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Uptake of Glycine from L-Alanylglycine into Renal Brush Border Vesicles

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Summary. Isolated renal brush border microvilli vesicles were employed to study the uptake of radiolabel from L-Ala·[^{3}H]Gly and D-Ala·[^{3}H]Gly as well as to determine the presence of dipeptidase activity. Microvilli vesicles were prepared from porcine kidney cortex by differential centrifugation through hypotonic Tris buffer containing Mg^{2+}. The microvilli vesicles transiently accumulated radiolabel from L-Ala·[^{3}H]Gly to higher levels than were initially present in the incubation medium (overshoot phenomenon). This accumulation was dependent on the presence of an inward-directed (extravesicular > intravesicular) Na^+ gradient and was osmotically sensitive and linear with respect to microvilli protein concentration. Analysis of intravesicular contents revealed that all ^{3}H uptake from L-Ala·[^{3}H]Gly appeared as free glycine. Hydrolysis studies demonstrated the rate of L-Ala·[^{3}H]Gly hydrolysis to free alanine and [^{3}H]glycine by the microvilli to be greatly in excess of their rate of radiolabel uptake from this dipeptide. In addition, the uptake profiles and kinetic constants for vesicular uptake of radiolabel from L-Ala·[^{3}H]Gly and free glycine were demonstrated to be identical when measured by double-labeling techniques in the same experiments. These results indicate that L-Ala·[^{3}H]Gly is hydrolyzed at the external surface of the microvilli with the [^{3}H]glycine released being transported into the vesicles by a Na^+ gradient-dependent system identical to that employed for free glycine.

Microvilli vesicle uptake of radiolabel from D-Ala·[^{3}H]Gly exhibited no Na^+ dependent "overshoot" effect. D-Ala·[^{3}H]Gly was completely resistant to microvilli-catalyzed hydrolysis.

Analysis of the microvilli for renal dipeptidase, an enzyme with hydrolytic activity against a wide range of L-dipeptides, revealed this enzyme to be enriched in the microvilli vesicles to a degree equivalent to that observed for marker enzymes for renal microvilli.

Renal dipeptidase catalyzed hydrolysis of L-Ala·Gly but not D-Ala·Gly, as was the case with microvilli-catalyzed hydrolysis of these dipeptides.

With its location in the renal brush border microvilli and its hydrolytic action against L-dipeptides, renal dipeptidase may act at the luminal surface of the proximal tubule cell to hydrolyze L-dipeptides present in the glomerular filtrate, with the resultant free amino acids transported across the brush border microvilli by Na^+ gradient-dependent processes.

The luminal membrane of kidney cortex proximal tubule cells is specialized to form a brush border composed of numerous finger-like projections called microvilli [25, 28]. These microvilli increase the surface area of the luminal membrane approximately 100-fold and greatly facilitate the primary function of the proximal tubule cell, which is the reabsorption of approximately 80% of the glomerular filtrate. The reabsorption of glomerular filtrate constituents entails active and/or passive transport systems [24].

Vesicular preparations of isolated renal brush border microvilli provide an excellent system for analysis of the molecular mechanisms involved in reabsorption of components of the glomerular filtrate, since they allow study of transport systems at a precise anatomic location in the absence of cellular metabolic processes.

Isolated renal brush border membrane vesicles have been shown to transport amino acids [15, 16] and sugars [5, 27] via Na^+ gradient-dependent, stereospecific, carrier-mediated systems. In the presence of an inward-directed Na^+ gradient (extravesicular > intravesicular) brush border vesicles transiently accu-

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mulate levels of amino acids and sugars substantially higher than those present in the external medium. Corresponding studies of uptake of amino acid residues from dipeptides using isolated renal brush border membrane vesicles are currently absent from the literature.

Studies [2, 3] in which dipeptides were intravenously administered to rats have demonstrated their rapid clearance from the blood, with the kidney having a high capacity for dipeptide uptake compared to other tissues. In addition, perfusion of rat kidney proximal tubules with radiolabeled glycyl-L-phenylalanine [38] demonstrated the presence of tubule dipeptidase activity as well as uptake of amino acid residues from the dipeptide. This evidence suggests that the proximal tubule brush border microvilli could be the site of dipeptidase activity and dipeptide clearance from the glomerular filtrate following dipeptide filtration from the blood by the renal glomeruli. Dipeptides are transiently present in the blood following a protein meal or infusion of peptides into the gut lumen [9, 36].

It was therefore decided to investigate microvilli vesicle uptake of radiolabel from radioactive dipeptides in order to determine the biochemical characteristics of any such uptake.

Materials and Methods

Methods

Preparation of Renal Microvilli. The procedure for isolation of renal brush border microvilli from porcine kidney cortex was that of Booth and Kenny [8], which utilizes a series of differential centrifugations in hypotonic Tris-HCl buffer containing Mg^{2+}. Protein concentrations were determined by the method of Bradford [10].

Marker Enzyme Assays and Electron Microscopy. The quality of the isolated brush border microvilli was checked by means of marker enzymes and electron microscopy. The subcellular organelles and their respective marker enzymes for which the membranes were assayed are as follows: brush border membrane – alkaline phosphatase [39], maltase and trehalase [13]; endoplasmic reticulum – NADPH cytochrome *c* reductase [32]; mitochondria succinate tetrazolium reductase [35]; lysosomes – β-galactosidase [30]; baso-lateral membrane – (Na^+/K^+) stimulated ATPase [29].

Synthesis of Radiolabeled Dipeptide. L-Ala·[^{3}H]Gly and D-Ala·[^{3}H]Gly were synthesized by a two-step procedure which entailed (i) production of the N-carboxy-α-amino acid anhyrides (NCAs) of L- and D-alanine and (ii) reaction of the NCA-alanine with 2·[^{3}H]glycine in cold alkaline solution to yield a dipeptide carbamate from which the dipeptide was released following acidification.

NCA-L- and D-alanine were prepared from carbobenzyloxy-L- or D-alanine by the procedure of Ben-Ishai and Katchalsky [6].

L-Ala·[^{3}H]Gly and D-Ala·[^{3}H]Gly were synthesized from [^{3}H]glycine and the corresponding NCA-α-amino acid by the procedure of Hirschman et al. [23].

Purification of Synthesized Dipeptide. Synthesized L-Ala·[^{3}H]Gly and D-Ala·[^{3}H]Gly were purified by ion-exchange chromatography on a column of AG50W-X2 resin (Bio-Rad Laboratories) with pyridine established as the resin cation. The column was eluted with a continuous gradient of pyridine-acetate, pH 4.0, which increased from 0.05–0.07 M pyridine over an elution volume of 400 ml.

Aliquots of ninhydrin-positive column fractions were subjected to thin-layer chromatography (TLC) on Eastman cellulose sheets (layer thickness 160 μm) using three solvent systems: isopropanol/2-butanone/1 N HCl (60:15:25), pyridine/H_2O (79:18), methanol/H_2O (99:1). Appropriate dipeptide and amino acid standards were also chromatographed.

Column fractions demonstrated by TLC to contain only Ala·[^{3}H]Gly were pooled and concentrated. Aliquots were taken for determination of mass by ninhydrin assay and ^{3}H radioactivity by liquid scintillation counting. The optical purity of the synthesized dipeptides was checked by incubation with pure renal dipeptidase, followed by analysis of the reaction products by cellulose TLC. Renal dipeptidase catalyzes hydrolysis of dipeptides containing N-terminal L-amino acids but not D-amino acids [11]. Renal dipeptidase completely hydrolyzed equal amounts of standard L-Ala·Gly and synthesized L-Ala·[^{3}H]Gly while no hydrolysis of standard D-Ala·Gly was observed. In a separate experiment the enzyme was unable to hydrolyze any of the synthesized D-Ala·[^{3}H]Gly. These results demonstrated that the synthesized D- and L-Ala·[^{3}H]Gly were optically pure.

Microvilli Uptake Assay System. All uptake experiments employed microvilli freshly prepared from porcine renal cortex.

Typically, 25 μl of microvilli (100–200 μg protein) suspended in 100 mM mannitol, 20 mM Tris-HCl, pH 7.6, were added to a 1.5 ml disposable Eppendorf tube containing 25 μl of an incubation buffer consisting of 100 mM mannitol, 200 mM NaCl, and 20 mM Tris-HCl, pH 7.6. Depending on the experiment performed, the incubation buffer also contained 2 mM L-Ala·[^{3}H]Gly, 2 mM D-Ala·[^{3}H]Gly, or 2 mM L-Ala·[^{3}H]Gly + 0.12 mM [^{14}C]Gly. The microvilli were rapidly mixed with the incubation buffer to give an overall concentration of 100 mM, 1 mM, and 0.06 mM for NaCl, dipeptide or [^{14}C]Gly, respectively, and incubated in a 37 °C water bath. Microvilli and incubation buffer were preincubated separately for several minutes in a 37 °C water bath immediately before each experiment. Uptake was stopped by pipetting each reaction mixture (50 μl) into 1 ml of ice-cold stopping solution composed of 155 mM NaCl, 20 mM Tris-HCl, pH 7.6. The resulting suspension was rapidly filtered through a Millipore filter (HAMK: 0.45 μm) and washed with three 1-ml aliquots of ice-cold stopping solution. The total time required for filtration and washing was approximately 25 sec. Each filter was placed in a scintillation vial containing 10 ml of Bray's solution and counted in a liquid scintillation counter. Controls were employed in which microvilli were added to ice-cold stopping solution containing incubation buffer and filtered immediately.

Uptake of tritiated D-mannitol was measured at the same concentration and under identical conditions as for dipeptide, except that sorbitol isoosmotically replaced mannitol in the incubation buffer.

For analysis of microvilli transport as a function of medium osmolarity, the osmolarity of the incubation medium was adjusted with sucrose. The osmolarity of the stopping solution was increased to that of the sample by adding sorbitol.

Studies of Na^+ gradient-independent uptake utilized sodium-free incubation medium and stopping solution, with mannitol isoosmotically replacing NaCl.

Kinetic constants for Na^+ gradient-dependent microvilli uptake of free [^{3}H]Gly and [^{3}H]Gly from L-Ala·[^{3}H]Gly were determined in duplicate experiments. Initial rates of radiolabel uptake by microvilli in the presence and absence of a Na^+ gradient were

Table 1. Marker enzyme analysis of renal brush border microvilli preparation[a]

Enzyme	Organelle	Homogenate		Brush Border Membranes		% Recovery	RSA[d]
		Tot. act.[b]	Sp. act.[c]	Tot. act.[b]	Sp. act.[c]		
Alkaline phosphatase	Brush border	1056±108	88 ±0.9	51.4±0.01	1190 ±10	4.86	13.5
Trehalase	Brush border	2616± 60	218 ±0.5	96.6±1.70	2237 ±40	3.70	10.3
NADPH-Cytochrome C reductase	Endoplasmic reticulum	262± 22	21.9±1.9	0.44±0.05	10.1± 1.2	0.17	0.46
Succinate-tetrazolium reductase	Mitochondria	764±105	63.7±8.8	0.26±0.06	6.8± 1.5	0.03	0.11
β-galactosidase	Lysosomes	86± 8	7.2±0.7	0.16±0.03	3.7± 0.6	0.18	0.51
Na^+/K^+ ATPase	Basolateral membrane	400± 40	33.4±3.4	0.81±0.037	18.7± 8.6	0.20	0.56

[a] Enzyme activities are expressed as μmol product/min/mg protein. Relative specific activity refers to the ratio of enzyme specific activity in the microvilli to that in the homogenate. Percent recovery refers to the percentage of homogenate enzyme activity recovered in the microvilli preparation. All assays were performed in triplicate on duplicate membrane preparations. Values for each enzyme activity are expressed as the mean ± SD.
[b] μmol/min [c] μmol/min/mg × 10^3. [d] Relative specific activity (microvilli/homogenate).

measured as a function of amino acid and dipeptide concentration (0.1–10.0 mM). The amount of Na^+ gradient-independent uptake was subtracted from that observed in the presence of an Na^+ gradient, and the kinetic constants for Na^+ gradient-dependent transport were obtained from Lineweaver-Burke plots.

Analysis of Intravesicular Contents. Microvilli were incubated with 1 mM L-Ala·[^{3}H]Gly in the uptake system previously described for periods of 30 sec and 20 min. The microvilli were collected via Millipore filtration. For each time point, twelve filters were extracted for 24 h at 4 °C with 15 ml of water. Each extract was concentrated and aliquots were subjected to cellulose TLC along with appropriate amino acid (including [^{14}C]Gly) and dipeptide standards. Isopropanol/2-butanone/1 N HCl (60:15:25) was used as the developing solvent. Following development, the chromatograms were sprayed with ninhydrin and cut into one centimeter sections; radioactivity was counted using a liquid scintillation counter.

Alanylglycine Hydrolysis Assay. Microvilli-catalyzed hydrolysis of radiolabeled alanylglycine was studied in the same incubation system employed for analysis of uptake. Hydrolysis was stopped by pipetting each raction mixture into tubes standing in a boiling water bath. Hydrolysis products were separated by cellulose thin layer chromatography using MEOH/H_2O (99:1) as the developing solvent. Hydrolysis products were visualized with ninhydrin spray, eluted from the TLC sheet with water, and quantitated by liquid scintillation counting.

Purification of Renal Dipeptidase. Renal dipeptidase was purified from porcine kidney cortex according to the method of Armstrong et al. [4]. The purified enzyme was homogenous as shown by analytical polyacrylamide gel electrophoresis [14].

Renal dipeptidase activity was assayed by hydrolysis of glycyldehydrophenylalanine according to the method of Rene and Campbell [37].

Materials

[2-^{3}H]Glycine (6.32 Ci/mmol) was obtained from ICN Pharmaceuticals. [2-^{14}C]Glycine (41 mCi/mmol) and D-[1-^{3}H]mannitol (22.4 Ci/mmol) were ordered from New England Nuclear. Glycyldehydrophenylalanine (GdP) was prepared from laboratory synthesis as described by Campbell et al. [12]. Reagents used in the enzyme assays were purchased from Sigma Chemical Co. and Fisher Scientific. Chemicals employed in dipeptide synthesis, as well as organic solvents used in TLC were ordered from Aldrich Chemical Co. Ion-exchange resin was obtained from Bio-Rad Laboratories. Cellulose TLC sheets and solvent tanks were ordered from Fisher Scientific. Uptake experiments utilized filters and a six-place vacuum-filtration manifold purchased from Millipore Corp.

Results

Purity of Renal Microvilli Preparation

Isolated renal microvilli and the crude cortical homogenates from which they were prepared were assayed for a number of enzymes serving as markers for various subcellular structures. The results of these assays are given in Table 1.

The relative specific activities of the brush border marker enzymes, alkaline phosphatase [42] and trehalase [7], were 13.5 and 10.3, respectively. This indicated preparations highly enriched in brush border microvilli. The microvilli were found to be devoid of maltase activity. Such an observation has also been reported by Vannier et al. [41] using brush borders isolated from pig kidney and may be peculiar to this species, since maltase is a marker for renal brush border in rabbit [7] and rat [19, 20].

Marker enzyme activities for mitochondria, endoplasmic reticulum, lysosomes, and baso-lateral membrane demonstrated that the microvilli were substantially free of contamination by these subcellular structures.

Electron micrographs of the microvilli revealed them to be vesicular and polydisperse in size, ranging from 0.2–0.8 μm in diameter. The microvilli appeared to be relatively free of other cell constituents and the vesicles were predominantly closed.

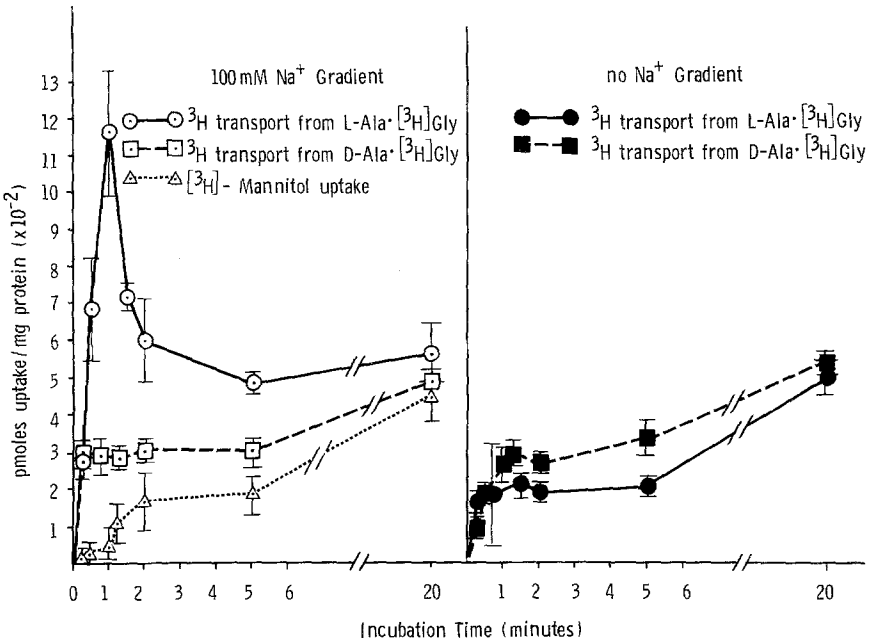

Fig. 1. Uptake of radiolabel from L-Ala·[^{3}H]Gly (⊙, ●), D-Ala·[^{3}H]Gly (⊡, ■), and [^{3}H] mannitol (△) by renal microvilli vesicles in the presence (⊙, ⊡, △) and absence (●, ■) of a Na^+ gradient (extravesicular > intravesicular). For radiolabel uptake by microvilli in a Na^+ gradient, the incubation medium (50 µl) contained 1 mM dipeptide or 1 mM [^{3}H] mannitol and microvilli (118–141 µg protein) in 100 mM mannitol (100 mM sorbitol for [^{3}H] mannitol uptake), 100 mM NaCl, 20 mM Tris-HCl, pH 7.6. For uptake in the absence of a sodium gradient, microvilli and dipeptide were in 50 µl of 300 mM mannitol, 20 mM Tris at pH 7.6. Assays were performed in triplicate using membranes prepared the day of the experiment. Values represent values ± SEM

The marker enzyme assays and electron microscopic examination revealed the microvilli to be in a substantially pure, homogeneous state. Haas et al. [21] and Vannier et al. [41] have employed electron microscopic, enzymological, and immunological techniques to demonstrate that renal microvilli vesicles isolated by the method of Booth and Kenny [8] are oriented "right-side-out," that is, in the same direction as their *in vivo* state.

Radiolabel Uptake from L-*Ala·[^{3}H]Gly,* D-*Ala·[^{3}H]Gly, and [^{3}H]mannitol*

Renal microvilli uptake of radiolabel from L-Ala·[^{3}H]Gly in the presence and absence of a 100 mM Na^+ gradient (extravesicular > intravesicular) is shown in Fig. 1. In the presence of the Na^+ gradient, microvilli uptake of radiolabel from L-Ala·[^{3}H]Gly exhibited an "overshoot" phenomenon with uptake peaking at 60 sec incubation and rapidly declining at longer incubation times. Radiolabel uptake from L-Ala·[^{3}H]Gly after 60 sec incubation was approximately twice that observed at equilibrium (20 min incubation). In the absence of a Na^+ gradient, microvilli uptake of radiolabel from L-Ala·[^{3}H]Gly demonstrated no overshoot. Uptake gradually increased with increasing incubation time to an equilibrium value (20 min incubation) approximately equal to that obtained in the presence of a Na^+ gradient. Thus the overshoot effect would appear to be due to the imposition of an inward-directed Na^+ gradient and represents an accumulation of radiolabel against a concentration gradient. This overshoot effect in the presence of a Na^+ gradient has also been observed for amino acid [15, 16] and sugar [5, 27] uptake by renal microvilli vesicles.

Microvilli uptake of 1 mM [^{3}H]mannitol in a sodium gradient is also shown in Fig. 1. Mannitol is not known to have a carrier in the renal brush border [40]. Therefore, the amount of microvilli uptake of this monosaccharide demonstrates the passage of solute into the vesicles as a result of diffusion, and indicates the degree of tightness of the vesicles. [^{3}H]mannitol uptake was quite small (< 50 pmol/mg protein) during the first 60 sec of incubation. This indicated that peak uptake of radiolabel from L-Ala·[^{3}H]Gly (1160 pmol/mg protein), which occurred at 60 sec incubation, was due to carrier-mediated transport rather than simple diffusion.

Microvilli uptake of radiolabel from D-Ala·[^{3}H]Gly was virtually the same whether or not a Na^+ gradient was present. No overshoot effect was observed. Uptake increased towards the same equilibrium value as that observed with L-Ala·[^{3}H]Gly. Therefore, microvilli uptake of radiolabel from D-Ala·[^{3}H]Gly does not appear to be Na^+ gradient-dependent.

Radiolabel Uptake as a Function of Microvilli Protein Concentration

Initial rates (30 sec incubation) of microvilli uptake of radiolabel from L-Ala·[^{3}H]Gly in a sodium gra-

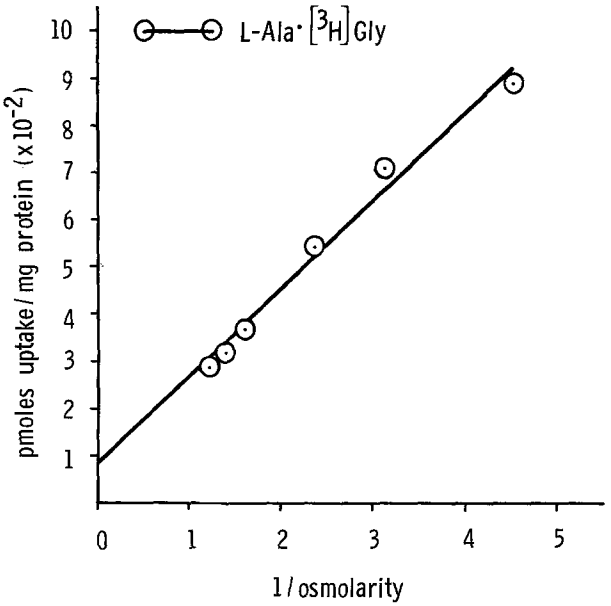

Fig. 2. Uptake of [^{3}H]Gly from L-Ala·[^{3}H]Gly (⊙) by renal microvilli as a function of incubation medium osmolarity. The incubation medium (50 µl) contained microvilli protein (135 µg per sample) and 1 mM L-Ala·[^{3}H]Gly in 100 mM NaCl, 20 mM Tris-HCL, at pH 7.6, with sucrose added to give the desired medium osmolarity. Incubation was allowed to proceed for 20 min at 37 °C. Values represent the means of triplicate assays

dient were followed as a function of membrane protein. Uptake was linear over the entire range of microvilli protein levels employed (0–330 µg protein/sample). The amount of microvilli protein normally employed in the transport studies was 115–200 µg/sample, which is well within the limits of linearity.

Uptake as a Function of Osmolarity

Microvilli uptake of radiolabel from L-Ala·[^{3}H]Gly at equilibrium (20 min incubation) as a function of medium osmolarity is demonstrated in Fig. 2. Medium osmolarity was adjusted by adding the impermeable solute sucrose to the incubation medium. The amount of radioactivity associated with the vesicles at infinite osmolarity represents surface binding rather than uptake into an intravesicular space, since at infinite osmolarity the vesicles would be flattened and the volume of their intravesicular space zero.

Microvilli uptake of radiolabel from L-Ala·[^{3}H]Gly was quite sensitive to changes in medium osmolarity, sharply decreasing with increases in osmolarity. When extrapolated to infinite medium osmolarity, uptake was 11.5±5.0% (95% confidence intervals) of that observed in the 320 milliosmolar incubation medium normally employed for transport studies. Such results indicate uptake into an osmotically reactive intravesicular space predominates, rather than binding to the surface of the vesicles.

Intravesicular Contents

Results obtained when microvilli vesicle contents were analyzed following incubation with L-Ala·[^{3}H]Gly in a sodium gradient are shown in Fig. 3. Cellulose TLC of the vesicular contents of microvilli incubated for 30 sec and 20 min in the presence of 1 mM L-Ala·[^{3}H]Gly revealed that the vesicular ^{3}H radioactivity had chromatographic properties identical to that of a free [^{14}C]glycine standard. This finding was very interesting as it indicated that radiolabel uptake from L-Ala·[^{3}H]Gly by the microvilli was in the form of free [^{3}H]Gly rather than intact dipeptide. Microvilli uptake of intact alanylglycine followed by intravesicular hydrolysis is unlikely, since all intravesicular radiolabel was in the form of free glycine after only 30 sec incubation. Two other possibilities remain: (i) hydrolysis of the L-Ala·[^{3}H]Gly at the external sur-

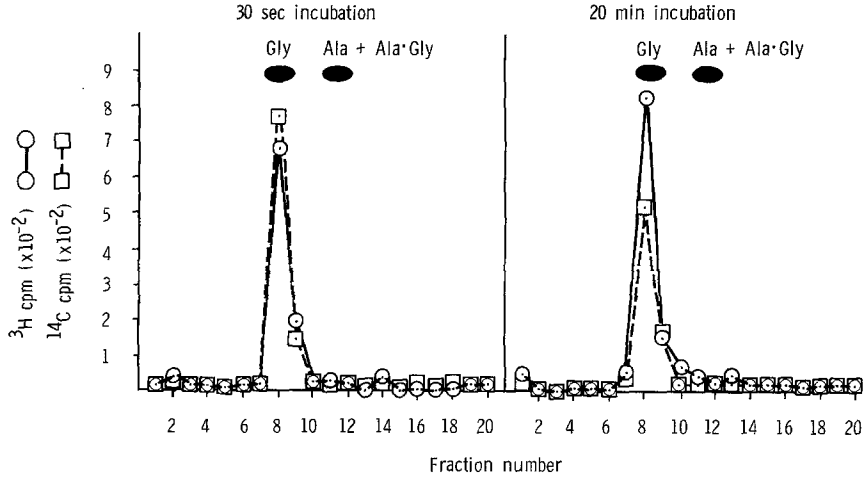

Fig. 3. Intravesicular contents of renal microvilli incubated with 1 mM L-Ala·[^{3}H]Gly in a Na^+ gradient. Microvilli (165 µg protein per sample) were incubated with dipeptide for 30 sec and 20 min in 50 µl of medium containing 100 mM mannitol, 100 mM NaCl, 20 mM Tris-HCl at pH 7.6 Analysis of microvilli vesicular contents was as described in Methods

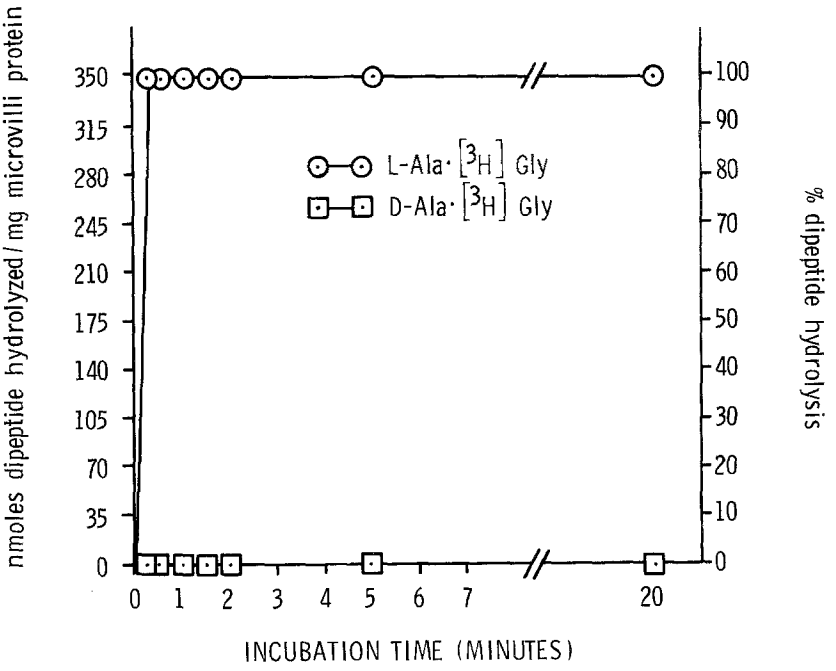

Fig. 4. Hydrolysis of L-Ala·[^{3}H]Gly (⊙) and D-Ala·[^{3}H]Gly (⊡) by renal microvilli vesicles. The incubation system (50 µl) contained 118 µg of microvilli protein and 1 mM dipeptide in 100 mM mannitol. 100 mM NaCl, 20 mM Tris-HCl at pH 7.6. Quantitation of dipeptide hydrolysis was as described in Methods. Values given are the means of duplicate assays

face of the vesicles, followed by uptake of the resultant free amino acids, or (ii) uptake of intact dipeptide at the external surface of the vesicle, followed by intramembrane hydrolysis and release of the resultant free amino acids to the interior of the vesicle.

It was decided to examine the hydrolysis of both L-Ala·[^{3}H]Gly and D-Ala·[^{3}H]Gly by isolated microvilli vesicles under the same conditions used in the uptake experiments in order to help elucidate the mechanism of microvilli uptake of dipeptide radiolabel.

Microvilli-Catalyzed Hydrolysis of D- *and* L-*Ala·[^{3}H]Gly*

The hydrolysis of L-Ala·[^{3}H]Gly and D-Ala·[^{3}H]Gly by microvilli in the same medium employed for studying uptake was followed as a function of time. The results are displayed in Fig. 4. An amount of microvilli protein approximately equal to that employed for studying tritium uptake from the dipeptides hydrolyzed all of the L-Ala·[^{3}H]Gly to free L-alanine and [^{3}H]glycine within 15 sec incubation at 37 °C. D-Ala·[^{3}H]Gly, however, was completely resistant to microvilli-catalyzed hydrolysis even after 20 min incubation.

The very rapid hydrolysis of L-Ala·[^{3}H]Gly by the microvilli made it obvious that this dipeptide was hydrolyzed at the external surface of the vesicle with the radiolabel then being transported into the vesicle as free [^{3}H]Gly. At 15 sec incubation, the time at which all of the L-Ala·[^{3}H]Gly in the incubation medium was hydrolyzed, microvilli uptake of ^{3}H from L-Ala·[^{3}H]Gly was less than one-fourth of that achieved at the peak incubation time (60 sec). Substantial microvilli uptake of intact L-Ala·[^{3}H]Gly is therefore doubtful, since intact dipeptide would have to be present at the external surface of the microvilli.

Uptake of radiolabel from D-Ala·[^{3}H]Gly by the microvilli apparently occurs in the form of intact dipeptide, since no hydrolysis was observed even after extended incubation. Whether radiolabel uptake from D-Ala·[^{3}H]Gly represents transport inside the microvilli vesicle or surface binding is not clear. Transport of D-Ala·[^{3}H]Gly inside the microvilli vesicles seems likely since this was the case with radiolabel uptake from L-Ala·[^{3}H]Gly (Fig. 2), but the possibility of radiolabel uptake from D-Ala·[^{3}H]Gly representing binding to the vesicle surface cannot be ruled out without further experimentation.

The dipeptidase activity of the incubation system was bound to the microvilli, since removal of the microvilli by filtration of the incubation system through Millipore filters (0.45 µm) produced a filtrate with insignificant dipeptidase activity.

Uptake from [^{14}C]Gly and L-*Ala·[^{3}H]Gly*

The next step in this investigation was to determine if [^{3}H]Gly obtained from microvilli-catalyzed hydrolysis of L-Ala·[^{3}H]Gly was transported into the vesicles by a carrier system identical to that employed

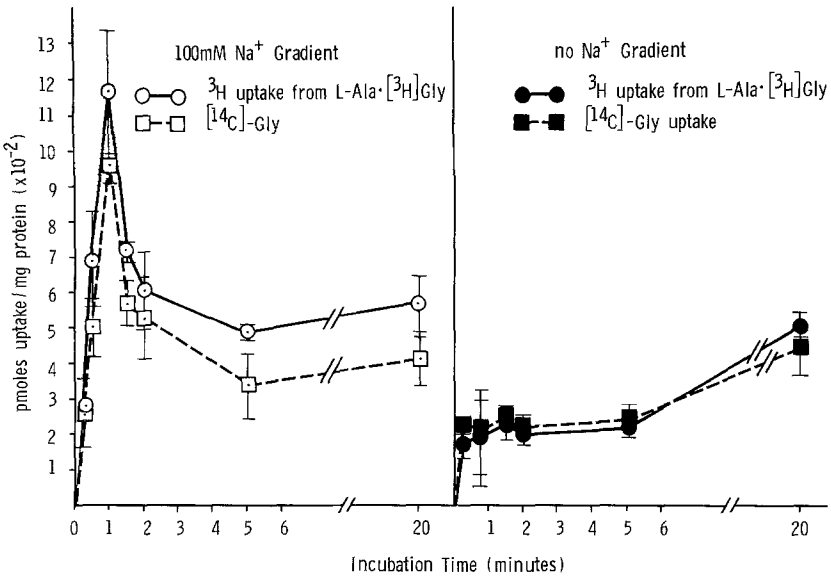

Fig. 5. Radiolabel uptake from 0.06 mM [^{14}C]Gly (□, ■) and 1 mM L-Ala·[^{3}H]Gly (⊙, ●) by renal microvilli vesicles in the presence (□, ⊙) and absence (■, ●) of a 100 mM Na^+ gradient. The incubation buffer was the same as given in the legend to Fig. 1. Amounts of microvilli protein per sample ranged from 118–141 μg. Assays were performed on freshly prepared microvilli. Values are mean values ± SEM and were calculated on the assumption that [^{14}C]Gly and [^{3}H]Gly from L-Ala·[^{3}H]Gly hydrolysis joined the same amino acid pool

for free glycine. Conceivably, microvilli transport of amino acid released as a result of microvilli-catalyzed dipeptide hydrolysis could differ from that of amino acid existing free in the incubation medium. For instance, hydrolysis of L-Ala·[^{3}H]Gly at the microvilli surface could confer a kinetic advantage to the transport of glycine released from the dipeptide as opposed to free glycine present in solution.

A small amount (0.06 mM) of [^{14}C]glycine was added to the microvilli incubation medium in addition to the 1 mM L-Ala·[^{3}H]Gly, and ^{14}C and ^{3}H uptake was observed as a function of incubation time. As shown in Fig. 5, the uptake profiles of [^{14}C]Gly and [^{3}H]Gly from L-Ala·[^{3}H]Gly were very similar. In the presence of a 100 mM Na^+ gradient, ^{14}C and ^{3}H uptake exhibited an overshoot effect with peak uptake occurring at 60 sec incubation. Uptake of both isotopes then declined at approximately the same rate until equilibrium levels were reached after 20 min. If the assumption is made that free [^{14}C]Gly and [^{3}H]Gly released from rapid microvilli hydrolysis of L-Ala·[^{3}H]Gly join the same amino acid pool, the amounts (pmol uptake/mg protein) of [^{14}C]Gly uptake and [^{3}H]Gly uptake from L-Ala·[^{3}H]Gly by the microvilli are nearly equivalent (Fig. 5), as would be expected. The similar patterns of radiolabel uptake from [^{14}C]Gly and L-Ala·[^{3}H]Gly suggest that [^{3}H]Gly released from the rapid hydrolysis of L-Ala·[^{3}H]Gly by the microvilli does indeed join the same amino acid pool as the free [^{14}C]Gly added to the incubation medium. In the absence of Na^+, uptake of ^{14}C and ^{3}H from glycine and L-alanylglycine was identical. No overshoot effect was seen. ^{3}H and ^{14}C uptake gradually increased with incubation time until approximately the same equilibrium values were reached as in the presence of the Na^+ gradient.

Kinetic Parameters of Transport

If [^{3}H]Gly resulting from hydrolysis of L-Ala·[^{3}H]Gly is transported into microvilli vesicles by the same system(s) used for free glycine, the kinetic constants for microvilli uptake of free glycine and glycine from the dipeptide should be identical. The kinetic constants for microvilli uptake of free and dipeptide glycine were therefore determined. In separate experiments the initial rates (30 sec incubation) of microvilli transport of free [^{3}H]Gly and [^{3}H]Gly from L-Ala·[^{3}H]Gly were measured in the presence of different concentrations of substrates (0.1–10.0 mM). Radiolabel uptake by microvilli in the absence of a Na^+ gradient was subtracted from that observed in the presence of a Na^+ gradient in order to obtain constants solely for Na^+ gradient-dependent uptake.

Before the determination of kinetic constants for microvilli transport of free and dipeptide glycine, the extent of microvilli-catalyzed hydrolysis of L-Ala·[^{3}H]Gly was observed for each of the dipeptide concentrations employed in the kinetic studies. Microvilli were incubated for 5 sec with 0.1–10.0 mM (5–500 mmol) L-Ala·[^{3}H]Gly. Amounts of microvilli normally employed in studying transport (118 μg protein) were able to hydrolyze all of the L-Ala·[^{3}H]Gly in the medium in a 5 sec incubation period. This was the case for each dipeptide concentration employed (0.1–10.0 mM).

It was apparent that [^{3}H]Gly uptake from L-

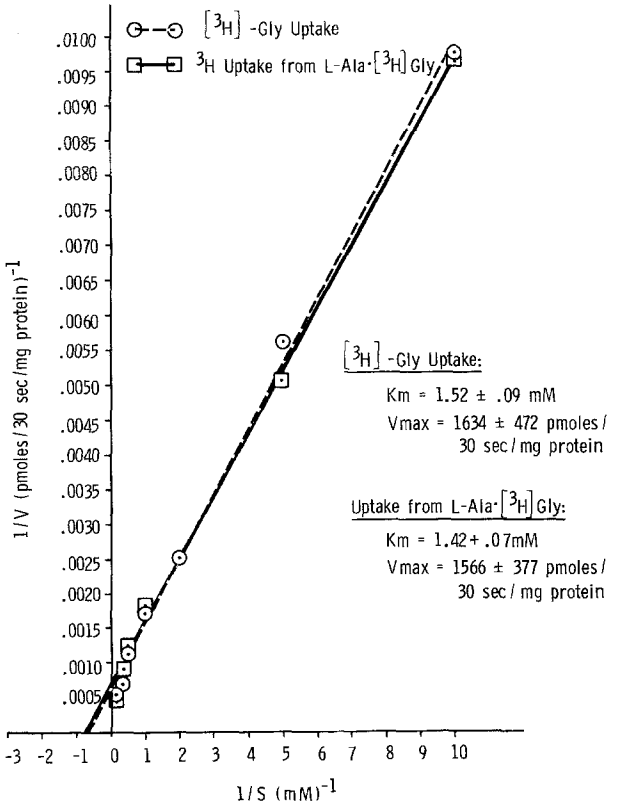

Fig. 6. Lineweaver-Burke plot of net sodium-dependent microvilli transport of free [^{3}H]Gly (⊙) and [^{3}H]Gly from L-Ala·[^{3}H]Gly (⊡). For radiolabel uptake in a Na^+ gradient, the incubation medium (50 µl) contained microvilli (179 µg protein) and 1 mM transport substrate (glycine or dipeptide in 100 mM mannitol, 100 mM NaCl, 20mM Tris-HCl, at pH 7.6. For microvilli uptake in the absence of sodium, microvilli (206 µg protein) and 1 mM substrate were incubated together in 50 µl of 300 mM mannitol, 20 mM Tris-HCl at pH 7.6. Transport in the absence of sodium was subtracted from that observed in the presence of a Na^+ gradient. Values given are the means of quadruplicate assays performed using freshly prepared membranes

Ala·[^{3}H]Gly by isolated renal microvilli consists of two separate events: (a) rapid hydrolysis of the dipeptide at the membrane surface to yield L-alanine and [^{3}H]glycine, and (b) a comparatively slow uptake of the resultant [^{3}H]glycine across the microvilli membrane into the intravesicular space.

The extremely rapid rate of microvilli-catalyzed L-Ala·[^{3}H]Gly hydrolysis meant that transport of [^{3}H]Gly into the intravesicular space and not dipeptide hydrolysis would be the rate-limiting step for [^{3}H]Gly uptake. In effect, the kinetics of microvilli uptake of [^{3}H]Gly from L-Ala·[^{3}H]Gly could be studied in the same manner as for free glycine, since hydrolysis of all intact dipeptide in the incubation system was virtually instantaneous (5 sec or less).

A Lineweaver-Burke plot of Na^+ gradient-dependent microvilli uptake of free [^{3}H]Gly and [^{3}H]Gly from L-Ala·[^{3}H]Gly is presented in Fig. 6. The K_m and V_{max} for microvilli transport of [^{3}H]Gly from L-Ala·[^{3}H]Gly were shown to be 1.42 ± 0.07 mM and 1566 ± 377 pmol/30 sec/mg microvilli protein, respectively. For microvilli uptake of free [^{3}H]Gly, the kinetic constants were $K_m = 1.52 \pm 0.09$ mM and $V_{max} = 1634 \pm 472$ pmol/30 sec/mg protein. Both the K_m and V_{max} values for microvilli uptake of free and dipeptide glycine were identical with the limits of experimental variation (95% confidence limits). Both free [^{3}H]Gly and [^{3}H]Gly from L-Ala·[^{3}H]Gly uptake by the microvilli was saturable, indicating the presence of a carrier-mediated process. Earlier research using kidney cortex slices [34] demonstrated the presence of two transport systems for glycine, a low K_m (0.1 mM) and a high K_m (2.7 mM) system. It was calculated that at glycine concentrations of 0.1 mM and higher the high K_m system predominates (65% of total glycine transport at 0.1 mM to 94% at 6.0 mM glycine). At the levels of free and dipeptide glycine used in our kinetic study, the high K_m system for glycine transport should dominate. Therefore the kinetic constants obtained would reflect glycine transport primarily by the high K_m system.

The finding that the kinetic constants for microvilli transport of free glycine and dipeptide glycine are identical furnishes strong support for the hypothesis that [^{3}H]Gly uptake from L-Ala·[^{3}H]Gly proceeds, following rapid membrane-catalyzed hydrolysis of the dipeptide, via the same system as that involved in uptake of free glycine.

Kinetic Parameters of Hydrolysis

The kinetic constants for microvilli-catalyzed L-Ala·[^{3}H]Gly hydrolysis were determined using the same incubation medium employed to study uptake. The initial rate of hydrolysis of L-Ala·[^{3}H]Gly was measured as a function of dipeptide concentration. Total hydrolysis of dipeptide in each reaction mixture was held to less than 15% by use of low amounts of microvilli protein, in order to keep the availability of dipeptide substrate from becoming rate-limiting. Dipeptide concentrations employed ranged from 0.5–10.0 mM. A Lineweaver-Burke plot of the hydrolysis data gave a K_m for dipeptide hydrolysis of 2.73 ± 0.68 mM and a V_{max} of 47.6 ± 19.6 µmol/min/mg membrane protein. These values are presented with 95% confidence limits.

Of particular interest is the finding that the V_{max} for L-Ala·[^{3}H]Gly hydrolysis is over 15,000 times greater than that observed for microvilli transport of [^{3}H]Gly from the dipeptide.

Renal Dipeptidase Activity in Microvilli

The next objective of this study was to investigate the microvilli enzyme(s) responsible for the high dipeptidase activity exhibited by the microvilli vesicles. A number of peptidases have been found to be localized in the renal brush border [25]: aminopeptidase A, aminopeptidase M, neutral endopeptidase, and dipeptidyl peptidase IV. Only aminopeptidase A and aminopeptidase M have any activity against dipeptides. Aminopeptidase A can catalyze hydrolysis of dipeptides but only if their N-terminal amino acid is L-glutamic acid or L-aspartic acid [18]. Aminopeptidase M catalyzes the hydrolysis of N-terminal amino acids from peptides of various sizes [31]. However, aminopeptidase M has very low catalytic activity against dipeptides compared to larger peptide substrates. Therefore, it is possible that another microvilli enzyme is responsible for the rapid depeptide hydrolysis exhibited by the microvilli.

Renal dipeptidase, a strict dipeptidase that catalyzes the hydrolysis of a wide variety of dipeptides (including L-Ala·Gly) has been shown to be restricted to the renal cortex [22]. Fractionation studies in this laboratory have revealed that renal dipeptidase is concentrated in the plasma membranes of the renal cortical tubules.[1,2] These findings raise the possibility that renal dipeptidase could be concentrated in the brush border microvilli of the cortical proximal tubules. Such an anatomic locus for this enzyme could suggest its involvement in the rapid hydrolysis of L-Ala·Gly by the microvilli vesicles.

Isolated brush border microvilli, as well as kidney cortex homogenate, were assayed for renal dipeptidase activity by the procedure of Rene and Campbell [37], which employs glycyldehydrophenylalanine (GdP) as the substrate. Renal cortex homogenate was found to catalyze hydrolysis of 11.0 ± 0.1 nmol GdP/min/mg protein while the isolated microvilli hydrolyzed 224 ± 14 nmol GdP/min/mg protein. Therefore the specific activity of renal dipeptidase in the microvilli is 20.4 times greater than that of kidney cortex homogenate.

The percent recovery of renal dipeptidase activity in the isolated microvilli was 7.33%. This is somewhat higher than the recovery of the brush border markers, alkaline phosphatase (4.86%) and trehalase (3.70%), and probably explains why renal dipeptidase is enriched to a greater degree in the microvilli than these two marker enzymes. The higher percent recovery of renal dipeptidase activity may be due to an activation of the enzyme during isolation of the microvilli. Another possibility is that renal dipeptidase may be localized in a particular region of the brush border membrane which is recovered in higher yield than other regions of this membrane. The membrane preparation procedure used in this study produces shearing of the renal microvilli from the rest of the brush border membrane. If renal dipeptidase is concentrated in the microvilli tips to a greater degree than alkaline phosphatase or trehalase, it could be more highly enriched in the microvilli preparation.

The high enrichment of renal dipeptidase in the microvilli preparation certainly suggests that this enzyme is a marker for these structures, but subcellular fractionation studies are necessary to establish this fact definitely.

Percentage of Microvilli Membrane Protein Present as Renal Dipeptidase

The specific activities of pure renal dipeptidase and the microvilli-bound enzyme were determined from measuring their hydrolysis of glycyldehydrophenylalanine according to the method of Rene and Campbell [37]. The percentage of microvilli protein due to renal dipeptidase was calculated by dividing the microvilli renal dipeptidase specific activity by that of the pure enzyme and multiplying by 100. The average specific activity for microvilli-bound renal dipeptidase was 0.22 μmol/min/mg membrane protein while that observed for the pure enzyme was 6.94 μmol/min/mg protein. Renal dipeptidase was therefore found to comprise 3.2% of the total microvilli protein.

Approximately the same amounts of enzyme protein have been reported for other peptidases shown to be located in the brush border microvilli. Aminopeptidase M has been shown to comprise 4.1–4.8% of the microvilli protein [17], while Kenny et al. [26] reported that dipeptidyl peptidase IV constituted 3.9% of the renal microvilli protein. Additional studies are necessary before the contribution of renal dipeptidase to microvilli hydrolysis of L-Ala·Gly (as well as other dipeptides) can be quantitated.

Discussion

In this study the uptake of radiolabel from L-Ala·[^{3}H]Gly and D-Ala·[^{3}H]Gly by isolated renal brush border microvilli vesicles was investigated. The renal microvilli employed in this research were checked by marker enzyme analysis (Table 1) and electron microscopy and found to be vesicular membrane preparations free from significant contamination by other subcellular organelles. In addition, renal

[1] L.J. Ferren, *unpublished observations.*
[2] J.M. Mullins, *unpublished observations.*

microvilli prepared by the method utilized in this study have been shown to have the proper orientation, that is, the brush border surface facing the exterior [21, 41].

Microvilli uptake of radiolabel from L-Ala·[^{3}H]Gly exhibited a Na^+ gradient-dependent "overshoot" which peaked after 60 sec incubation at a level approximately twice that attained at equilibrium (Fig. 1). Radiolabel uptake from D-Ala·[^{3}H]Gly exhibited no Na^+ gradient-dependent "overshoot", being virtually the same in the presence or absence of a Na^+ gradient (Fig. 1).

Uptake of L-Ala·[^{3}H]Gly radiolabel increased linearly with the amount of microvilli protein employed and represented transport into an intravesicular space rather than surface binding to the microvilli (Fig. 2).

Analysis of microvilli intravesicular contents following incubation with L-Ala·[^{3}H]Gly revealed that the vesicles contained free [^{3}H]Gly rather than intact dipeptide. This was the case even after only 30 sec incubation with the radiolabeled dipeptide (Fig. 3). Hydrolysis studies demonstrated that all L-Ala·[^{3}H]Gly present in the microvilli transport system was hydrolyzed to L-Ala and [^{3}H]Gly within 15 sec incubation (Fig. 4). Such rapid hydrolysis suggested that radiolabel uptake from L-Ala·[^{3}H]Gly by the microvilli represented uptake of free [^{3}H]Gly rather than intact dipeptide. Further experiments comparing radiolabel uptake as a function of time, as well as the kinetic constants for microvilli transport of free glycine and [^{3}H]glycine from L-Ala·[^{3}H]Gly (Figs. 5–6), indicate that free and dipeptide glycine are taken into the microvilli vesicles by the same transport system. Transport of free glycine and glycine from L-Ala·Gly appears to occur via a carrier-mediated system that exhibits a Na^+ gradient-dependent "overshoot" effect. The absolute values of the kinetic constants for transport of free glycine and glycine from L-Ala·Gly hydrolysis could conceivably be affected by the changing driving forces of the Na^+ gradient as it decays. However, the kinetic data for transport of free glycine and glycine from L-Ala·Gly were obtained under identical Na^+ gradient conditions and incubation time, so the fact that their kinetic constants are identical would not be changed.

D-Ala·[^{3}H]Gly is completely resistant to microvilli-catalyzed hydrolysis (Fig. 4) and exhibits no Na^+ gradient-dependent "overshoot" (Fig. 1). Microvilli uptake of radiolabel from D-Ala·[^{3}H]Gly is in the form of intact dipeptide. Further experiments are necessary to establish whether this uptake represents transport, as is the case for radiolabel uptake from L-Ala·[^{3}H]Gly, or simply D-Ala·[^{3}H]Gly binding to the microvilli surface.

The data presented in this paper suggests that uptake of L-dipeptide amino acids by renal microvilli occurs by hydrolysis of the dipeptides at the external surface of the membrane, followed by transport of the released amino acids by the same Na^+ gradient-stimulated, carrier-mediated processes known to exist for free amino acids [15, 16]. Further uptake studies using a variety of radiolabeled dipeptides are necessary before these microvilli systems for transport of amino acids from dipeptides can be definitely established.

The high enrichment of renal dipeptidase in renal brush border microvilli raises two possibilities: (i) renal dipeptidase may be a marker enzyme for renal brush border microvilli, as it is enriched in the microvilli to a higher degree than such known brush border markers as alkaline phosphatase and trehalase. However, the presence of renal dipeptidase in other renal subcellular organelles cannot yet be definitely ruled out. (ii) renal dipeptidase may be involved in the rapid hydrolysis of L-Ala·Gly (and possibly other dipeptides) exhibited by the microvilli. Renal dipeptidase has been demonstrated to catalyze the hydrolysis of a wide range of dipeptides, with L-Ala·Gly being the dipeptide against which the enzyme has the greatest hydrolytic activity [22]. Earlier work has shown renal dipeptidase to have no catalytic activity against dipeptides containing D-amino acids [11], as is supported in this study by its inability to hydrolyze D-Ala·[^{3}H]Gly. The renal microvilli also did not catalyze hydrolysis of D-Ala·[^{3}H]Gly.

Although several enzymes capable of degrading peptides have been found to be localized in the renal brush border, renal dipeptidase appears to be the only strict dipeptidase thus far located in this membrane fraction. The peptidases found in renal brush border membranes are aminopeptidase A, aminopeptidase M, neutral endopeptidase and dipeptidyl peptidase IV [25]. Only aminopeptidase A and aminopeptidase M have any dipeptidase activity and, as mentioned earlier, this activity is of a limited nature. Therefore, renal dipeptidase could be responsible for at least a portion of the dipeptidase hydrolytic capacity exhibited by renal brush border microvilli.

With its enrichment in the brush border microvilli and its activity against a large number of dipeptides, renal dipeptidase could participate in the hydrolysis of dipeptides filtered from the blood by the renal glomeruli and passed into the lumen of the proximal tubules. Amino acids released from dipeptide hydrolysis could then be transported across the brush border into the proximal tubule cells by the same Na^+ gradient-stimulated transport systems available to free amino acids, thus making separate dipeptide transport systems unnecessary.

Probably the most likely source of circulatory di-

peptides would be from the intestine following a protein meal. Although protein digestion products enter the portal blood from the intestine predominately in the form of free amino acids [33], studies have demonstrated elevated plasma levels of dipeptides following a protein meal [36] or the infusion of dipeptides into the intestinal lumen [1, 9]. Renal dipeptidase could be involved in renal microvilli salvaging of dipeptide amino acids that otherwise would be lost in the urine. This could be of particular importance in retaining essential amino acids present in the plasma dipeptides.

Another possible function for renal dipeptidase would involve completion of the hydrolysis of small peptides filtered from the blood by the kidneys. The combined specificities of the microvilli enzymes aminopeptidase A, aminopeptidase M, dipeptidyl peptidase IV, neutral endopeptidase, and renal dipeptidase would allow complete degradation of a variety of peptides present in the proximal tubules following filtration from the blood. Dipeptides produced from the hydrolytic action of the four brush border peptidases could be hydrolyzed to free amino acids by renal dipeptidase and absorbed by the proximal tubule cells. Such a coordinated enzyme system could play a particularly important role in the hydrolysis and removal of peptide hormones passing from the glomeruli into the proximal tubules.

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