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The High and Low Affinity Transport Systems for Dipeptides in Kidney Brush Border Membrane Respond Differently to Alterations in pH Gradient and Membrane Potential*

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The principal aim of the present study was to investigate the effects of variation in proton gradient and membrane potential on the transport of glycyl-L-glutamine (Gly-Gln) by renal brush border membrane vesicles. Under our conditions of transport assay, Gly-Gln was taken up by brush border membrane vesicles almost entirely as intact dipeptide. This uptake was mediated by two transporters shared by other dipeptides and characterized as the high affinity (K_t = 44.1 ± 11.2 μM)/low capacity (V_{max} = 0.41 ± 0.03 nmol/mg protein/5 s) and low affinity (K_t = 2.62 ± 0.50 mM)/high capacity (V_{max} 4.04 ± 0.80 nmol/mg protein/5 s) transporters. In the absence of a pH gradient, only the low affinity system was operational, but with a reduced transport capacity. Imposing a pH gradient of 1.6 pH units increased the V_{max} of both transporters. Kinetic analysis of the rates of Gly-Gln uptake as a function of external pH revealed Hill coefficients of close or equal to 1, indicating that transporters contain only one binding site for the interaction with external H^+. The effects of membrane potential on Gly-Gln uptake were investigated with valinomycin-induced K^+ diffusion potentials. The velocity of the high affinity system but not of the low affinity system increased linearly with increasing inside-negative K^+ diffusion potentials ($p < 0.01$). The K_t of neither system was affected by alterations in either pH gradient or membrane potential. We conclude that (a) the high affinity transporter is far more sensitive to changes in proton gradient and membrane potential than the low affinity transporter and (b) in the presence of a pH gradient, transport of each dipeptide molecule requires cotransport of one hydrogen ion to serve as the driving force.

A metabolically important function of kidney and intestine is the uptake of dipeptides and tripeptides by an active transport system. The uniqueness of this function in kidney and intestine is underscored by the results of recent studies which have shown lack of such a system in tissues such as liver, skeletal muscle, and red blood cells (1-4).

The initial discovery of an active transport system for dipeptides and tripeptides in the intestine (5) prompted questions regarding its driving force. An obvious candidate appeared to be the Na^+ gradient, because it was shown to be necessary for transport of amino acids and glucose. Studies on the Na^+ dependence of dipeptide transport, using preparations of intestinal segments, however, produced conflicting results (6). Recently, Ganapathy *et al.* (7) and Takuwa *et al.* (8), using brush border membrane vesicles of intestine, clearly show that transport of dipeptides is not dependent on a Na^+ gradient, but rather on a H^+ gradient. A H^+ gradient may stimulate dipeptide transport by increasing the apparent affinity (K_t), increasing the reaction velocity (V_{max}), or both. Studies aimed at discerning these mechanisms of H^+ stimulation of dipeptide transport in intestinal brush border membrane vesicles have come to different conclusions. Ganapathy *et al.* (7) found that an inwardly directed proton gradient stimulates intestinal glycylsarcosine transport by increasing the V_{max} of the transport system, whereas the apparent K_t was unaffected. On the other hand, the opposite was found for the intestinal transport of glycylglycine by Takuwa *et al.* (8).

Dipeptide transport by brush border membrane vesicles of kidney, like intestine, is stimulated by a proton gradient (9). However, the effect of a proton gradient on the kinetic parameters of dipeptide transport has not been studied yet in kidney. In view of the conflicting observations in the intestine, as reviewed above, the present studies were performed in kidney brush border membrane vesicles to investigate the mechanism of stimulation of dipeptide transport by decreasing the extravesicular pH. These studies were performed in the presence and absence of a membrane potential. Although membrane potential is known to affect dipeptide transport (9), its impact on the kinetics of transport has not yet been studied in either intestine or kidney.

We used glycylglutamine to characterize peptide transport in the kidney. Glutamine is a major substrate for kidney metabolism. Due to its instability (10), glutamine currently is not included in amino acid solutions; on the other hand, glycylglutamine is quite stable and currently is being considered as a source of glutamine for inclusion in intravenous solutions (11). In addition, recent studies have shown that during intravenous infusion of glycylglutamine, kidney plays a greater role than either liver, muscle, or intestine in removing this dipeptide from plasma (12). A knowledge of glycylglutamine transport by the kidney, therefore, may have useful clinical applications.

Because glycylglutamine was a novel substrate, we initially performed a series of studies to characterize the renal transport of this dipeptide. These included investigation of: (a) membrane binding, (b) the optimal conditions for transport, (c) hydrolysis outside and inside of the vesicles, and (d) effect of substrate concentration on the transport kinetics. These studies, besides being necessary for the present investigation,

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advanced the current knowledge regarding functional characteristics of peptide transport.

EXPERIMENTAL PROCEDURES

Materials—Adult male Sprague-Dawley rats (250–300 g) were purchased from Zivic-Miller (Allison Park, PA). Custom-synthesized [glutamine-3,4-^{3}H]glycyl-L-glutamine (49 Ci/mmol) and [alanyl-U-^{14}C]glycyl-L-alanine (168 mCi/mmol), as well as D-[U-^{14}C]glucose (340 mCi/mmol) were obtained from Du Pont-New England Nuclear. All unlabeled compounds were from Sigma or Bachem BioScience Inc. (Philadelphia, PA). Reagents for protein-dye-binding were obtained from Bio-Rad. Enzyme kits for γ-glutamyltransferase and leucine-aminopeptidase activity were also obtained from Sigma.

Membrane Vesicle Preparation—Rats were fed Purina rat chow ad libitum. After the animals were killed, kidneys were removed quickly and decapsulated. Brush border membrane vesicles were prepared from kidney tissue comprising the outer medulla and cortical regions by Mg^{2+} aggregation in the presence of EGTA[1] (13). For each preparation tissues from six rats were homogenized 1:10 (w/v) in ice-cold buffer (12 mM Tris/NaOH, 5 mM EGTA, and 300 mM mannitol, pH 7.5) using an Ultra-Turrax at a speed of 50 for 90 s. Ice-cold distilled water was added to the homogenate (1:1, v/v) and 1 M $MgCl_2$ was added to give a final concentration of 10 mM. After stirring for 1 min, the suspension was allowed to stand for 15 min and then centrifuged at 3000 × *g* for 10 min. The supernatant was then centrifuged at 38,000 × *g* for 30 min. The pellets were suspended in a suitable preloading buffer using a 26-gauge needle and centrifuged at 38,000 × *g* for 30 min. This procedure was repeated twice.

The final pellets containing the brush border membranes were loaded for most of the experiments with 50 mM Hepes, 75 mM Tris, and 100 mM K_2SO_4 (pH 8.3) or 45 mM Hepes, 40 mM Mes, 40 mM Tris, and 100 mM K_2SO_4 (pH 7.4). The protein concentration of the membrane suspensions was adjusted to 7.5 mg/ml and vesicles were stored in liquid nitrogen until use. Storage of the vesicles for at least 3 months did not cause any loss in transport activity.

Uptake Measurements—Uptake studies were performed at 22–24 °C by using a rapid filtration technique with Millipore filters (HAWP type, 0.45 μm pore size). Uptake was initiated by rapidly mixing 40 μl of membrane suspension (300 μg of protein) with 160 μl of medium containing [glutamine-3,4-^{3}H]glycyl-L-glutamine (2–4 μCi/ml) or [alanine-U-^{14}C]glycyl-L-alanine (1–3 μCi/ml). The composition of the uptake buffer varied depending upon the experiment, but in most cases it was 50 mM Hepes, 50 mM Mes, 25 mM Tris (pH 6.0), and 300 mM mannitol. Mixing 40 μl of membrane vesicles preloaded with buffer (pH 8.3), with 160 μl of medium (pH 6.0), resulted in a final incubation pH of 6.7. When transport studies were performed in the presence of a K^+ diffusion potential or a voltage clamp, the K^+-specific ionophore valinomycin (12.5 μg/mg membrane protein) was added in ethanol. Carrier ethanol was added to the corresponding controls. Incubation of vesicles was terminated by addition of 5 ml of ice-cold stop solution comprising 2 mM Hepes/Tris (pH 7.5) and 210 mM KCl followed by filtration. The filter was washed twice with 5 ml of ice-cold stop buffer, and the radioactivity associated with the filter was counted in 10 ml of Ecoscint H (National Diagnostics, Manville, NY) in a β-scintillation counter (Packard TriCarb, Downers Grove, IL).

Hydrolysis of Gly-Gln—To determine the extent of hydrolysis of Gly-Gln in both the incubation medium and the intravesicular contents, vesicles (preloaded with pH 8.3 buffer) were incubated as described above for different time intervals with 100 μM Gly-[^{3}H]Gln in buffer, pH 6.0 (final pH 6.7), at 24 °C. Incubation of vesicles was terminated by 5 ml of ice-cold stop buffer and filtration. The filtrate was trapped in 2.5 ml of 18% sulfosalicylic acid and filters were extracted with 1 ml of 6% sulfosalicylic acid. The filtrate and the filter extract were diluted with 6% sulfosalicylic acid to contain approximately 10,000 dpm/ml. 100 μl of the diluted medium or vesicular contents were applied to a Varian chromatograph and eluted (12). Fractions were collected for 1-min intervals and counted in 10 ml of Aquasol-2 (Du Pont-New England Nuclear). The radioactivity associated with the dipeptides and their constituent amino acids was quantified using the standard elution profile.

Calculations and Statistics—Absolute velocity data as a function of substrate concentration were first corrected for the diffusional component as measured at 4 °C (K_d: 0.89 ± 0.08 μmol/mg/5 s/mM). The resulting data were fitted by iteration to an equation involving the sum of two saturable components. Analysis was performed by using INPLOT (Graph Pad, San Diego, CA) with initial estimates as derived from inspection of Eadie-Hofstee plots.

Linear regression analysis of the uptake data as a function of medium osmolarity and membrane potential was performed by the method of least-squares.

All experiments were carried out with at least three preparations in triplicate. The values presented, therefore, represent the mean ± S.E. from at least three preparations. Comparison of data for significance were performed by analysis of variance followed by the Student-Neuman-Keuls test.

Purity of the Brush Border Membrane Vesicle Preparations—The purity of brush border membrane preparations was assayed by the marker enzymes leucine aminopeptidase (EC 3.4.11.1), γ-glutamyltransferase (EC 2.3.2.2), and K^+-stimulated *p*-nitrophenylphosphatase (EC 3.6.1.3) (14). There was a 23-fold enrichment in the specific activity of leucine aminopeptidase in the final membrane fraction compared with the initial homogenate (2.89 ± 0.96 *versus* 66.62 ± 6.70 units/mg of protein) and a 14-fold enrichment in γ-glutamyltransferase activity (1.33 ± 0.16 *versus* 18.80 ± 3.22 units/mg of protein). *p*-Nitrophenylphosphatase, a marker enzyme of the basolateral membrane, was not enriched in BBMV compared with the crude homogenate (0.166 ± 0.013 and 0.230 ± 0.011 units/mg protein, respectively).

Validation of Transport Function—The time course of [^{14}C]glucose uptake by brush border membrane vesicles was used as a parameter for the validation of vesicle function and integrity. Studies of the time-dependent uptake of glucose into BBMV were performed in media containing 45 mM Hepes, 20 mM Tris, 100 mM mannitol (pH 7.4), 100 μM glucose, and either 100 mM NaCl or 100 mM KCl. The stop solution contained 10 mM Hepes/Tris (pH 7.4), 300 mM mannitol, and 0.1 mM phloridzin. Uptake studies were performed at 24 °C as described above. A 5-fold overshoot at 15 s of incubation compared to the equilibrium value (60 min) was observed when vesicles were incubated in a medium containing 100 mM NaCl. In the KCl medium glucose entry into the BBMV was slow and reached the equilibrium value after about 20 min. The distribution volume of glucose after 30 min of incubation at increasing medium mannitol concentrations was found to be 1.27 ± 0.32 μl/mg of protein.

RESULTS

Time Course of Gly-Gln Uptake—To measure uptake as a function of time, BBMV were incubated with Gly-Gln for a time period of 2 s to 30 min (Fig. 1). Incubation was performed in the presence of a pH gradient (pH_{in} 8.3/pH_{out} 6.7) and in the absence of a pH gradient (pH_{in} 8.3/pH_{out} 8.3).

In the presence of a pH gradient, uptake was linear up to 8 s and exhibited an overshoot of six times the equilibrium value (0.962 ± 0.062 *versus* 0.169 ± 0.019 pmol/mg protein). In the absence of a pH gradient, influx of Gly-Gln never exceeded the equilibrium value. To study uptake when it was linear with respect to time, an incubation time of 5 s was chosen for all subsequent transport studies.

Effect of Medium Osmolarity on Gly-Gln Uptake—To distinguish between transport into an osmotically reactive space and binding to the membrane, Gly-Gln uptake was studied in the presence of increasing concentrations of mannitol. Uptake was terminated by ice-cold stop buffer adjusted to the same osmolarity as the incubation medium. Linear regression analysis of uptake *versus* 1/osm (Fig. 2) revealed no binding of Gly-Gln to the membrane (intercept not significantly different from zero).

Hydrolysis of Gly-Gln by BBMV—As will be shown below, brush border membranes of renal tubular cells possess hydrolase activity against Gly-Gln. Therefore, it is possible that glycyl[^{3}H]glutamine, when incubated with BBMV, could be hydrolyzed prior to transport. If the radioactivity entering BBMV were free glutamine, then uptake should be increased in the presence of Na^+ or reduced in the presence of unlabeled

[1] The abbreviations used are: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; BBMV, brush border membrane vesicles; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

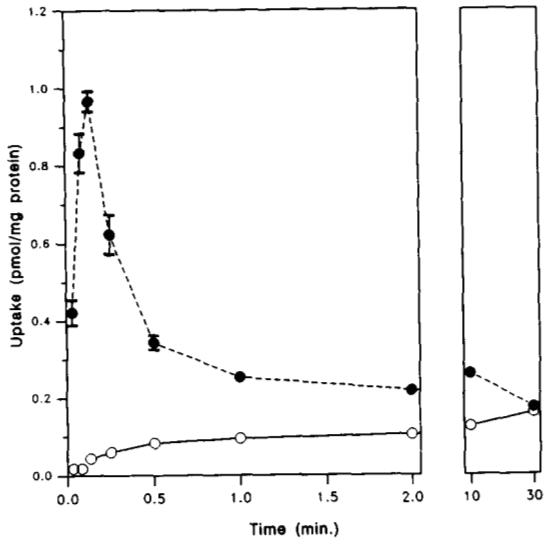

FIG. 1. **Time course of Gly-Gln uptake in the presence and absence of an inwardly directed pH gradient.** BBMV were preloaded with 50 mM Hepes, 75 mM Tris buffer (pH 8.3), containing 100 mM K_2SO_4. Uptake of Gly-Gln (0.1 μM) was measured either in 50 mM Hepes, 75 mM Tris buffer (pH 8.3), and 300 mM mannitol (*open circles*) or 50 mM Hepes, 50 mM Mes, 25 mM Tris, pH 6.0 (final pH 6.7), and 300 mM mannitol (*filled circles*). The data represent mean ± S.E. of three membrane preparations.

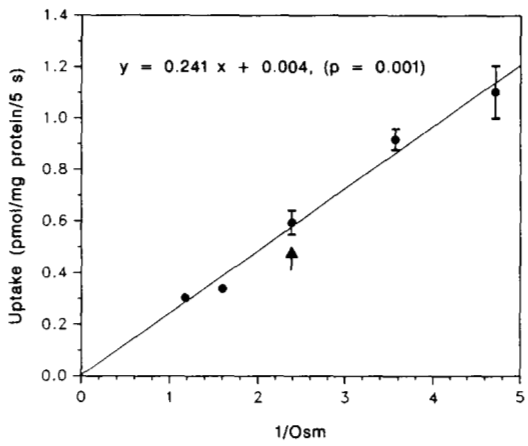

FIG. 2. **Effect of osmolarity on Gly-Gln uptake.** BBMV were preloaded with 50 mM Hepes, 75 mM Tris buffer (pH 8.3), containing 100 mM K_2SO_4. Uptake of 0.1 μM Gly-Gln was measured at 5 s of incubation in buffer containing 50 mM Hepes, 50 mM Mes, 25 mM Tris, pH 6.0 (final pH 6.7) with varying concentrations of mannitol. Incubation was terminated by adding 5 ml of ice-cold stop solution containing 2 mM Hepes/Tris (pH 7.5) adjusted with mannitol to the same osmolarity as the different incubation solutions. The data represent mean ± S.E. of three membrane preparations. The *arrow* indicates the medium osmolarity under normal uptake conditions.

free glutamine in the incubation medium. To determine whether the radioactivity which enters BBMV during transport studies was in the form of free glutamine or Gly-Gln, the following studies were performed.

Glutamine uptake from Gly-Gln (0.05 μM) was studied in the presence and absence of Na^+ (100 mM) and in the presence and absence of free glutamine (1 and 5 mM). The effects of Na^+ and free glutamine were studied in the absence and the presence of a pH gradient (Table I). Neither Na^+ nor free glutamine had any significant effect on the uptake of glutamine from Gly-Gln either in the presence or the absence of a pH gradient (Table I). These results suggest that, under our conditions of transport assay, there was very little or no

TABLE I

Effect of a Na^+ gradient and free glutamine on the uptake of Gly-Gln in the absence and the presence of a pH gradient

Without pH gradient, BBMV preloaded with 45 mM Hepes, 20 mM Tris, 320 mM mannitol (pH 7.4) were incubated for 5 s in 0.05 μM Gly-Gln, 45 mM Hepes, 20 mM Tris (pH 7.4), and either 320 mM mannitol or 1 mM glutamine, and 319 mM mannitol, 5 mM glutamine, and 315 mM mannitol, 100 mM NaCl, and 120 mM mannitol. With pH gradient, BBMV preloaded with 50 mM Hepes, 75 mM Tris, 100 mM K_2SO_4 (pH 8.3) were incubated in media comprising 0.05 μM Gly-Gln, 50 mM Hepes, 50 mM Mes, 25 mM Tris (pH 6.0) containing 300 mM mannitol or 1 mM glutamine and 299 mM mannitol, 5 mM glutamine and 295 mM mannitol, or 100 mM NaCl and 100 mM mannitol. The data represent mean ± S.E. of three membrane preparations.

Conditions of incubation		pH gradient	
NaCl	Gln	Without	With
mM		*pmol/mg protein/5 s*	
0	0	0.057 ± 0.016	0.245 ± 0.022
100	0	0.064 ± 0.020	0.220 ± 0.013
0	1	0.070 ± 0.015	0.266 ± 0.034
0	5	0.065 ± 0.022	0.236 ± 0.029

TABLE II

Recovery of intact Gly-Gln in the incubation medium and intravesicular contents

BBMV were preloaded with 50 mM Hepes, 75 mM Tris, 100 mM K_2SO_4 (pH 8.3). Recovery of intact Gly-Gln was measured as described after incubation of BBMV in medium containing 50 mM Hepes, 50 mM Mes, 25 mM Tris, 300 mM mannitol (pH 6.0) and 100 μM Gly-Gln. The data represent mean ± S.E. of two membrane preparations.

Incubation time	Medium	BBMV
	% peptide remaining intact	
5 s	98.3 ± 2.3	95.8 ± 1.8
30 s	92.4 ± 2.3	88.8 ± 2.6
1 min	85.7 ± 5.2	83.7 ± 1.4
30 min	67.9 ± 1.2	56.0 ± 1.7
60 min	48.5 ± 2.0	39.8 ± 3.1

hydrolysis of Gly-Gln prior to transport.

To investigate further the validity of this conclusion, the recovery of Gly-Gln as dipeptide was determined in the incubation medium and intravesicular contents as a function of time. As shown in Table II, free glutamine accounted for only 5–15% of the total tracer within the first minute of incubation both in the medium and in the vesicles. After 60 min of incubation, Gly-Gln was hydrolyzed by about 50% in the medium and about 60% in the vesicles. The point which is pertinent to emphasize is that at 5 s of incubation, the time period for measuring transport activity, over 95% of radioactivity in both the incubation medium and the intravesicular contents was in the form of intact dipeptide. Thus, uptake under the conditions of our transport assay represents almost completely transport of intact peptide.

Uptake of Gly-Gln as a Function of Substrate Concentration—To investigate the mechanism of uptake, BBMV were incubated with a range of concentrations (0.05 μM to 10 mM) of Gly-Gln in the presence of a pH gradient (pH_{in} 8.3/pH_{out} 6.7). As shown in Fig. 3, Gly-Gln uptake was saturable as a function of substrate concentration. The Eadie-Hofstee plot of the data shown in the inset to Fig. 3 reveals the presence of a high affinity and a low affinity transport system. The kinetic constants of the two affinity sites were determined by nonlinear regression analysis and least-squares fitting (Table III). These results indicate that the two transporters differ by

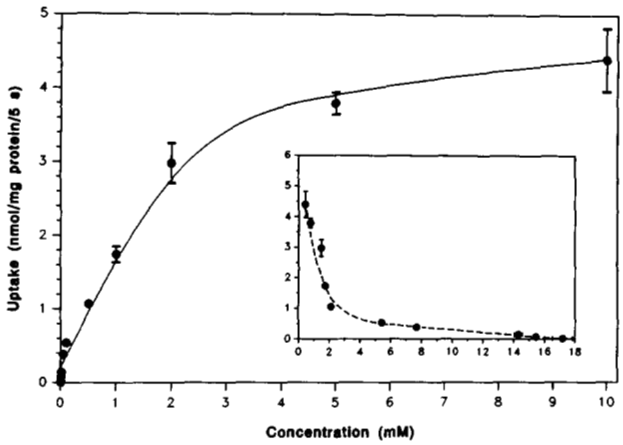

FIG. 3. **Kinetics of Gly-Gln uptake as a function of Gly-Gln concentration in the presence of a pH gradient.** Membrane vesicles were preloaded with 50 mM Hepes, 75 mM Tris, 100 mM K_2SO_4 (pH 8.3) and incubated in buffer containing 50 mM Hepes, 50 mM Mes, 25 mM Tris, pH 6.0 (final pH 6.7), and 290–300 mM mannitol according to the concentration of Gly-Gln (0.1 μM to 10 mM) to equalize medium osmolarity. Uptake data represent only the saturable component after subtraction of the diffusional component as measured under the same experimental conditions except that the incubation temperature was 4 °C. *Inset*: Eadie-Hofstee plot (*v versus v/S*) of the saturable Gly-Gln uptake where *v* is the rate of uptake and *S* the Gly-Gln concentration in millimolar. The data represent mean ± S.E. of three membrane preparations.

TABLE III

Kinetic parameters of Gly-Gln and Gly-Ala uptake

BBMV were incubated in the presence of a pH gradient (pH_{in} = 8.3, final pH_{out} = 6.7) with Gly-Gln (0.1 μM to 10 mM) and Gly-Ala (25 μM to 10 mM). Kinetic constants were derived as described under "Experimental Procedures." The rate of Gly-Ala hydrolysis in the medium and the intravesicular contents was determined under the same experimental conditions as described for Gly-Gln (see "Experimental Procedures"). Gly-Ala recovery after 5 s of incubation was 96.0 ± 0.8% in the medium and 84.8 ± 2.6% in intravesicular contents. The data represent mean ± S.E. of three membrane preparations. HA and LA refer to the high affinity and low affinity transport systems, respectively.

Dipeptide	K_t		V_{max}	
	HA	LA	HA	LA
	μM	*mM*	*nmol/mg protein/5 s*	
Gly-Gln	44.1 ± 11.2	2.62 ± 0.50	0.41 ± 0.03	4.04 ± 0.80
Gly-Ala	101.5 ± 20.9	3.07 ± 0.68	0.65 ± 0.20	3.72 ± 1.27

50-fold with respect to substrate affinity and by 10-fold with respect to maximal velocity.

Relevance of Gly-Gln Transport to Other Dipeptides—To investigate whether the existence of two transport systems is unique to Gly-Gln or is common to other dipeptides, we investigated the mechanism of uptake of Gly-Ala exactly as we did that of Gly-Gln. BBMV were incubated with a range of concentrations (25 μM to 10 mM) of Gly-Ala in the presence of a pH gradient (pH_{in} 8.3/pH_{out} 6.7). Gly-Ala transport was also found to be mediated by a high affinity system and a low affinity system. As shown in Table III, the K_t and V_{max} values of Gly-Ala transport were not significantly different from the corresponding values of Gly-Gln transport.

To investigate whether Gly-Gln and other dipeptides share the same transport system, the effect of other dipeptides on Gly-Gln uptake was studied. The concentrations of Gly-Gln and dipeptides in the incubation medium were 0.1 μM and 1 mM, respectively. The incubation was maintained for 5 s in the presence of a pH gradient (pH_{in} 8.3/pH_{out} 6.7). All dipeptides tested inhibited uptake of Gly-Gln (Table IV). The inhibition ranged from 63 to 86%.

TABLE IV

Effect of other dipeptides on Gly-Gln uptake

BBMV preloaded with 50 mM Hepes, 75 mM Tris, 100 mM K_2SO_4 (pH 8.3), were incubated for 5 s in buffers comprising 50 mM Hepes, 50 mM Mes, 25 mM Tris, 300 mM mannitol (pH 6.0), and 0.1 μM Gly-Gln either in the absence (control) or the presence of 1 mM other dipeptide. The data represent mean ± S.E. of two membrane preparations.

Dipeptide	Uptake	Control
	pmol/mg protein/5 s	%
None	0.861 ± 0.045	100.0
Gly-Lys	0.319 ± 0.014	37.1
Gly-Glu	0.242 ± 0.020	28.1
Gly-Pro	0.198 ± 0.012	23.0
Gly-Gly	0.165 ± 0.008	19.2
Gly-His	0.162 ± 0.010	18.8
Gly-Thr	0.149 ± 0.024	17.3
Gly-Ala	0.132 ± 0.009	15.4
Gly-Tyr	0.122 ± 0.008	14.3

TABLE V

Effect of different pH gradients in the presence of a voltage clamp on the kinetic parameters of Gly-Gln uptake

BBMV preloaded with 45 mM Hepes, 75 mM Tris, 100 mM K_2SO_4, 10 mM mannitol (pH 8.3) were incubated in media comprising either 50 mM Hepes, 50 mM Mes, 20 mM Tris (final pH 6.7); 40 mM Hepes, 40 mM Mes, 45 mM Hepes, 40 mM Tris (final pH 7.1); 40 mM Hepes, 35 mM Mes, 50 mM Tris (final pH 7.4) as well as 100 mM K_2SO_4 and 12.5 μg of valinomycin/mg of protein and Gly-Gln in concentrations of 0.1 μM to 10 mM. Kinetic constants were derived as described under "Experimental Procedures." The data represent mean ± S.E. of three membrane preparations. HA and LA refer to the high affinity and low affinity transport systems, respectively; ND is not determined.

pH gradient	K_t		V_{max}	
	HA	LA	HA	LA
pH units	*μM*	*mM*	*nmol/mg protein/5 s*	
	ND	2.14 ± 0.80	ND	1.02 ± 0.31
0.9	90.2 ± 19.4	3.61 ± 0.97	0.07 ± 0.02	1.25 ± 0.29
1.2	61.3 ± 19.2	4.00 ± 0.26	0.15 ± 0.02[a]	2.03 ± 0.33
1.6	42.0 ± 11.7	3.22 ± 0.47	0.30 ± 0.03[a]	3.78 ± 0.73[a]

[a] $p < 0.01$ compared with other values in the same column.

Effect of Different pH Gradients on Gly-Gln Transport—The data shown in Fig. 1 demonstrate that the transient accumulation of Gly-Gln in the kidney BBMV, above its medium concentration, depends on the presence of a transmembrane pH gradient. The following studies were performed to investigate the impact of pH gradients on the kinetic parameters of Gly-Gln transport under voltage-clamp conditions. The concentration of Gly-Gln in the incubation medium was varied from 0.1 μM to 10 mM. Vesicles were preloaded with a buffer (pH 8.3) and incubated in media with a final pH of 6.7, 7.1, or 7.4. For kinetic studies performed in the absence of a pH gradient, vesicles were preloaded with a buffer (pH 7.4) and incubated in a medium (pH 7.4) containing 75 μM H^+-specific ionophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

In the absence of a pH gradient, only the low affinity system was operational (Table V, Fig. 4). In the presence of a pH gradient (0.9 pH units), both systems were operational (Table V). Increasing the transmembrane pH gradient from 0.9 to 1.2 units increased the V_{max} of the high affinity system by approximately 2-fold, but was without a significant effect on the low affinity system. A further increase in pH gradient to 1.6 pH units further increased the V_{max} of the high affinity system and also significantly increased the V_{max} of the low affinity system. The K_t values of both systems were not

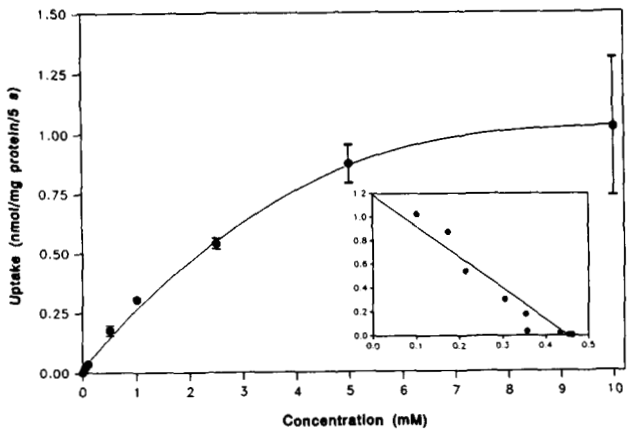

FIG. 4. **Kinetics of Gly-Gln uptake as a function of Gly-Gln concentration in the absence of a pH gradient.** The membrane vesicles were preloaded with 45 mM Hepes, 40 mM Mes, 40 mM Tris, 100 mM K_2SO_4, 10 mM mannitol (pH 7.4), and incubated in 45 mM Hepes, 40 mM Mes, 40 mM Tris (pH 7.4), and 290–300 mM mannitol according to the addition of Gly-Gln (0.1 μM to 10 mM) to equalize medium osmolarity. Prior to the incubation the membrane vesicles were preequilibrated with 75 μM of the H^+-specific ionophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Uptake data represent the saturable uptake after subtraction of the uptake as measured under identical conditions but at 4 °C. *Inset*: Eadie-Hofstee plot of the data plotted as *v versus v/S* where *S* represents the Gly-Gln concentration in millimolar. The data represent mean ± S.E. of three membrane preparations.

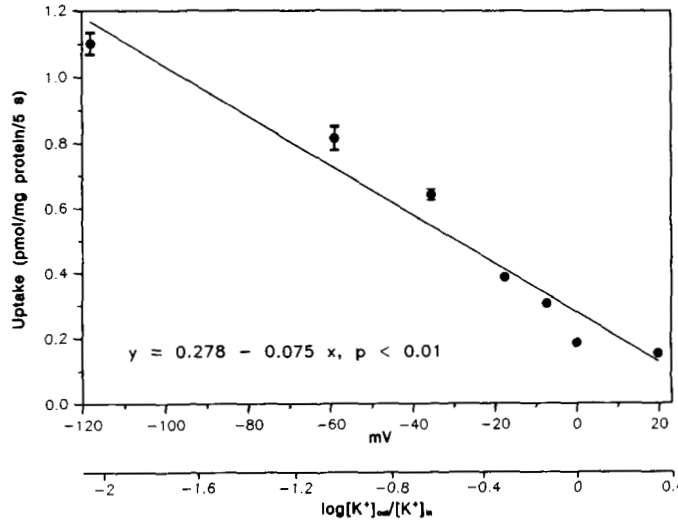

FIG. 5. **Effect of different K^+ diffusion potentials on Gly-Gln uptake in the presence of a pH gradient.** Uptake of Gly-Gln (0.1 μM) was measured in BBMV preloaded with 45 mM Hepes, 75 mM Tris (pH 8.3), and incubated in buffers containing 50 mM Hepes, 50 mM Mes, 20 mM Tris, pH 6.0 (final 6.7). The calculated Nernst K^+-diffusion potential was varied in the presence of various intravesicular and extravesicular K_2SO_4 concentrations ($K^+_{in:out}$ (mM): 100:1, 100:6.5, 100:100, and 45:100) and 12.5 μg of valinomycin/mg of protein. All media were adjusted with mannitol to a final osmolarity of 430 mosm/liter.

affected by alterations in the pH gradient.

Effect of Membrane Potential on Gly-Gln Uptake—The effect of membrane potential was examined systematically by varying the intravesicular and extravesicular K^+ concentrations in the presence of valinomycin. Assuming that the actual intravesicular K^+ concentration was equal to the concentration with which the vesicles were loaded, apparent Nernst K^+ diffusion potentials were calculated and were used as operational values. Maintaining transmembrane K^+ gradients between 100:1 and 45:100 resulted in Nernst K^+ diffusion potentials ranging from −118 to +20 mV. As shown in Fig. 5, in the presence of a pH gradient, Gly-Gln uptake was affected significantly by alterations in the K^+ diffusion potentials. All the studies involving membrane potential were performed in the presence of a pH gradient (pH_{in} 8.3/pH_{out} 6.7). Increasing the K^+ diffusion potential from 0 to −118 mV increased the rate of transport by 6-fold from 0.185 ± 0.015 to 1.101 ± 0.057 pmol/mg protein/5 s. In contrast, the rate of Gly-Gln uptake decreased only slightly as the membrane was depolarized.

To study the impact of the K^+ diffusion potentials on the kinetic constants of Gly-Gln transport, the following experiment was performed. Uptake of Gly-Gln as a function of substrate concentration was studied over a concentration range of 0.05 μM to 10 mM. Valinomycin-induced K^+ diffusion potentials were adjusted to −118 mV, −70 mV, 0 mV, and +20 mV by varying the intra- and extravesicular K^+ concentrations. Uptake assays were performed in the presence of a transmembrane pH gradient of 1.6 units (pH_{in} 8.3/pH_{out} 6.7). As shown in Table VI, the V_{max} of the high affinity system increased significantly by increasing the inside-negative K^+ diffusion potential. In contrast, the V_{max} of the low affinity system was independent of the K^+ diffusion potentials. The K_t of neither the high affinity nor the low affinity system was affected by alterations in the membrane potential.

Kinetic Analysis of the Interaction of External H^+ with the Dipeptide Transport System—To investigate the number of binding sites for hydrogen ions on the peptide transporters, as well as their dissociation constants (pK_a), Gly-Gln uptake

TABLE VI

Effect of membrane potential on kinetic parameters of Gly-Gln uptake

Effect of different K^+ diffusion potentials on the kinetics of Gly-Gln uptake in the presence of a pH gradient. Uptake of Gly-Gln (0.1 μM to 10 mM) was measured in BBMV preloaded with 45 mM Hepes, 75 mM Tris (pH 8.3) and incubated in buffers containing 50 mM Hepes, 50 mM Mes, 20 mM Tris (pH 6.0). The calculated Nernst K^+ diffusion potential was varied in the presence of various intravesicular and extravesicular K_2SO_4 concentrations ($K^+_{in:out}$ (mM): 100:1, 100:6.5, 100:100, and 45:100) and 12.5 μg of valinomycin/mg of protein. All media were adjusted with mannitol to a final osmolarity of 430 mosm/liter. The kinetic constants were derived as described under "Experimental Procedures." The data represent mean ± S.E. of four membrane preparations. HA and LA refer to the high affinity and low affinity transport system, respectively.

K^+ diffusion potential	K_t		V_{max}	
	HA	LA	HA	LA
mV	*μM*	*mM*	*nmol/mg protein/5 s*	
−118	60.1 ± 15.2	2.54 ± 0.43	1.10 ± 0.10	4.69 ± 1.09
−70	27.6 ± 9.8	1.98 ± 0.81	0.52 ± 0.03[a]	4.78 ± 0.87
0	25.1 ± 11.4	3.18 ± 0.92	0.40 ± 0.05[a]	4.70 ± 1.08
+20	40.2 ± 16.1	3.20 ± 1.13	0.29 ± 0.05[a]	3.24 ± 0.42

[a] $p < 0.01$ compared with −118 mV.

was determined as a function of external pH at a fixed internal pH of 8.3. This experiment was performed at Gly-Gln concentrations of 0.1 μM and 1 mM either in the presence or the absence of a voltage clamp. At a Gly-Gln concentration of 0.1 μM the high affinity transport system predominates with about 85% the total uptake, whereas at a substrate concentration of 1 mM both systems contribute about 50% to total uptake.

Fig. 6, *A* and *B*, shows the uptake of 0.1 μM or 1 mM Gly-Gln as a function of extravesicular pH. In all cases uptake increased significantly with decreasing pH, but the effect was much more pronounced in the absence of a voltage clamp. The rate of uptake of Gly-Gln plotted against the external H^+ concentration showed a saturable process. The apparent V_{max} values as a function of external H^+, as calculated from

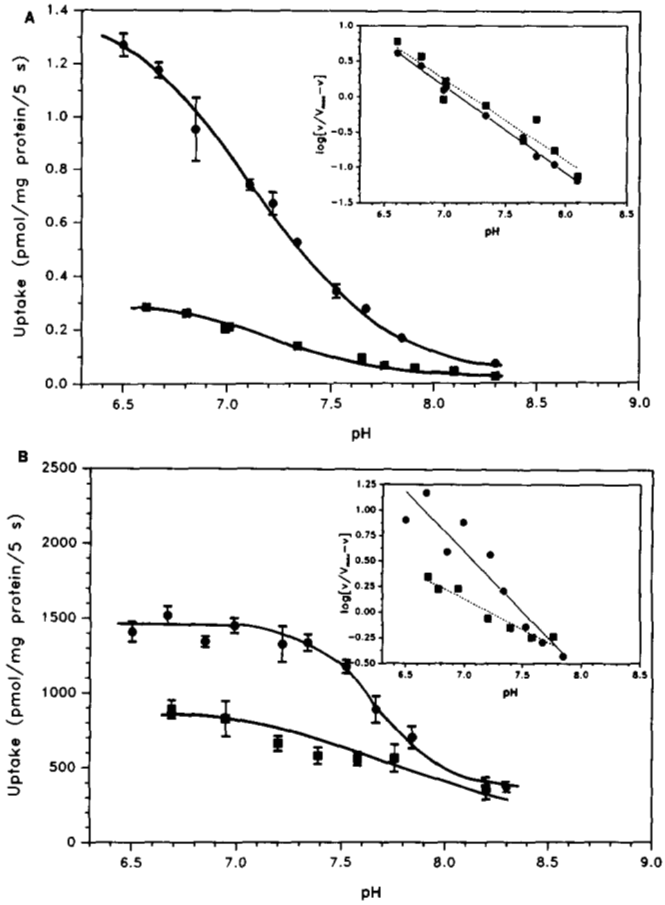

FIG. 6. **Dependence of Gly-Gln uptake on external pH.** BBMV preloaded with 45 mM Hepes, 75 mM Tris, 100 mM K_2SO_4 (pH 8.3) were incubated with either 0.1 μM Gly-Gln (*A*) or 1 mM Gly-Gln (*B*) in buffers containing 300 mM mannitol (*squares*) or 100 mM K_2SO_4 and 12.5 μg of valinomycin/mg of protein (*circles*). The initial external pH was varied with different concentrations of Hepes, Mes, and Tris to a final concentration of 125 mM for the buffers. Uptake of Gly-Gln is plotted *versus* the final incubation pH which resulted from mixing 40 μl of vesicle suspension preloaded with buffer pH 8.3 with 160 μl of medium. The *insets* show the Hill plots of the uptake data in the main panel plotted as log (v/V_{max}-v) *versus* final pH after subtraction of the uptake rates measured in the absence of a pH gradient. The data represent mean ± S.E. of three membrane preparations.

Eadie-Hofstee plots (v *versus* $v/[H^+]$), were affected significantly by the membrane potential. At 0.1 μM Gly-Gln, the maximal uptake rate in the absence of a voltage clamp was 5 times higher than in its presence (1.78 ± 0.24 *versus* 0.34 ± 0.06 pmol/mg protein/5 s). At 1 mM Gly-Gln, V_{max} was increased from 900 ± 83.3 pmol/mg protein/5 s in the presence of a voltage clamp to 1574 ± 105.0 pmol/mg protein/5 s in the absence of a voltage clamp.

Based on these uptake rates as a function of external pH, we performed a kinetic analysis of the interaction between H^+ and dipeptide transport according to the Hill equation (15). Prior to the analysis all transport rates were corrected for the uptake of Gly-Gln in the absence of a pH gradient ($pH_{out} = pH_{in} = 8.3$). Hill plots of these data were constructed by plotting $\log(v/V_{max} - v)$ against the extravesicular pH (the *insets* in Fig. 6). The V_{max} values were derived from the corresponding Eadie-Hofstee plots and used to calculate the Hill parameters. As shown in Table VII, under all experimental conditions, Hill coefficients of close or equal to one were obtained. Altering the V_{max} values within the range of their standard deviations still showed Hill coefficients of close or equal to one (0.786 to 1.261). By applying the Hill equation derived by Miller and Pollock (16), almost identical Hill coefficients were obtained.

The extravesicluar pH at which the rate of H^+/dipeptide cotransport is half-maximal was determined by solving the Hill equation for $\log(v/V_{max} - v) = 0$. The calculated pH values at which the H^+-driven dipeptide transport was half-maximal ($pH_{out} = pK_a$ for $v = 0.5\ V_m[H^+]$) ranged between 6.96 ± 0.05 and 7.32 ± 0.15.

These results suggest that the high affinity type H^+/dipeptide cotransporter contains a single site for its interaction with external H^+ having an apparent pK_a of approximately 7.0. The observation that the Hill analysis of the experiments with 1 mM Gly-Gln did not change the Hill coefficients significantly compared to the experiments with 0.1 μM Gly-Gln suggests that the low affinity site probably also contains only a single H^+ binding site with a similar pK_a value.

DISCUSSION

The results of the present study provided new insight into the mechanism and the driving force of dipeptide transport by the kidney. Gly-Gln uptake by kidney BBMV appeared to be mediated by two carrier systems. One carrier system was characterized as the high affinity, low capacity type and the other as the low affinity, high capacity type. The operation of the high affinity transporter was strictly dependent on the presence of a transmembrane pH gradient. This requirement did not seem to be as critical for the low affinity transporter. In fact, the low affinity transport system may operate in the absence of a proton gradient, but with reduced velocity. This conclusion was suggested by detection of only one saturable uptake process in the absence of a pH gradient, which had a K_t which was not significantly different from that of the low affinity transporter (Table V).

A hypothesis to explain the stimulation of dipeptide transport in the presence of a pH gradient has been advanced by Ganapathy *et al.* (7) and Takuwa *et al.* (8). They propose that the downhill movement of H^+ along its chemical or electrochemical gradient provides the driving force for concentrative electrogenic dipeptide uptake. Our data on the pH-dependent uptake of Gly-Gln in the presence of a voltage clamp demonstrated the importance of the chemical driving force of H^+. However, when an inside-negative K^+ diffusion potential was imposed and the chemical driving force was converted into an electrochemical driving force, the V_{max} of the high affinity system was increased almost three times (Table VI). Because Gly-Gln was almost completely in the zwitterionic form in the pH range investigated, the cotransport of H^+ with a dipeptide molecule into the BBMV appeared to be electrogenic.

Along this line, we studied the effect of variation in pH gradient on kinetic parameters of dipeptide transport in the kidney. Increasing the transmembrane pH gradient progressively increased the transport capacity of the high affinity system. The V_{max} of the low affinity system increased significantly only when a transmembrane pH gradient of 1.6 units was applied.

Previously, dipeptides such as glycylproline and glycylsarcosine were used for characterizing peptide transport in the kidney. The use of these dipeptides was to circumvent the problem of hydrolysis prior to transport because both dipeptides are relatively resistant to hydrolysis by membrane peptide hydrolases. Gly-Gln, unlike Gly-Pro, Gly-Sar and carnosine, could be hydrolyzed by the membrane peptide hydrolases. Therefore, the use of Gly-Gln in our study required

TABLE VII

Kinetic parameters derived from Hill analysis

Gly-Gln concentration	Voltage clamp condition	V_{max}	pK_a	Hill coefficient	Regression coefficient
		pmol/mg protein/5 s			
0.1 μM	−	1.54 ± 0.21	6.96 ± 0.05	1.20 ± 0.09	0.991
0.1 μM	+	0.32 ± 0.05	7.12 ± 0.08	1.22 ± 0.14	0.993
1.0 mM	−	1316 ± 94.2	7.32 ± 0.15	0.89 ± 0.20	0.786
1.0 mM	+	704 ± 102.2	7.12 ± 0.18	0.91 ± 0.08	0.914

validation as an appropriate probe for characterizing dipeptide transport in the kidney. This was accomplished by showing that, under our conditions of transport assay, the glutamine residue of Gly-Gln was transported as dipeptide and not as free amino acid and, moreover, over 98% of Gly-Gln remained as dipeptide in the incubation medium. This recovery is comparable to recovery of either Gly-Pro or carnosine reported by others (17, 18). In addition, we showed recovery of over 95% of Gly-Gln in the intravesicular content. Therefore, an outcome of the present study is that dipeptides which are not resistant to hydrolysis could be used as substrates for the peptide transporters, as long as the incubation time is kept short.

Our study also established that Gly-Gln can be used as a model substrate for investigating peptide transport phenomena. Evidence includes the following: (*a*) The use of Gly-Ala as a substrate also resulted in detection of two dipeptide transporters, namely, the high affinity/low capacity and the low affinity/high capacity transporter. The kinetic constants for transport of Gly-Ala were similar to those for Gly-Gln. (*b*) The inhibition studies showed that Gly-Gln shares the high affinity system with other dipeptides. (*c*) The K_t of Gly-Gln transport (44 μM) by the high affinity system was comparable to the K_t values previously reported (19) for transport of Phe-Pro-Ala (36 μM) and Gly-Sar (94 μM) in kidney BBMV. On the other hand, the K_t for the low affinity system has only been reported in one study (20) using Gly-Pro as substrate; a value of 22 mM was found. This value is considerably different from the 2–4 mM which we report for Gly-Gln. Perhaps this discrepancy can be attributed to the fact that the previous study (20) did not correct uptake values for a diffusional component, thereby leading to an overestimation of K_t.

The presence of two binding sites for Gly-Gln transport led to the following question: Are the binding sites on the same or two separate carriers? The results of the present study were suggestive of two separate carriers. The evidence is based on the observation that alteration in the membrane potential had a different impact on the kinetics of Gly-Gln uptake by the high affinity and the low affinity system. A linear relationship was found between the inside-negative K^+ diffusion potentials and the velocity of the high affinity system. In contrast, the low affinity system did not respond significantly to changes in membrane potential.

The above suggestion that there are two distinct carriers for Gly-Gln transport leads to the following question: Are both carriers present in the brush border membrane of the same or different renal tubular cells? Our BBMV preparation was composed predominantly of cortical tissue and only a small amount of outer medulla. Moreover, other investigators using BBMV prepared only from the cortex of rat kidney also concluded the existence of the high and low affinity peptide transporters (20). Therefore, it appears that both systems are present in the membrane of the same cell.

Finally, our study provided information on the stoichiometric coupling between proton and dipeptide fluxes which previously has not been studied in the kidney. Calculations of the Hill coefficients of the uptake rate as a function of external H^+ performed at 0.1 μM and 1 mM Gly-Gln showed consistently a 1:1 ratio, both in the absence and the presence of a voltage clamp. These data suggested that the transporters contain only one binding site for the interaction with external H^+. However, this suggestion was more certain for the high affinity than the low affinity transporter. At a substrate concentration of 0.1 μM, 85% of Gly-Gln was transported by the high affinity system, whereas at a concentration of 1 mM, only 50% of Gly-Gln was transported by the high affinity system.

It is pertinent to note that our estimation of a 1:1 ratio between proton and dipeptide fluxes in the kidney differed from the same estimate in the intestine. Electrophysiological studies in the toad intestine and a fluoroscopic technique using a potential-sensitive dye to monitor changes in membrane potential caused by dipeptide uptake into the intestinal BBMV, revealed a ratio (H^+:dipeptide) of 1.87:1 (21) and 2:1 (22), respectively. The basis for the difference between the present finding in the kidney and the previous findings in the intestine was not entirely clear. It could be that there were some differences between dipeptide transport in BBMV preparation prepared from kidney and intestine. This suggestion is based on the following observations. (*a*) Studies in BBMV (9, 15), like the present one, have shown that H^+-dependent dipeptide uptake is concentrative with overshoot values exceeding the equilibrium values by severalfold, but this has not been observed in the intestine (7, 8). (*b*) The K_t values reported for the pH gradient-dependent and -independent uptake of oligopeptides into intestinal BBMV vary between 0.4 and 19.5 mM (7, 8, 23). These values are comparable to the K_t value for the low affinity system of kidney that we characterized by using Gly-Gln or Gly-Ala. (*c*) Studies in kidney BBMV using thiol group-specific reagents have shown that thiol groups are involved in pH-dependent dipeptide translocation (24) but the same reagents, when used in intestinal BBMV, were ineffective in modifying the oligopeptide transporter (25). Therefore, it remains to be established whether intestine, like kidney, has a high affinity dipeptide transporter.

In conclusion, our studies of brush border membrane of renal tubular cells showed the presence of two dipeptide transporters with different responses to changes in proton gradient and membrane potential. The high affinity transporter appeared to be far more sensitive to these changes than the low affinity transporter. Finally, in the presence of a pH gradient, transport of each neutral dipeptide molecule appeared to require cotransport of one hydrogen ion to serve as the driving force.

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