and hippuryl-glycylglycine were from Schwarz-Mann (Orangeburg, NY). The synthetic converting-enzyme inhibitor SQ 20881 was obtained from Dr. Z. Horovitz of Squibb Inc. (Princeton, NJ). All other reagents used were of analytical grade.

RESULTS

Similar to the results of Meezan et al. (21), isolation of glomeruli with the procedures employing iron oxide or tantalum powder yielded preparations with an estimated purity of better than 97%. When physiological perfusion pressures were used (<150 mm Hg), approximately 30% of the glomeruli were recovered after homogenization and repeated sedimentation. Yield increased to about 45% with high perfusion pressures (200 mm Hg). Our estimates of yield are based on the data of Arataki (2) that a normal kidney of a 250g rat contains approximately 28,000 glomeruli.

SEM revealed that many of the glomeruli had intact Bowman's capsule. In the glomeruli without intact capsules, the capillary loops were seen to be covered with intact epithelium with no evidence of damage. The podocytes demonstrated intact, branching trabeculae and normal interdigitating foot processes. (Fig. 1 & 2).

TEM of isolated glomeruli (Fig. 3-5) revealed that although there was occasional lysis of cells, especially endothelial cells, more than 75%

Fig. 1
Fig. 2

of the endothelial cells were intact. Granular iron particles were present in about one-fourth of the capillary loops and endothelial cell damage tended to be localized where the iron particles were densely packed in capillary lumina. Cell lysis, when found, was not prominent in the thicker perinuclear cytoplasm. The thicker portions of the epithelial and endothelial cells revealed some intracellular edema and swelling of the endoplasmic reticulum and mitochondria. The attenuated portions of these cells, consisting of the foot processes of the epithelial cells and the fenestrated filtration membranes of the endothelial cells, were consistently intact (Fig. 5). The fibrillar material of the glomerular basal lamina was less densely packed than in intact glomeruli. The fibrils were probably separated by fluid. The visualization of the lamina lucida and lamina densa as distinct entities in the basal lamina was obscured by the separation of fibrils. Slit membrane preservation between adjacent foot processes was variable.

Figures 3 and 4 compare the freeze etch appearance of the glomeruli in situ in the kidney, and the isolated glomeruli. Both pictures reveal tangential fractures through the epithelial podocytes showing interdigitating foot processes. The only differences between glomeruli in situ and isolated glomeruli is an apparent increase in the small hillocks in the isolated glomeruli.

Although we do not know of specific marker enzymes for glomeruli, glomeruli do contain membrane bound enzymes such as glucose-6-phosphatase and alkaline phosphatase and soluble enzymes such as LDH (38). Table 1 shows the relative specific activities of these enzymes in isolated glomeruli. Their activities per mg protein were close to that of crude homogenate of renal cortex. Assuming a relatively uniform distribution of these enzymes throughout the kidney, these results indicated that the isolated glomeruli had not lost a significant amount of either their soluble or membrane-bound enzyme content. Table 2 shows the relative specific activities of kallikrein,

Fig. 3
Fig. 4
Fig. 5

Table 1

Table 2

kininase, angiotensin I converting enzyme and angiotensinase. Kallikrein, as determined by bioassay and with ^3H -TAME ester, was present in variable low concentrations in isolated glomeruli. The range of relative specific activities (0.2 to 1.8: cortex = 1) was not related to the method of extraction or perfusion pressure. Since glomeruli represent less than one percent of the protein of the renal cortex, hypothetically the relative specific activity of an enzyme confined to the glomeruli would be over 100 in purified preparation.

Microscopic examination showed that a small proportion of the isolated glomeruli contained afferent and efferent vascularelements. Renin activity was therefore assayed to determine the amount of juxtaglomerular cells present in the purified glomerular preparation. In the two preparations when iron oxide was used and in the one where tantalum was employed, the average recovery of renin was similar to that of kallikrein, approximately one percent of the total activity of the cortex.

Small amounts of kininase, angiotensin I-converting enzyme and angiotensinase were present in every glomerular preparation examined. However, their specific activities remained at the level or below that of crude homogenate. In addition, their relative specific activities in the glomeruli were less than one tenth of that found in purified brush border of proximal tubule.

Figures 6 and 7 contrast the appearance of proximal tubule brush border in situ to that of brush border concentrated and purified from kidney homogenate by differential centrifugation. SEM revealed that the isolated brush border was better than 90% pure. TEM showed that most of the isolated brush border consisted of membranes uncontaminated by underlying cytoplasm. The brush border preparation was devoid of core material and appeared empty (Figs. 8 & 9). Freeze etching of unfixed brush border revealed a significant decrease in the number of embedded membrane particles in the isolated microvilli. Intact microvilli on the average had 2,200 particles per square

Fig. 6
Fig. 7

Fig. 8
Fig. 9

micrometer while isolated microvilli had 400 particles per square micrometer. There was no significant difference between the number of particles on the outer or inner faces of these fractured membranes (Figs. 10 & 11).

Enzyme assay of the isolated brush border preparation produced results similar to those previously reported (33, 35). The marker enzyme of brush border, alkaline phosphatase, was enriched fifteen fold with no enrichment of endoplasmic reticulum marker enzyme, glucose-6-phosphatase. Kallikrein activity was undetectable in the purified microvilli. Kininase, angiotensin I converting enzyme and angiotensinase were highly enriched in the brush border membranes with relative specific activities of 8.3, 9.6 and 8.2 respectively.

DISCUSSION

Changes in the levels of intra-renal kinin and angiotensin have been demonstrated to alter renal function (23, 25). Because of the short half-life of these peptides, we have studied the intra-renal concentration and distribution of the enzymes that can determine their activities by metabolizing them.

A purified preparation of kidney glomeruli, free of tubular contamination, was prepared from renal cortex. SEM and TEM demonstrated that the purified glomeruli were morphologically similar to glomeruli in situ. In addition, the isolated glomeruli had similar marker enzyme activities as glomeruli isolated by others (31). The present studies demonstrate that kallikrein is not localized in glomerular corpuscles. In a previous paper (35), we demonstrated that kallikrein is present on both plasma membrane and endoplasmic reticulum enriched fractions missing from preparations of brush border of proximal tubules. Kallikrein is probably localized in distal tubules. Kallikrein may be localized in an area close to the glomerular corpuscle such as the macula densa of the distal tubule. Such a localization could explain the changes in kallikrein excretion caused by changes in renal

Fig. 10
Fig. 11

perfusion pressure (23) and its apparent entrance into tubular filtrate at the level of the distal tubule (4, 30).

Kinins and angiotensins are rapidly inactivated in a variety of vascular beds, including the kidney (1, 7, 32). The angiotensinase activity described here is probably due to angiotensinase A (35). This enzyme cleaves the N-terminal Asp⁻¹ of angiotensin I and II, thus it can release angiotensin III from angiotensin II. The kininase activity in the extract represented the total kininase activity on bradykinin. Kininase II is the angiotensin I converting enzyme, that either cleaves Phe-Arg from the C-terminal end of bradykinin, or His-Leu from angiotensin I. By this action it can inactivate the hypotensive peptide or liberate the hypertensive one (39,40). Converting enzyme has been localized in vascular endothelial cells of the lung (29), in human endothelial cells grown in culture (14) and all along the nephron (11). Therefore, endothelial cells of the glomerular corpuscles were thought to be a major renal site of conversion of angiotensin I and inactivation of kinins and angiotensin II. The present experiments, however, demonstrate that although converting enzyme, kininase and angiotensinase are present in isolated glomeruli, the concentration of these enzymes is less than one-tenth that found in isolated brush border of proximal tubule (35). The relative distribution of these enzymes in renal glomeruli, and brush border of proximal tubules (11, 35) indicates that the majority of peptide inactivation occurs at the luminal membrane of the proximal tubules after filtration. This system may prevent extrarenal kinin (and also angiotensins) released in plasma or infused into the kidney from altering intrarenal kinin concentration in the distal tubule. Such a system would appear to be necessary if the release of intrarenal kinin is important in controlling some renal functions.

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Figures 1 and 2. Low and high magnification SEM pictures of isolated glomeruli. Some glomeruli retain portions of Bowman's capsule. Figure 2 demonstrates that the podocytes and their interdigitating processes remain intact through the isolation procedure. Magnification 860X and 7800X.

Figure 3. TEM of freeze-etch replica of tangential fracture through interdigitating processes of podocytes in an intact glomerulus. The glomerular basement membrane and endothelium can be seen along the left margin. Magnification 14,500X.

Figure 4. TEM of freeze-etch replica showing a similar tangential fracture through the interdigitating processes of podocytes in an isolated glomerulus. There is an increase in the number of hillocks on the membranes but otherwise the epithelium appears unchanged. Magnification 18,000X.

Figure 5. TEM of a representative section of an isolated glomerulus showing the presence of iron particles. The peripheral portions of the fenestrated endothelium were generally intact. Approximately 25% of the endothelium was disrupted mainly in areas of heavy iron deposits and thicker portions of the endothelial cell cytoplasm. The podocytes are also largely intact but show focal areas of swelling and lysis (see arrows). The basal lamina in the isolated glomeruli is not as dense as the basal lamina in untreated glomeruli. Magnification 6,800X.

Figure 6. SEM photomicrograph of normal brush border from intact proximal convoluted tubules of rat kidney. Note the branching and fusion of brush border villi along the exposed surface. Magnification 20,000X.

Figure 7. Clumps of isolated brush border prepared as described in text. The preparation is estimated to be approximately 95% pure. The microvilli are unchanged in size and shape and in general resemble to microvilli of the normal kidney except that they have become tangled during the isolation procedure. Magnification 18,000X.

Figure 8. TEM photomicrograph of normal intact brush border. The microvilli can be seen to branch and fuse. Only branching is demonstrated in the section. The microvilli appear to have a core of dense granular material and contain microtubules. Magnification 25,000X.

Figure 9. TEM of isolated brush border membranes. The surface membrane is intact but the core material and the microtubules are missing. The microvilli appear empty. Magnification 15,000X.

Figure 10. Freeze-etch replica of intact brush border. Both the inner and outer surfaces of the plasma membrane are studded with particles. Core structure cannot be seen with the preparation. Magnification 36,400X.

Figure 11. Freeze-etch replica of isolated brush border. Note that the membrane surfaces appear intact but there has been a striking loss of the embedded particles in both the inner and outer membrane surfaces. Particle counts revealed a 82% decrease in the number of embedded particles in the isolated brush border. Magnification 42,700X.

TABLE 1

Perfusion Material	Iron Oxide		Tantalum Powder	
Perfusion Pressure (mm Hg)	120		120	
Experiments (No.)	2		2	
	Specific Activity	Relative Specific Activity	Specific Activity	Relative Specific Activity
Glucose-6-Phosphatase ¹	0.06	1.1	0.07	1.3
Alkaline Phosphatase ¹	0.06	0.7	0.05	0.6
LDH				0.6

Enzyme content of isolated glomeruli purified from rat kidney cortex by iron oxide or tantalum powder extraction. Relative specific activity is (mean specific activity in the glomeruli preparation)/(mean specific activity in the homogenate).

¹umole of substrate hydrolysed per min per mg of protein

TABLE 2

Perfusion Material	Iron Oxide			Tantalum Powder		
	Perfusion Pressure (mm Hg)	SA	RSA	Perfusion Pressure (mm Hg)	SA	RSA
Perfusion Pressure (mm Hg)	120			120		
Experiments (No.)	4			3		
	200			200		
	6			4		
<u>Enzyme:</u>						
Kallikrein ²	2.4 ± 1.4	1.0	1.2	2.0 ± 1.1	0.8	1.2 ± 0.5
³ H-TAME Esterase			1.1			0.8
Kininase ³	100 ± 30	0.3	0.4	180 ± 50	0.6	70 ± 30
Angiotensin I ⁴ Converting Enzyme	120 ± 40	0.9	1.1	80 ± 40	0.5	100 ± 40
Angiotensinase ³	90 ± 20	1.1	0.8	90 ± 30	1.0	

Enzyme content of isolated glomeruli purified from rat kidney cortex by iron oxide or tantalum powder extraction. All values given as means ± S.E.M. Relative specific activity is (mean specific activity in the glomeruli preparation)/(mean specific activity in the homogenate).

²ng of bradykinin equiv. released per min per mg of protein

SA = Specific Activity

³ng of bradykinin (angiotensin II) hydrolysed per min per mg of protein

RSA = Relative Specific Activity

⁴pmole of hippurylglycylglycine hydrolysed per min per mg of protein

This study was supported in part by a grant from The American Heart Association - Texas Affiliate, by the N.I.H.U.S.P.L. 16320 and HL 14187, and by the ONR N00014-75-0807. Patrick E. Ward is a National Institutes of Health Postdoctoral Fellow (N.I.H.U.S.P.H.S., 1F22 HL 00643).

