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Methodological Aspects of Measuring Amino Acid Uptake in Studies with Porcine Jejunal Brush Border Membrane Vesicles

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Summary

With L-glutamine, as a representative amino acid this study was undertaken to examine the effects of substrate concentrations on initial and equilibrium amino acid uptake and intravesicular volume determined with porcine jejunal brush border membrane vesicles prepared by Mg^{2+}-aggregation and differential centrifugation. Transport measurements (24 °C) were conducted by the rapid filtration manual procedure. Glutamine uptake was shown to occur into an osmotically-active space ranging between 1.09-1.58 μl/mg protein with little non-specific membrane binding. At different concentrations (in parentheses), the duration of initial glutamine uptake in both Na^+ gradient and Na^+-free conditions was 10 s (0.01 mM), 15 s (0.17 mM), and 20 s (1.9 and 9.4 mM), respectively. Substrate concentrations affected the duration of initial uptake, with lower substrate concentrations giving shorter duration for initial amino acid uptake. At different substrate concentrations (in parentheses), the time required to reach equilibrium glutamine uptake was 5 min (0.01 mM), 10 min (0.17 mM), and 60 min (1.9 and 9.4 mM), respectively. Thus, substrate concentrations also affected the time required to reach equilibrium uptake. The higher the substrate concentration, the longer the incubation time needed to reach equilibrium amino acid uptake. At the glutamine concentrations of 0.01, 0.17, 1.9, and 9.4 mM, the average intravesicular volume was estimated to be 1.58±0.21, 1.09±0.28, 1.24±0.18, and 1.36±0.21 μl/mg protein, respectively. Substrate concentrations had no effect ($p>0.05$) on the intravesicular volume of membrane vesicles. In conclusion, in the experiments on amino acid transport kinetics measured with the rapid filtration manual procedure, the incubation time used for measuring the initial uptake rate should be determined from the time course experiments conducted at the lowest substrate concentration used, whereas the intravesicular volume can be obtained from equilibrium uptake measured at any substrate concentrations.

Key words
L-glutamine • Initial uptake • Equilibrium uptake • Brush border membrane vesicles • Pigs

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Introduction

Enterocyte brush border membrane vesicles, as prepared by either Mg^{2+}- or Ca^{2+}-aggregation and differential centrifugation, are widely used to characterize nutrient transport across the intestinal brush border membrane (Stevens *et al.* 1984, Hopfer 1987). The uptake of nutrients in isolated enterocyte membrane vesicles is usually determined by rapid filtration manual procedure and, in a very few cases, is measured by the semi-automatic and automatic apparatus (Berteloot *et al.* 1991, Kessler *et al.* 1978a,b). The advantages and disadvantages of the rapid filtration manual procedure and the automatic apparatus procedure have been previously compared and discussed (Berteloot and Semenza 1990, Berteloot *et al.* 1991).

In transport kinetic studies with the automatic apparatus procedure, the initial rate of uptake is obtained by regression analysis of nutrient uptake determined at multiple time points (Berteloot *et al.* 1991). However, in kinetic studies with the rapid filtration manual procedure, it is not feasible and practical to measure the initial rates of uptake from multiple time points over a broad range of substrate concentrations. Thus the initial uptake is often determined with one fixed time period assuming that the duration of initial nutrient uptake is similar for all substrate concentrations. Furthermore, the intravesicular volume, i.e. internal volume of membrane vesicles, is a characteristic parameter of membrane vesicles and is usually determined from the equilibrium uptake at one substrate concentration. However, it is not known whether substrate concentrations affect the duration of initial and equilibrium amino acid uptake and the subsequent determination of intravesicular volume. We feel that these questions are important methodological aspects of measuring amino acid uptake in membrane vesicles by the fast filtration manual procedure and need to be clarified.

Therefore, this study was designed to examine the effects of substrate concentrations on the duration of initial and equilibrium amino acid uptake and on the determination of intravesicular volume. Because glutamine transport across the enterocyte brush border membrane had been well characterized in several mammalian species (e.g. Bulus *et al.* 1989, Said *et al.* 1989), we used L-glutamine as a representative amino acid in the present study.

Method

Chemicals

L-[G-^{3}H]glutamine (specific activity: 1.63-1.70 TBq/mmol) was from Amersham Corporation. Ecolume scintillant was from IGN. Bio-Rad dye reagent was from Bio-Rad Laboratories. Phenylmethylsulfonyl fluoride (PMSF), ouabain, Na_2ATP, *p*-nitrophenyl phosphate, and other chemicals were from Sigma Chemical Co.

Animals and preparation of mucosal scrapings

The intestinal mucosal samples used in these studies were collected from four pigs of average weight of 110 kg obtained from the Purdue University Swine Research Center (a cross of Yorkshire-Landrace dams and Hampshire-Duroc sires). The entire small intestine was removed after sacrificing each pig and a 2-meter jejunal segment was dissected at six meters proximal to the ileo-cecal sphincter and flushed with ice-cold saline (154 mM NaCl, 0.1 mM PMSF, HEPES-Tris, pH 7.4). The jejunum was divided into 15-cm segments which were opened longitudinally and freed of mucus by patting with paper towel. Mucosa was collected by scraping the luminal surface firmly with a spatula. The mucosal scrapings from an individual pig were pooled, placed in tightly capped tubes, and stored at −70 °C. The experimental protocols were approved by Purdue University Animal Care and Use Committee.

Preparation of brush border membrane vesicles

Brush border membrane vesicles were prepared by Mg^{2+}-aggregation and differential centrifugation according to the procedure of Maenz and Patience (1992). Specifically, about 15 g of mucosal scraping was thawed in 300 ml of ice-cold homogenate buffer (50 mM D-mannitol, PMSF, HEPES-Tris, pH 7.4) and homogenized for 1 min using a polytron homogenizer. The resulting homogenate was pooled and centrifuged on a Sorvall SS-34 rotor at 2000 x *g* for 15 min. After removing the top foam layer and discarding pellets, the supernatant was mixed with 1 M $MgCl_2$ to a final concentration of 10 mM $MgCl_2$, stirred for 15 min at 4 °C, and then centrifuged at 2400 x *g* for 15 min. After removing the top foam layer, pellets were discarded and the resultant supernatant was centrifuged at 19000 x *g* for 30 min to generate crude brush border membrane pellets. The supernatant was discarded and 1 ml of the buffer (300 mM D-mannitol, 50 mM Hepes, pH 7.4) was added

to the centrifuge tubes. The pellets were resuspended by repeated passage through a 25-gauge needle and pooled.

For a given uptake experiment, the crude brush border membrane vesicle suspension was diluted in 60 ml of the buffer (14.4 mM D-mannitol, 150 mM KCl, HEPES-Tris, pH 7.4) and centrifuged at 39000 x *g* for 30 min to generate the final brush border membrane vesicle pellets. The final pellets were resuspended with a 25-gauge needle in a suitable volume of the same buffer to give the final brush border membrane vesicle suspension. Aliquots were taken for enzyme assays. The final vesicle suspension was assayed for protein content and diluted to 8 mg protein/ml with the same buffer for uptake measurements.

Protein and enzyme assays

Protein was determined according to the method of Bradford (1976) using Bio-Rad protein dye reagent and bovine serum albumin (fraction V) as standard. Alkaline phosphatase (EC 3.1.3.1) was assayed according to Engström (1964) with *p*-nitrophenyl phosphate as substrate. Sucrase (EC 3.2.1.48) was determined according to the procedure of Dahlgvist (1964). Ouabain-sensitive Na^+/K^+-ATPase (EC 3.6.1.3) was assayed by the method of Schwartz *et al.* (1969). The ouabain-sensitive Na^+/K^+-ATPase activity was determined as the difference between inorganic phosphate concentrations in the presence and absence of ouabain (5 mM).

Transport measurements

Transport experiments were carried out according to the rapid filtration manual procedure (Tsang and Cheeseman 1994). A fifty microliter uptake buffer was first placed at the bottom of a polystyrene tube (100 x 15 mm), and then 10 μl of the brush border membrane vesicle suspension was spotted onto the side of the tube in two separate drops immediately above the uptake buffer without contact with the ^{3}H-glutamine-containing uptake buffer. After 20-s warming up at the room temperature (24 °C), uptake incubation, i.e., the mixing of membrane vesicle suspension and uptake buffer, was initiated by a foot switch-activated vibromixer, the process was terminated by the injection of 1.125 ml of ice-cold wash solution (14.4 mM D-mannitol, 150 mM NaCl, 0.1 mM $HgCl_2$, HEPES-Tris, pH 7.4). One milliliter of the mixture solution was then rapidly pipetted onto the center of 0.45 μm cellulose acetate filters (pre-soaked with 20 mM L-glutamine, HEPES-Tris, pH 7.4) mounted in a Manifold filtration unit, which was connected to a vacuum source. Timing was controlled with an electronic GraLab model 545 timer/intervalometer. The filters were immediately rinsed four times with 5 ml of ice-cold wash solution. To avoid the disruption of the membrane vesicles, the rinsing buffer was injected into each filtration hole along the internal edge rather than directly on the center of the membrane filters. The remaining uptake mixture in the incubation tubes was collected and, at the end of the experiment, pooled and counted for the average initial radioactivity in the uptake media. After 30 min extraction in 5 ml of the scintillation fluid, the radioactivity associated with the filters was measured using a liquid scintillation analyzer with automatic quench correction. In the time course experiments, values of non-specific binding to filters and membrane vesicles were corrected by subtracting time-zero counting from the total counting. In order to correct for the non-specific membrane binding, the time-zero incubations were conducted by injecting the same amount of stop and wash buffer prior to the mixing of vesicle resuspensions and uptake buffers (Tsang and Cheeseman 1994).

Uptake incubations were performed in triplicate for each uptake experiment. Four uptake experiments were conducted using four batches of the vesicle suspension prepared from the mucosal scrapings of four pigs. Compositions of uptake buffers are described in detail in the legends to figures.

Calculations and statistical analyses

Glutamine uptake into the membrane vesicles was calculated according to the isotope dilution principle as:

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

where V is the amount of glutamine transported into the membrane vesicles (nmol/mg protein), R_F is radioactivity in disintegration per minute (DPM) of the filters (DPM/filter), R_B is the radioactivity for non-specific retention by filters or membrane vesicles (DPM/filter), [S] is glutamine concentration in the uptake media (nmol/μl), R_I is radioactivity in the uptake media (DPM/μl), and W is the amount of vesicle protein provided for the incubation (mg protein).

The average intravesicular volume of the porcine brush border membrane vesicles was calculated according to the principles described by Hopfer *et al.* (1973) and Maenz *et al.* (1991). When glutamine uptake reached equilibrium, the extravesicular glutamine concentration was equal to the intravesicular concentration. Therefore, intravesicular volume was calculated as:

$$V_O = [((R_F - R_B) \times [S])/R_I]/([S] \times W) \quad (2)$$

V_O is intravesicular volume of porcine jejunal brush border membrane vesicles under equilibrium glutamine uptake (μl/mg protein); R_F, R_B, R_I, [S], and W are as previously defined for equation (1).

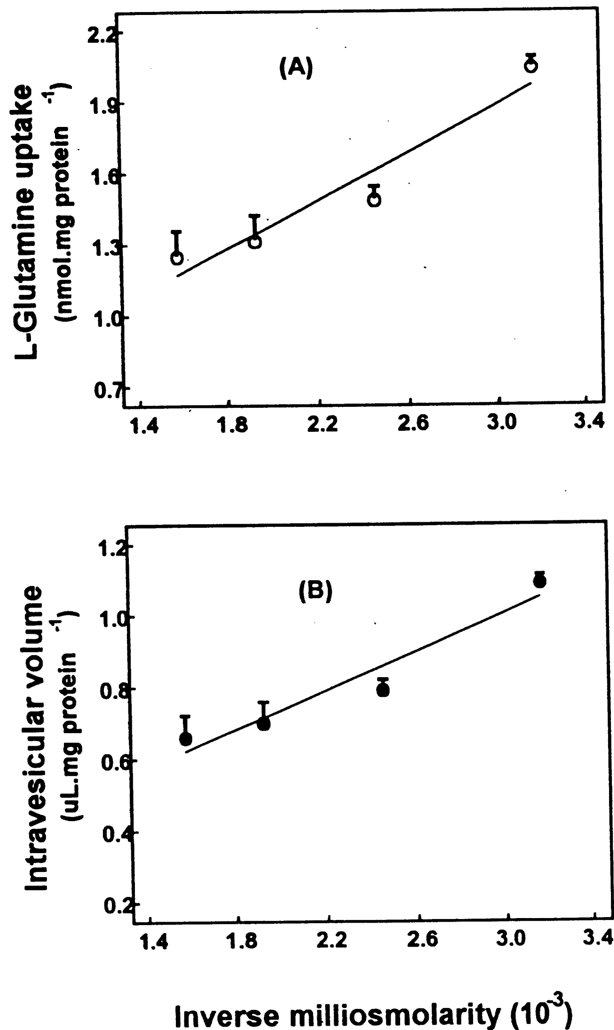


Fig. 1. *Effect of extravesicular inverse osmolarity gradient on equilibrium uptake of 1.9 mM L-glutamine in the presence of a Na^+ gradient (A) and on intravesicular volume of porcine jejunal brush border membrane vesicles (B). The membrane vesicles were pre-loaded with a buffer containing 14.4 mM D-mannitol, 150 mM KCl, 10 mM Trizma·HCl, pH 7.4. The four uptake buffers consisted of 150.0 mM NaCl, 10.0 mM Trizma·HCl, 2.3 mM L-glutamine, 0.64 uM L-[G-^{3}H]glutamine, pH 7.4, and graded concentrations of D-mannitol at 12.1, 95.2, 208.2, and 321.1 mM, respectively. Uptake incubations were conducted by mixing 10 μl the membrane vesicle suspension with 50 μl corresponding uptake buffer for 60 min. The resultant uptake media (60 μl) contained 1.9 mM L-glutamine, 125.0 mM NaCl, 25.0 mM KCl, and graded concentrations of D-mannitol. Each point represents the mean and standard error (n=4) of four uptake experiments using vesicle suspension prepared from the mucosal scrapings of four pigs. The linear relationship between the equilibrium glutamine uptake and inverse osmolarity gradient was $y=0.38+500.10*x$; $r^2=0.87$, $P<0.05$, $n=16$; the linear relationship between intravesicular volume and inverse osmolarity gradient was $y=0.20+266.60*x$; $r^2=0.87$, $P<0.05$, $n=16$.*

Results and Discussion

Relative to the mucosal homogenate, the brush border membrane fraction had an average of 12.8-fold enrichment in alkaline phosphatase specific activities and 0.39-fold enrichment in ouabain-sensitive Na^+/K^+-ATPase specific activity, indicating that the brush border membrane vesicle preparation had very little contamination with the basolateral membrane.

To ascertain that glutamine is transported into an osmotically active vesicular space but not mere non-specific binding to the vesicular membrane, the effects of extravesicular osmotic pressure on equilibrium glutamine uptake are shown in Figure 1. In this experiment, the initial extravesicular osmolarity was 315 mOsm. Extravesicular osmotic pressure was elevated by increasing the concentration of D-mannitol from 12.1 to 321.1 mM in the uptake buffers. This resulted in graded levels of extravesicular osmolarities of 315, 407, 520, and 633 mOsm. The intravesicular osmolarity was maintained at 315 mOsm. With increases in extravesicular osmolarity, 60-min equilibrium glutamine uptake decreased linearly ($P<0.05$) from 2.04 to 1.25 nmol/mg protein at the substrate concentration of 1.9 mM (Fig. 1A). The linear relationship between the equilibrium glutamine uptake and inverse osmolarity gradient was $y = 0.38 + 500.10*x$, $r^2 = 0.87$. In addition, at the concentration of 1.9 mM, the non-specific membrane binding of glutamine was extrapolated to be 0.38 nmol/mg protein from this linear relationship. Similarly, the intravesicular volume was linearly decreased ($P<0.05$) from 1.09 to 0.66 μl/mg protein with increasing extravesicular osmolarity (Fig. 1B). The linear relationship between the intravesicular volume and

inverse osmolarity gradient was y = 0.20 + 266.60*x, r^2 = 0.87. The linear effect of extravesicular osmolarity gradient on equilibrium glutamine uptake and intravesicular volume indicated that glutamine was transported into an osmotically active intravesicular space.

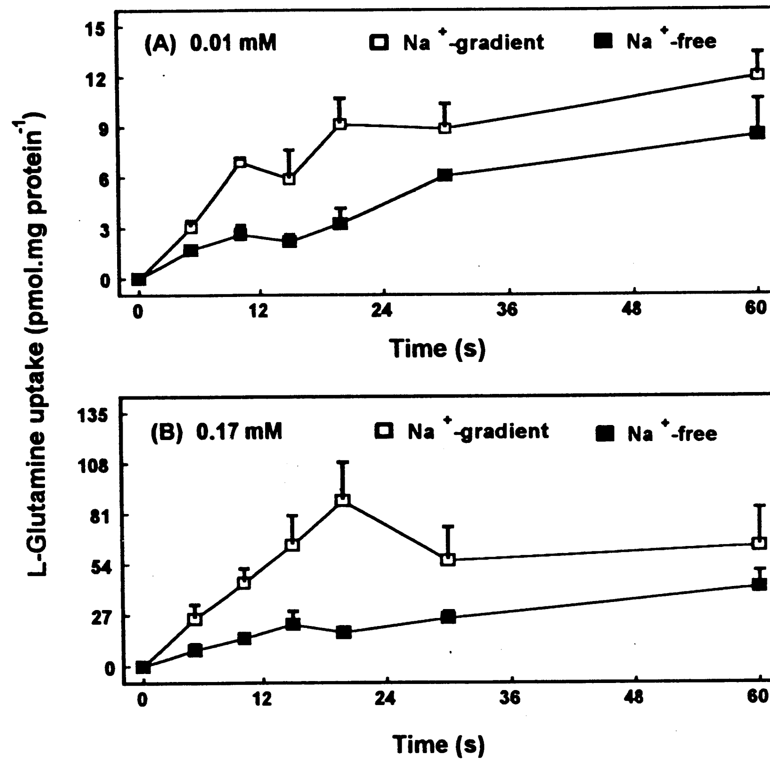


Fig. 2. *Initial L-glutamine uptake at concentrations of 0.01 and 0.17 mM under Na^+ gradient and Na^+-free conditions. The membrane vesicles were pre-loaded with a buffer containing 14.4 mM D-mannitol, 150 mM KCl, 10 mM Trizma·HCl, pH 7.4. The uptake buffers consisted of 150.0 mM NaCl, 10.0 mM Trizma·HCl, 14.39/14.20 mM D-mannitol, 0.012/0.20 mM L-glutamine, and 0.64/1.28 μM L-[G-^{3}H]glutamine at pH 7.4. Uptake incubations were conducted at the time points of 0, 5, 10, 15, 20, 30 and 60 s, respectively, by mixing 10 μl the membrane vesicle suspension with 50 μl corresponding uptake buffer. The resultant uptake media (60 μl) contained 0.01/0.17 mM L-glutamine, 125.0 mM NaCl, 25.0 mM KCl, and 10 mM Trizma·HCl. Each point represents the mean and standard error (n=4) of four uptake experiments using the vesicle suspension prepared from the mucosal scrapings of four pigs.*

The effects of substrate concentrations on initial and equilibrium glutamine uptake were examined at the concentrations of 0.01, 0.17, 0.19, and 9.4 mM, respectively. At the concentration of 0.01 mM in the presence and absence of a Na^+ gradient (Fig. 2A), glutamine uptake was linear approximately up to 10 s, i.e. the duration for initial uptake being 10 s. Furthermore, glutamine uptake reached an equilibrium at 5-min incubation in both Na^+ gradient and Na^+-free conditions and the equilibrium did not change for up to 60 min (Fig. 3A). Before reaching the equilibrium, glutamine uptake was enhanced in the presence of a Na^+ gradient in comparison with the Na^+-free condition (Fig. 2A).

At the concentration of 0.17 mM, glutamine uptake appeared to be linear for 15 s in both Na^+ gradient and Na^+-free conditions (Fig. 2B). Thus, the duration for initial glutamine uptake was 15 s at this concentration. Glutamine uptake reached an equilibrium at 10-min incubation under the Na^+ gradient and Na^+-free conditions and the equilibrium was maintained for up to 60 min (Fig. 3B). Before reaching equilibrium, glutamine uptake was stimulated by the Na^+ gradient (Fig. 2B).

At concentrations of 1.9 and 9.4 mM, glutamine transport appeared to be linear for 20 s, and the duration for initial glutamine uptake was 20 s at these concentrations (Fig. 4). Glutamine transport into membrane vesicles did not reach an equilibrium until 60-min incubation and the equilibrium did not change for up to 120 min (Fig. 5). Glutamine uptake was higher in the Na^+ gradient than in the Na^+-free condition before reaching the equilibrium (Fig. 4).

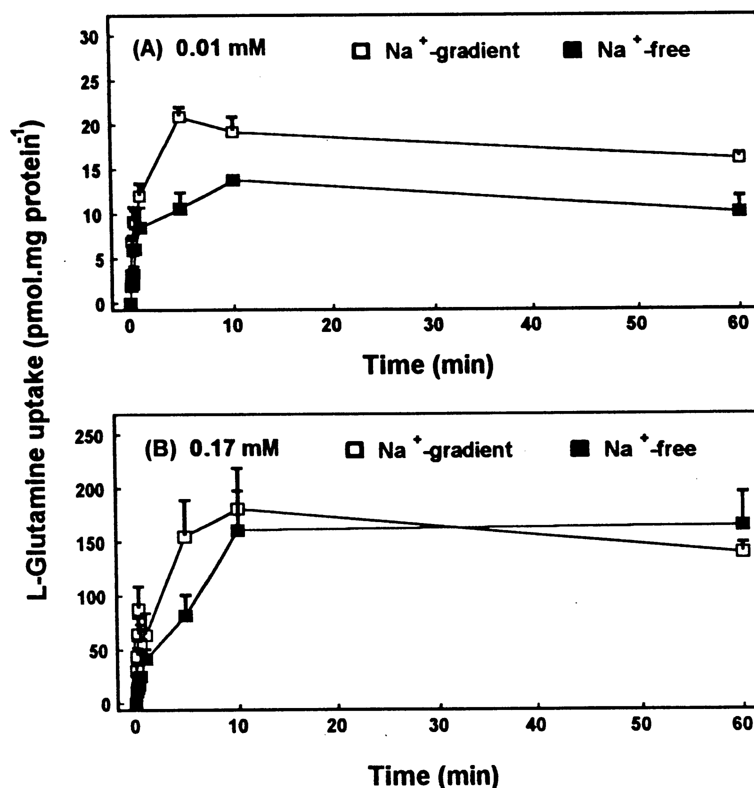
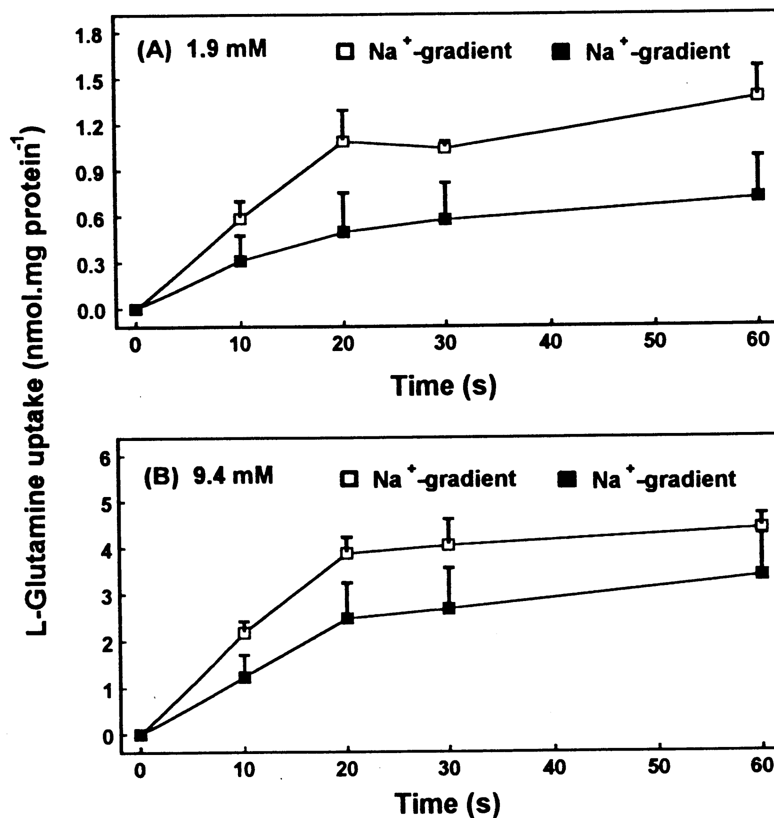


Fig. 3. *Equilibrium L-glutamine uptake at concentrations of 0.01 and 0.17 mM under Na^+ gradient and Na^+-free conditions. Uptake incubations were carried out at the time points of 0, 5, 10, 15, 20, 30, 60 s and 5, 10 and 60 min, respectively. Experimental conditions were the same as described for Fig. 2. Each point represents the mean and standard error (n=4) of four uptake experiments using vesicle suspension prepared from the mucosal scrapings of four pigs.*

Fig. 4. *Initial L-glutamine uptake at the concentrations of 1.9 and 9.4 mM under Na^+ gradient and Na^+-free conditions. The membrane vesicles were pre-loaded with a buffer containing 14.4 mM D-mannitol, 150 mM KCl, 10 mM Trizma·HCl, pH 7.4. The uptake buffers consisted of 150.0 mM NaCl, 10.0 mM Trizma·HCl, 12.1/3.1 mM D-mannitol, 2.3 or 11.3 mM L-glutamine, and 1.28 or 1.28 μM L-[G-^{3}H]glutamine, pH 7.4. Uptake incubations were conducted at the time points of 0, 10, 20, 30 and 60 s, respectively, by mixing 10 μl the membrane vesicle suspension with 50 μl corresponding uptake buffer. The resultant uptake media (60 μl) contained 1.9/9.4 mM L-glutamine, 125.0 mM NaCl, 25.0 mM KCl, and 10 mM Trizma·HCl. Each point represents the mean and standard error (n = 4) of four uptake experiments using vesicle suspension prepared from the mucosal scrapings of four pigs.*



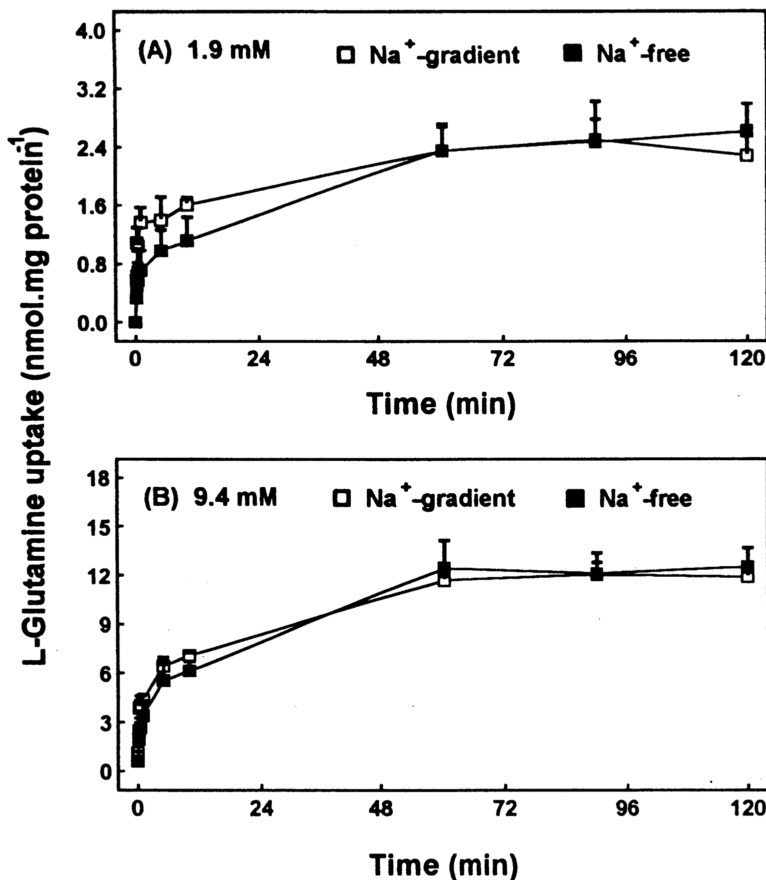


Fig. 5. *Equilibrium L-glutamine uptake uptake at concentrations of 1.9 and 9.4 mM under Na^+ gradient and Na^+-free conditions. Uptake incubations were carried out at the time points of 0, 10, 20, 30, 60 s and 5, 10, 60, 90 and 120 min, respectively. Experimental conditions were the same as described for Fig. 4. Each point represents the mean and standard error (n = 4) of four uptake experiments using vesicle suspension prepared from the mucosal scrapings of four pigs.*

Taken together, these results clearly demonstrated that substrate concentrations affected the duration of initial amino acid uptake. The lower the substrate concentration, the shorter the duration of initial amino acid uptake. Furthermore, substrate concentrations also affected the amount of time required for reaching equilibrium amino acid uptake. The higher the substrate concentration, the longer the incubation time needed to reach equilibrium uptake.

Intravesicular volume is an important parameter characterizing prepared enterocyte membrane vesicles. Based on the equilibrium glutamine uptake, the volume of intravesicular space of the porcine jejunal brush border membrane vesicles was estimated at different substrate concentrations in both Na^+ gradient and Na^+-free conditions, ranging between 1.09-1.58 μl/mg protein (Table 1). Except for the glutamine concentration of 0.01 mM, at which equilibrium glutamine uptake and intravesicular volume measured were relatively higher in the Na^+ gradient condition than in the Na^+-free condition, there were no differences in estimated intravesicular volume values between the Na^+ gradient and Na^+-free conditions. Therefore, the measured intravesicular volume values were pooled for both Na^+ gradient and Na^+-free conditions. At the glutamine concentrations of 0.01, 0.17, 1.9 and 9.4 mM, the average intravesicular volume was estimated to be 1.58±0.21, 1.09±0.28, 1.24±0.18, and 1.36±0.21 μl/mg protein, respectively. There were no differences in the intravesicular volume values of the vesicles determined at different glutamine concentrations. The intravesicular volume, 1.09-1.58 μl/mg protein, determined in this study was similar to the values (1-2 μl/mg protein) reported by Crooker and Clark (1986), Wolffram *et al.* (1986), and summarized by Hopfer (1987). However, studies by Johnston and Freeman (1988) observed much smaller intestinal brush border membrane vesicular volume of 0.42-0.64 μl/mg protein, as these were prepared from the rat jejunum. In a recent study by Buddington and Malo (1996), intravesicular volume of intestinal brush border membrane vesicles from 8-week porcine fetuses was reported to be 1.5-3.6 μl/mg protein and the measurements were affected by temperature. Therefore, temperature may be partly responsible for the differences in the intravesicular volume values reported in the different studies. Additionally, variation in the intravesicular volume between various studies probably resulted from their differences in the process of mucosal homogenization including types of homogenizer, time and speed applied.

Table 1. Intravesicular volume of porcine jejunal enterocyte brush border membrane vesicles determined from equilibrium L-glutamine uptake at different concentrations

	L-glutamine concentrations (mM)			
	0.01	0.17	1.90	9.40
Equilibrium time[1]	5	10	60	60
Equilibrium uptake[2]	15.75±2.07	185.58±47.35	2354.65±340.15	12739.60±1957.60
Intravesicular volume[3]	1.58±0.21	1.09±0.28	1.24±0.18	1.36±0.21

Data are means ± S.E.M., n=8, [1]Equilibrium time: min, [2]Equilibrium uptake: pmol/mg protein., [3]Intravesicular volume: µl/mg protein.

In summary, with glutamine as a representative amino acid, this study has shown that substrate concentrations affected the duration of time required for both initial and equilibrium amino acid uptake into enterocyte brush border membrane vesicles. However, substrate concentrations appeared to have no effects on the determination of intravesicular volume. Therefore, in transport kinetic studies with rapid filtration manual procedure, the rates of initial uptake should be measured with the duration of initial amino acid uptake obtained from the lowest substrate concentration used, whereas intravesicular volume can be obtained from equilibrium uptake measured at any substrate concentrations. Furthermore, these principles that were clearly demonstrated in the current study may be applicable to nutrient uptake in studies with various membrane vesicles, such as enterocyte basolateral membrane vesicles, renal proximal tubular membrane vesicles, hepatic membrane vesicles, and muscle sarcolemmal membrane vesicles, when uptake is measured with the fast filtration manual procedure.

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Reprint requests

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