Physiol. Res. 50: 373-381, 2001

Estimation of Apparent L-Amino Acid Diffusion in Porcine Jejunal Enterocyte Brush Border Membrane Vesicles

M. Z. FAN[1], O. ADEOLA[2], E. K. ASEM[3]

Department of Animal and Poultry Science[1], University of Guelph, Guelph, Ontario, Canada, Departments of Animal Sciences[2] and Basic Medical Sciences[3], Purdue University, West Lafayette, Indiana, USA

Received August 9, 2000
Accepted November 30, 2000

Summary

There is an overlap of carrier-mediated L-amino acid transport and apparent simple diffusion when measured in intestinal brush border membrane vesicles. Using L-threonine and L-glutamine as representative amino acids, this study was undertaken to estimate apparent simple diffusion of L-amino acids and to establish the effective dosage of $HgCl_2$ for completely blocking carrier-mediated L-amino acid transport in porcine jejunal enterocyte brush border membrane vesicles. Jejunal mucosa was scraped from three pigs weighing 26 kg. Enterocyte brush border membrane vesicles, with an average enrichment of 24-fold in sucrase specific activity, were prepared by Mg^{2+}-precipitation and differential centrifugation. *In vitro* uptake was measured by the fast filtration manual procedure. $HgCl_2$ blocked the carrier-mediated initial transport of L-threonine and L-glutamine under Na^+-gradient condition in a dose-dependent manner. At the minimal concentration of 0.165 μmol $HgCl_2$ mg^{-1} protein, carrier-mediated L-threonine and L-glutamine transport was completely inhibited. The apparent L-threonine and L-glutamine diffusion was estimated to be 8.6±0.7 and 12.4±1.0 % of the total uptake at the substrate concentrations of 5 μM (L-threonine) and 50 μM (L-glutamine). Therefore, the treatment of porcine brush border membrane vesicles with a minimum of 0.165 μmol $HgCl_2$ mg^{-1} protein completely blocks carrier-mediated L-amino acid transport and enables the direct estimation of apparent L-amino acid diffusion in enterocyte brush border membrane vesicles.

Key words
Amino acids • Enterocyte • Brush border membrane vesicles • Diffusion • Pigs

Introduction

Amino acid absorption in the small intestine proceeds by two routes: the widely accepted transcellular route (Hopfer 1987) and a paracellular route (Madara and Pappenheimer 1987). The transcellular route involves three steps: transport across the brush border membrane of enterocytes, intracellular movement and extrusion across the basolateral membrane. Transport of amino acids across the brush border membrane occurs *via* Na^+-dependent (secondary active transport) or Na^+-independent (facilitated diffusion) systems. Under normal physiological conditions, only a small portion of the

Fax+4202 24920590
E-mail physres@biomed.cas.cz http://www.biomed.cas.cz /physiolres

transmembrane movement occurs by simple diffusion (Mailliard *et al.* 1995).

Isolated enterocyte membrane vesicles have been widely used to study transcellular nutrient transport across the membranes of enterocytes (Stevens *et al.* 1984). The uptake of nutrients in membrane vesicles is usually determined by the fast filtration manual procedure and, very occasionally, by employing the automatic apparatus procedure (Stevens *et al.* 1984). In studies on intestinal brush border membrane vesicles with the fast filtration manual procedure, a significant linear component, i.e., apparent simple diffusion, was often observed in the characterization of amino acid transport kinetics (Hopfer 1987, Madara and Pappenheimer 1987).

In order to partition carrier-mediated L-amino acid transport in intestinal brush border membrane vesicles, it is essential to determine the apparent simple diffusional component. However, there are no specific inhibitors available to block L-amino acid transporters. Furthermore, unlike the case of strict stereo-specificity in D-glucose transport, most intestinal amino acid transport systems are not very stereo-specific, although L-amino acids are preferentially transported (Daniels *et al.* 1969, Brachet *et al.* 1987). Thus, the apparent simple diffusion of L-amino acids can not be reliably determined by using their respective enantiomorphs. Alternatively, as demonstrated in two earlier studies (Maenz and Cheeseman 1986, Cheeseman 1993), the treatment of intestinal basolateral membrane vesicles with $HgCl_2$ inhibited the basolateral membrane-associated sugar transporter (GLUT2) and the estimated D-glucose diffusion resembled the values determined with L-glucose or GLUT2-specific inhibitors (phlorizin and cytochalasin B). The results of these authors indicated that $HgCl_2$ is an effective non-specific inhibitor of amino acid transporters. However, information on the direct determination of apparent diffusion of L-amino acids in the intestinal brush border membrane vesicles is lacking. Therefore, we employed the $HgCl_2$-inhibition approach, using L-glutamine and L-threonine as representative amino acids, to determine the apparent L-amino acid diffusion. We established the effective dosage of $HgCl_2$ for complete blockade of carrier-mediated L-amino acid transport in porcine jejunal brush border membrane vesicles. Because L-glutamine and L-threonine transport in porcine intestinal brush border membrane vesicles were well characterized in previous studies (Maenz and Patience 1992, Fan *et al.* 1998,1999), these two amino acids have been used in this study.

Material and Methods

Chemicals

L-[3-^{3}H]threonine (specific activity: 562 GBq mmol^{-1}) and L-[G-^{3}H]glutamine (specific activity: 1.63-1.70 TBq mmol^{-1}) were obtained from the Amersham Corporation. Ecolume scintillant was purchased from IGN and the Bio-Rad dye reagent was from Bio-Rad Laboratories. Bovine serum albumin (fraction V), L-threonine, L-glutamine, D-mannitol, Trizma•HCl, Trizma•base, phenylmethylsulfonyl fluoride (PMSF), Na_2ATP, sucrose and all other chemicals were purchased from Sigma Chemical Co.

Animals and preparation of mucosal scrapings

The intestinal mucosal samples used in these studies were collected from three 26-kg pigs obtained from the Purdue University Swine Research Center (a cross of Yorkshire-Landrace dams and Hampshire-Duroc sires). While pigs were under anesthesia, the abdomen was opened and the small intestine, transected 5 cm from both the pyloric and the ileo-cecal sphincter, was removed. A 100-cm jejunal segment was dissected at 250 cm peripherally to the pyloric sphincter and flushed with ice-cold saline (154 mM NaCl, 0.1 mM PMSF at pH 7.4). The jejunum was divided into 15-cm segments that were opened longitudinally and freed of mucus by patting with a paper towel. The mucosa was scraped with a spatula. The mucosal scrapings were pooled within each individual pig, placed in screw-capped plastic tubes and stored at –70 °C. The experimental protocol was approved by Purdue University Animal Care and Use Committee.

Preparation of brush border membrane vesicles

Jejunal brush border membrane vesicles were prepared from frozen mucosa by Mg^{2+}-precipitation and differential centrifugation according to an established procedure (Maenz and Patience 1992, Fan *et al.* 1999). Specifically, about 15 g of mucosal scrapings were thawed in 300 ml of ice-cold homogenate buffer (50 mM D-mannitol, 10 mM Trizma•HCl, 0.1 mM PMSF at pH 7.4) and homogenized with a polytron homogenizer for 1 min. The resulting homogenate was pooled and centrifuged in a Sorvall SS-34 rotor at 2 000 x *g* for 15 min. After removing the top foam layer and discarding the pellets, the supernatant was mixed with 1 M $MgCl_2$ to a final concentration of 10 mM $MgCl_2$, stirred at 4 °C for

15 min, and then centrifuged at 2 400 x *g* for 15 min. After discarding top foam layer, the resultant supernatant was centrifuged at 19 000 x *g* for 30 min to generate crude brush border membrane pellets which were resuspended in a buffer containing 14.4 mM D-mannitol, 150 mM KSCN, Trizma•HCl at pH 7.4. The resulting suspension was centrifuged at 39 000 x *g* for 30 min to generate the final brush border membrane vesicle pellets that were then resuspended in a suitable amount of the same buffer. Aliquots of the vesicle suspension were taken for enzyme assays. The final vesicle suspension was assayed for protein content and diluted to contain 10 mg protein ml^{-1} with the same buffer.

Protein and enzyme assays

Protein was determined according to the Bradford method (1976) with the Bio-Rad dye reagent and bovine serum albumin (Fraction V) as the standard. All the following enzyme activity assays were carried out under conditions in which enzyme reactions were linear. Sucrase (EC 3.2.1.48) was assayed according to the procedure of Dahlgvist (1964). As a brush border membrane marker, sucrase activities were assayed at 37 °C for 10 min in a final volume of 0.050 ml containing brush border membrane vesicle suspension (6.3 μg protein), or mucosal homogenate (12.6 μg protein), 50 mM sodium maleate, 28 mM sucrose at pH 6.0. To check the orientation of prepared brush border membrane vesicles, sucrase activities were also regularly assayed with the addition of 0.20 mM sodium deoxycholate (to lyse membrane vesicles) according to the aforementioned sucrase assay procedure. Na^+/K^+-ATPase (*EC* 3.6.1.3) was assayed by the method of Schwartz *et al.* (1969). In the Na^+/K^+-ATPase activity assay, incubations were conducted in a final volume of 1 ml containing membrane suspensions (12.5 μg protein), or mucosal homogenate (25.0 μg protein), 5.0 mM $MgCl_2$, 100.0 mM NaCl, 20.0 mM KCl, 6.0 mM Na_2ATP, 0.20 mM sodium deoxycholate (to lyse membrane vesicles) at pH 7.4 and 37 °C for 20 min in the presence or absence of 5 mM ouabain. Inorganic phosphate was measured by the method of Heinoen and Lahti (1981).

Treatment of membrane vesicles

For each $HgCl_2$-inhibition experiment, nine batches (50 μl each batch) of the jejunal brush border membrane vesicle suspension (10 mg protein ml^{-1}) were respectively treated with 5 μl of nine different $HgCl_2$ stock solutions (0, 1, 4, 8, 16, 24, 36, 64 and 96 mM $HgCl_2$ at pH 7.4) to obtain graded concentrations of $HgCl_2$ (0, 0.09, 0.36, 0.73, 1.46, 2.18, 3.27, 5.82 and 8.73 mM at the protein content of 9.1 mg ml^{-1}). The treated vesicle suspensions were then mixed and incubated at 4 °C for 30 min prior to the uptake measurements.

Uptake measurements

Uptake experiments were carried out by the rapid filtration manual procedure (Tsang *et al.* 1994, Fan *et al.* 1999). Fifty microliters of the uptake buffer were first placed at the bottom of a polystyrene tube (100 x 15 mm) and then 10 μl of the brush border membrane vesicle suspension were spotted onto the side of the tube in two separate drops immediately above the uptake buffer. After 20 s warming at the room temperature (24 °C), uptake incubation was initiated by a foot switch-activated vibromixer, and the process was terminated by the addition of 1.125 ml of ice-cold wash solution (14.4 mM D-mannitol, 150 mM NaSCN, 10 mM Trizma•HCl, 0.1 mM $HgCl_2$ at pH 7.4). One milliliter of the uptake mixture was then rapidly pipetted onto 0.45-um cellulose acetate filters (soaked with 20 mM L-threonine or L-glutamine solutions at pH 7.4) mounted on an Manifold filtration unit, which was connected to a vacuum source. Exact timing was monitored with an electronic GraLab model 545 timer-intervalometer. The filters were immediately washed four times with 5 ml of ice-cold wash solution. Our preliminary testing showed that quadruple washing with 5 ml per washing was ideal to minimize non-specific radioactive binding to the membrane filters. The washing solution was injected into the filtration hole along the edge with a 50-ml syringe to avoid direct flushing of the membrane vesicles. The remaining uptake mixture in the incubation tubes was collected, pooled and counted to determine the average initial radioactivity in the uptake media. After 30-min extraction in 5 ml of the ecolume scintillant, filters were counted on a model 1900 TR Tri-Carb liquid scintillation analyzer with automatic quench correction. The non-specific binding of L-threonine and L-glutamine to filters and membrane vesicles was corrected by subtracting the time-zero radioactivity. In the inhibition experiments, the non-specific binding of L-threonine and L-glutamine to filters was subtracted from the total counting values.

Each uptake experiment was conducted in triplicate. Three separate uptake experiments were conducted using three different batches of membrane vesicle suspensions prepared from the mucosal scrapings from three pigs. Composition of the uptake buffers is described in detail in the legends to the figures.

Calculations and statistical analyses

L-threonine and L-glutamine uptake into the membrane vesicles was calculated according to the isotope dilution principle as follows:

$$V = [((R_F - R_B) \times [S])/R_I]/W \quad (1)$$

where V is the amount of L-threonine or L-glutamine transported into the membrane vesicles (nmol•mg^{-1} protein), R_F is radioactivity in disintegrations per minute (DPM) of the filters (DPM per filter), R_B is the radioactivity for non-specific retention by filters or membrane vesicles (DPM per filter), [S] is L-threonine or L-glutamine concentration in the uptake media (nmol•ul^{-1}), R_I is radioactivity in the uptake media (DPM•ul^{-1}), and W is the amount of vesicle protein provided for the incubation (mg protein).

$HgCl_2$ inhibition of initial threonine or glutamine uptake was analyzed according to Tsang and Cheeseman (1994) using the Eadie-Hofstee plot:

$$J = I_{MIN} - IC_{50}*(J/[I]) \quad (2)$$

$$I_{MAX} = I_C - I_{MIN} \quad (3)$$

where J is the initial rate of L-threonine or L-glutamine uptake in the presence of a Na^+-gradient (pmol•mg^{-1} protein•s^{-1} or % of the control); I_{MIN} is the minimal initial uptake rate as inhibited by $HgCl_2$ (pmol•mg^{-1} protein•s^{-1} or % of the control); IC_{50} is the inhibitor ($HgCl_2$) concentration at half maximal inhibition (mM); [I] is the inhibitor ($HgCl_2$) concentration (mM); I_{MAX} is maximal extent of inhibition in initial L-threonine or L-glutamine uptake rate (pmol•mg^{-1} protein•s^{-1} or % of the control); I_C are the mean values of initial rates of L-threonine and L-glutamine uptake measured with $HgCl_2$-free membrane vesicles (pmol•mg^{-1} protein•s^{-1} or % of the control).

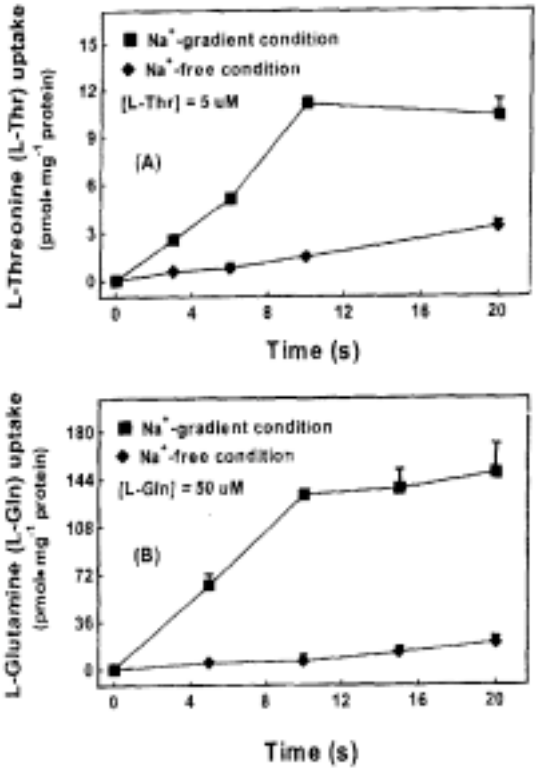

Fig. 1. *Time course of 5.0 μM L-threonine (A) and 50 μM L-glutamine (B) uptake into porcine jejunal brush border membrane vesicles under the Na^+-gradient condition. The uptake buffer contained 150.0 mM NaSCN, 10.0 mM Trizma·HCl, 14.4 mM D-mannitol, 6.0 μM L-[3-^{3}H]threonine (for L-threonine uptake) or 60 μM L-glutamine including 0.96 μM L-[G-^{3}H]glutamine plus non-labeled L-glutamine (for L-glutamine uptake), pH 7.4. Uptake incubations were conducted by mixing 10 μl of the membrane vesicle suspension (9.1 mg protein•ml^{-1}) with 50 μl corresponding uptake buffer. The resultant uptake media (60 μl) contained 5.0 μM L-[3-^{3}H]threonine or 50 μM L-glutamine, 125.0 mM NaSCN, 25.0 mM KSCN. Each point represents the mean and standard error derived from three experiments (triplicate observations per experiment).*

Results and Discussion

Relative to mucosal homogenate, the final brush border membrane preparation had an average of 24-fold enrichment in sucrase specific activity. The enrichment in specific activity of Na^+/K^+-ATPase in the final brush border membrane preparation was less than twofold relative to the mucosal homogenate, suggesting relatively little contamination with the basolateral membrane fraction. The membrane vesicles were regularly checked for orientation by measuring sucrase activity with non-lysed and lysed membrane vesicle preparations. There were no effects of membrane-lysing on sucrase activity, suggesting that the brush border membrane vesicles were completely outside-out or in membrane sheets.

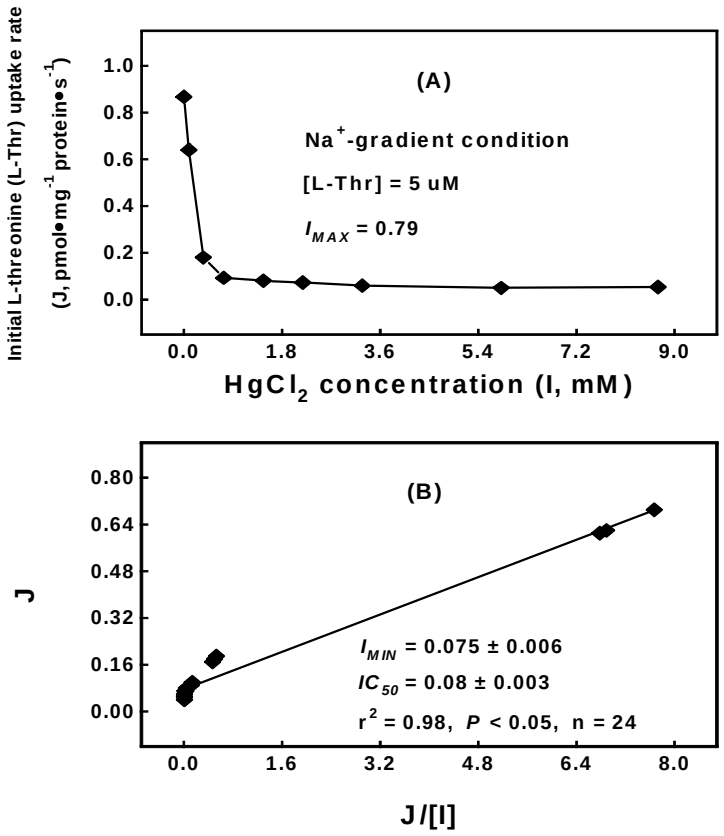

Fig. 2. *$HgCl_2$ inhibition of initial rates of 5.0 μM L-[3-^{3}H]threonine uptake into porcine jejunal brush border membrane vesicles under the Na^+-gradient conditions: (A). Inhibition relationship; (B). Eadie-Hofstee plot of the inhibition relationship. The uptake buffer contained 150.0 mM NaSCN, 10.0 mM Trizma·HCl, 14.4 mM D-mannitol, 6.0 μM L-[3-^{3}H]threonine, pH 7.4. Uptake incubations were conducted by mixing 10 μl of the corresponding $HgCl_2$-treated membrane vesicle suspension (9.1 mg protein•ml^{-1}) with 50 μl of the uptake buffer. The resultant uptake media (60 μl) contained 5.0 μuM L-[3-^{3}H]threonine, 125.0 mM NaSCN and 25.0 mM KSCN. I_{MIN}: the minimal L-threonine uptake rate (pmol•mg^{-1} protein•s^{-1}); IC_{50}: $HgCl_2$ concentration at the half maximal inhibition (mM).*

At the concentrations of 5 μM (for L-threonine) and 50 μM (for L-glutamine), time courses of L-threonine and L-glutamine uptake were determined in both Na^+-gradient and Na^+-free conditions (Figs 1A and 1B). L-threonine uptake appeared to be linear up to 6 s while L-glutamine uptake was linear for 10 s in the Na^+ gradient condition. The uptake of L-threonine and L-glutamine in the presence of an inwardly directed Na^+ gradient (125 mM) displayed enhanced transport, indicating the existence of Na^+-dependent L-threonine and L-glutamine transport components. In previous studies, transport of L-threonine and L-glutamine across porcine jejunal brush border membranes was demonstrated to occur *via* both Na^+-dependent B° and Na^+-independent L transporters (Berteloot *et al.* 1991, Stevens *et al.* 1982). Therefore, the initial L-threonine and L-glutamine uptake in the Na^+-gradient condition was overlapped by Na^+-dependent and Na^+-independent transport as well as by simple diffusion. Thiocyanate is lipophilic and can cross cell membrane rapidly (Gunther

et al. 1987). Therefore, the inclusion of thiocyanate in both uptake and vesicle resuspension buffers effectively clamped the membrane potential. Since vesicle membrane potentials were clamped, the Na^+-dependent transport of these L-amino acids was driven by the Na^+ gradient alone, whereas their Na^+-independent transport and simple diffusion were energized by the substrate concentration gradients.

It was established in a previous study that $HgCl_2$ irreversibly binds to membrane proteins and inhibits nutrient transporters (Wright *et al.* 1980). Therefore, the logic behind the $HgCl_2$-inhibition approach for the determination of simple diffusion is that, under the conditions of maximal inhibition, $HgCl_2$ can virtually block all transporters and the residual amino acid uptake will represent the simple diffusional component. As the determined diffusional component includes non-specific membrane binding, it should be referred to as apparent simple diffusion.

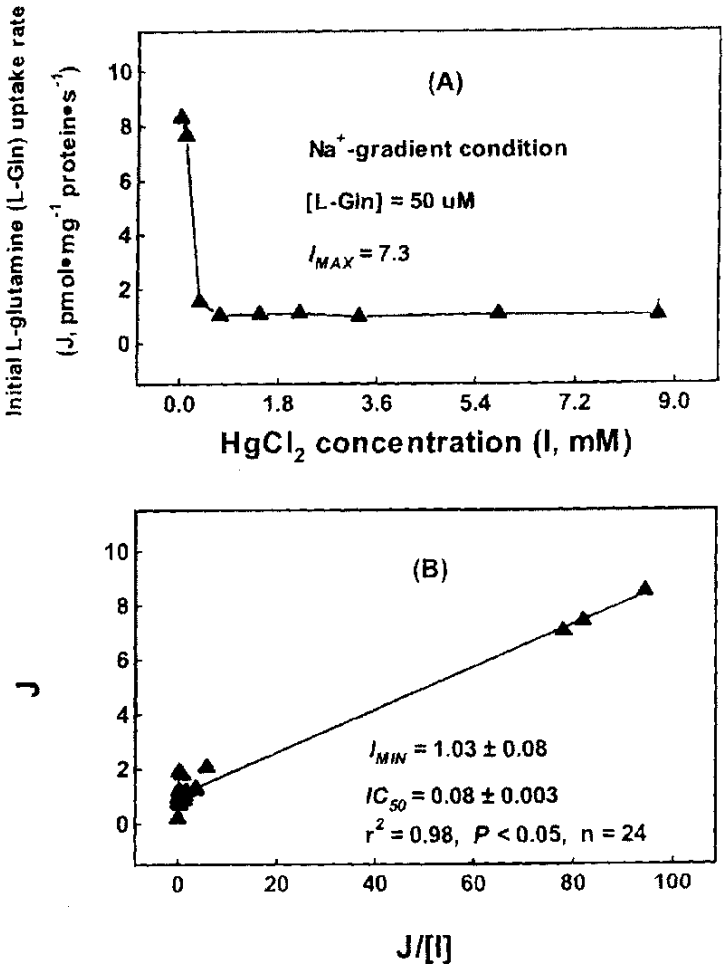

Fig. 3. *$HgCl_2$ inhibition of initial uptake of 50 μM L-glutamine uptake into porcine jejunal brush border membrane vesicles under the Na^+-gradient conditions: (A). Inhibition relationship; (B). Eadie-Hofstee plot of the inhibition relationship. The uptake buffer contained 150.0 mM NaSCN, 10.0 mM Trizma·HCl, 14.4 mM D-mannitol, 60 μM L-glutamine including 0.96 μM L-[G-^{3}H]glutamine plus non-labeled L-glutamine, pH 7.4. Uptake incubations were conducted by mixing 10 μl of the corresponding $HgCl_2$-treated membrane vesicle suspension (9.1 mg protein•ml^{-1}) with 50 μl of the uptake buffer. The resultant uptake media (60 μl) contained 50 μM L-glutamine, 125.0 mM NaSCN and 25.0 mM KSCN. I_{MIN}: the minimal L-glutamine uptake rate (pmol•mg^{-1} protein.s^{-1}); IC_{50}: $HgCl_2$ concentration at the half maximal inhibition (mM).*

The relationship between initial rate of L-threonine uptake measured in the Na^+-gradient condition and increased $HgCl_2$ concentrations is illustrated in Figure 2. Increases in $HgCl_2$ concentration effectively blocked carrier-mediated L-threonine uptake (both Na^+-dependent and Na^+-independent transport). Inhibition kinetic analysis was conducted by the Eadie-Hofstee plot (Fig. 2B). The maximal inhibition in the L-threonine transport (I_{MAX}) was 0.79 when expressed in pmol•mg^{-1} protein•s^{-1} (see Table 1). When expressed as a percentage of the control values (no $HgCl_2$), the maximal inhibition (I_{MAX}) was 91.4 %; the corresponding IC_{50} was estimated to be 0.080±0.003 mM. Therefore, as shown in Figure 2B, the minimal residual L-threonine uptake (I_{MIN} = 0.075 pmol•mg^{-1} protein•s^{-1} or 8.6 % of the control) at maximal $HgCl_2$ inhibition represented apparent threonine diffusion, whereas the maximal inhibition of uptake (I_{MAX} = 0.79 pmol•mg^{-1} protein·s^{-1} or 91.4 % of the control) reflected the carrier-mediated transport components,

including both the Na^+-dependent and Na^+-independent L-threonine transport.

The relationship between initial L-glutamine uptake rate determined in the Na^+-gradient condition and increased $HgCl_2$ concentrations is illustrated in Figure 3. Increases in $HgCl_2$ concentration also effectively blocked carrier-mediated L-glutamine uptake (both Na^+-dependent and Na^+-independent transport). Inhibition kinetic analysis was again conducted by the Eadie-Hofstee plot (Fig. 3B). As summarized in Table 1, when expressed in pmol•mg^{-1} protein•s^{-1}, the maximal amount of inhibition in the L-glutamine transport (I_{MAX}) was 7.28. When expressed as a percentage of the control values (no $HgCl_2$), the maximal inhibition (I_{MAX}) was 87.6 %; the corresponding IC_{50} was estimated to be 0.080±0.003 mM. Therefore, as presented in Figure 3B, the minimal residual L-glutamine uptake (I_{MIN} = 1.03 pmol•mg^{-1} protein•s^{-1} or 12.4 % of the control) at maximal $HgCl_2$ inhibition represented apparent glutamine diffusion. On the other hand, the maximal inhibition of uptake (I_{MAX} = 7.28 pmol•mg^{-1} protein•s^{-1} or 87.6 % of the control) reflected the carrier-mediated transport components, including both the Na^+-dependent and Na^+-independent L-glutamine uptake.

Table 1. The inhibition parameter estimates of $HgCl_2$ inhibition of initial L-threonine and L-glutamine uptake in the Na^+-gradient condition

	Inhibition kinetic parameter estimates		
	I_{MAX}	I_C	I_{MIN}
L-Threonine uptake rate (J)			
J (pmol•mg^{-1} protein•s^{-1})	0.79±0.01	0.87±0.01	0.075±0.006
J (% of the control)	91.40±0.73	99.60±1.53	8.62±0.73
L-Glutamine uptake rate (J)			
J (pmol•mg^{-1} protein•s^{-1})	7.28±0.08	8.31±0.32	1.03±0.08
J (% of the control)	87.60±0.08	100.00±3.82	12.36±0.95

Data are means ± S.E.M. IC_{50} is $HgCl_2$ concentration at the half maximal inhibition of the initial rate of L-amino acid uptake. The estimated IC_{50} value was 0.08±0.003 mM for both L-threonine and L-glutamine ($P<0.05$, $n=24$). I_{MAX} is the maximal magnitude of inhibition in initial L-threonine or L-glutamine uptake rate (pmol•mg^{-1} protein•s^{-1} or % of the control, $P<0.05$, $n=24$). I_C are the mean initial rates of L-threonine and L-glutamine uptake of control groups, measured with $HgCl_2$-free membrane vesicle suspension (pmol•mg^{-1} protein•s^{-1} or % of the control, $n=3$). I_{MIN} is the minimal L-threonine or L-glutamine uptake rate (pmol•mg^{-1} protein•s^{-1} or % of the control, $P<0.05$, $n=24$).

The minimal $HgCl_2$ concentration required for the complete inhibition of carrier-mediated L-threonine and L-glutamine transport was observed to be 1.5 mM at the protein content of 9.1 mg•ml^{-1}, i.e., 0.165 μmol $HgCl_2$ mg^{-1} protein. This effective $HgCl_2$ threshold level (1.5 mM) was relatively higher than the dosage of $HgCl_2$ (1.0 mM) used in the measurement of D-glucose diffusion (Wright *et al.* 1980, Maenz and Cheeseman 1986). As L-threonine and L-glutamine are typical substrates for intestinal brush border membrane-associated neutral amino acid transporters, the results of this study can, at least, be extended, to the measurements of apparent diffusion of the L-form of other neutral amino acids.

It should, however, be pointed out that the apparent simple diffusional component is particularly associated with the initial nutrient uptake measured in enterocyte membrane vesicles by the fast filtration manual procedure (Berteloot *et al.* 1991). In several studies in which the initial nutrient uptake in enterocyte brush border membrane vesicles was measured by the fast filtration manual procedure, significant diffusional component was observed for L-proline and L-phenylalanine in rabbits (Stevens *et al.* 1982), D-glucose in rats (Maenz and Cheeseman 1986) and humans (Malo and Berteloot 1991), polyamines in rabbits (Brachet *et al.* 1995), and L-leucine and D-glucose in pigs and sheep (Wolfram *et al.* 1986). In order to obtain carrier-mediated saturable transport kinetics, the apparent

diffusional contribution was eliminated by computing the linear component in the kinetic analysis (Wolfram *et al.* 1986, Brachet *et al.* 1995).

When initial nutrient uptake in intestinal brush border membrane vesicles was measured by the automatic apparatus procedure, initial nutrient uptake rates were derived from the multiple-time point uptake values by polynomial regression analysis, i.e., slope of the linear fraction of the time course is the estimated initial nutrient uptake rate (Maenz and Cheeseman 1986, Berteloot and Semenza 1990). This process of determination of initial uptake rate also eliminated the non-saturable component, namely, apparent simple diffusion including non-specific binding. Therefore, the initial nutrient uptake in intestinal brush border membrane vesicles, as determined by the automatic apparatus procedure, is not associated with apparent simple diffusion (Maenz and Cheeseman 1986).

As was pointed out by Berteloot and Semenza (1990), the apparent simple diffusional component measured with enterocyte brush border membrane vesicles in various studies included non-specific membrane binding and simple diffusion. The non-specific binding is the result of substrate binding onto the vesicular membrane surface and unsealed membrane sheets or trapping in the aqueous space surrounding the membrane vesicles, all of which may not be washed out during the rapid filtration procedure (Berteloot and Semenza 1990). Pure diffusion represents the intrinsic leaky permeability of the vesicular membranes to different substrates (Gains and Hauser 1984). However, it is technically difficult to further quantitatively differentiate the apparent diffusion into the components simple diffusion and non-specific binding.

The significant contributions of L-amino acid diffusion observed in studies with enterocyte brush border membrane vesicles seem to be contradictory to the widely accepted concept. Under normal physiological conditions, enterocyte brush border membranes are highly impermeable to hydrophilic solutes including L-amino acids and only a small portion of transmembrane uptake of L-amino acids occurs through simple diffusion (Mailliard *et al.* 1995). This discrepancy between *in vivo* and *in vitro* membrane diffusion may be largely due to the fact that the process of membrane vesicle preparation may have destroyed the normal extracellular matrix and the cytoskeleton of enterocyte. Thus, this might have resulted in changes in membrane fluidity and increases in the brush border membrane permeability of the vesicles. In addition, it was also shown that a significant fraction of brush border membrane vesicles was usually not sealed completely, which could cause significant leaky diffusion (Gains and Hauser 1984).

In conclusion, the treatment of porcine intestinal brush border membrane vesicles with a minimum of 0.165 μmol $HgCl_2$ mg^{-1} vesicle protein completely blocks the carrier-mediated L-threonine and L-glutamine uptake and allows direct estimation of the apparent L-amino acid diffusion in intestinal brush border membrane vesicles.

Acknowledgements

We acknowledge the assistance of Charles Thomas with the mucosa collection. This report was submitted under No. 16412 of the Purdue University Agricultural Research Programs.

References

BERTELOOT A, SEMENZA G: Advantages and limitations of vesicles for the characterization and kinetic analysis of transport systems. *Methods Enzymol* **192:** 409-437, 1990.

BERTELOOT A, MALO C, BRETON S, BRUNTTE M: Fast sampling, rapid filtration apparatus: principal characteristics and validation from studies of D-glucose transport in human jejunal brush-border membrane vesicles. *J Membr Biol* **122**: 111-125, 1991.

BRACHET P, ALVARADO F, PUIGSERVER A: Kinetic evidence for separate systems in transport of D- and L-methionine by rat small intestine. *Am J Physiol* **252**: G320-G324, 1987.

BRACHET P, DEBBABI H, TOMÉ D: Transport and steady-state accumulation of putrescine in brush-border membrane vesicles of rabbit small intestine. *Am J Physiol* **269**: G754-G762, 1995.

BRADFORD M: A rapid and sensitive method for the determination of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal Biochem* **72**: 248-254, 1976.

CHEESEMAN CI: GLUT2 is the transporter for fructose across the rat intestinal basolateral membrane. *Gastroenterology* **105**: 1050-1056, 1993.

DAHLGVIST A: Method for assay of intestinal disaccharidases. *Anal Biochem* **7**: 18-25, 1964.

DANIELS VG, NEWEY H, SMYTH DH: Stereochemical specificity of neutral amino acid transfer systems in rat small intestine. *Biochim Biophys Acta* **183**: 637-639, 1969.

FAN MZ, ADEOLA O, MCBURNEY MI, CHEESEMAN CI: Kinetic partitioning of L-glutamine transport into porcine jejunal brush border membrane vesicles. *Comp Biochem Physiol A* **121**: 411-422, 1998.

FAN MZ, ADEOLA O, TUREK JJ, ASEM EK: Methodological aspects of measuring amino acid uptake in studies with porcine jejunal brush border membrane vesicles. *Physiol Res* **48:** 281-289, 1999.

GAINS N, HAUSER H: Leakiness of brush border vesicles. *Biochim Biophys Acta* **772**: 161-166, 1984.

GUNTHER RD, SCHELL RE, WRIGHT EM: Ion permeability of rabbit intestinal brush border membrane vesicles. *J Membr Biol* **78**: 119-127, 1987.

HEINOEN JK, LAHTI RJ: A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphate. *Anal Biochem* **133**: 313-317, 1981.

HOPFER U: Membrane transport mechanisms for hexoses and amino acids in the small intestine. In: *Physiology of the Gastrointestinal Tract.* Vol. 2. LR JOHNSON (ed), Raven Press, New York, 1987, pp 1499-1526.

MADARA JL, PAPPENHEIMER JR: Structural basis for physiological regulation of paracellular pathways in intestinal epithelia. *J Membrane Biol* **100**: 149-164, 1987.

MAENZ DD, CHEESEMAN CI: Effect of hyperglycemia on D-glucose transport across the brush border and basolateral membrane of rat small intestine. *Biochim Biophys Acta* **860**: 277-285, 1986.

MAENZ DD, PATIENCE JF: L-Threonine transport in pig jejunal brush border membrane vesicles. *J Biol Chem* **267**: 22079-22086, 1992.

MALO C., BERTELOOT A: Analysis of kinetic data in transport studies: new insights from kinetic studies of Na^+-D-glucose cotransport in human intestinal brush border membrane vesicles using a fast sampling filtration apparatus. *J Membr Biol* **122**: 127-141, 1991.

MAILLIARD ME, STEVENS BR, MANN GE: Amino acid transport by small intestinal, hepatic, and pancreatic epithelia. *Gastroenterology* **108**: 888-910, 1995.

SCHWARTZ A, JULIUS JC, HARIGAYA S: Possible involvement of cardiac Na^+/K^+ adenosine triphosphatase in the mechanism of action of cardiac glycosides. *J Pharmacol Exp Ther* **168**: 31-41, 1969.

STEVENS BR, ROSS HJ, WRIGHT EM: Multiple transport pathways for neutral amino acids in rabbit jejunal brush border vesicles. *J Membr Biol* **66**: 213-225, 1982.

STEVENS BR, KAUNITZ JD, WRIGHT EM: Intestinal transport of amino acids and sugars: advances using membrane vesicles. *Annu Rev Physiol* **46**: 417-433, 1984.

TSANG R, AO Z, CHEESEMAN CI: Influence of vascular and luminal hexoses on rat intestinal basolateral glucose transport. *Can J Physiol Pharmacol* **72**: 317-326, 1994.

WOLFFRAM S, EGGENBERGER E, SCHARRER E: Kinetics of D-glucose and L-leucine transport into sheep and pig intestinal brush border membrane vesicles. *Comp Biochem Physiol A* **84**: 589-593, 1986.

WRIGHT EM, VAN OS CH, Mircheff AK: Sugar uptake by intestinal basolateral membrane vesicles. *Biochim Biophys Acta* **597**: 112-124, 1980.

Reprint requests

Ming Z. Fan, Ph.D., Room 250, #70 Animal Science/Nutrition Building, Department of Animal and Poultry Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1, Fax: (519) 836-9873, E-mail: mfan@uoguelph.ca