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## Differentiating Passive from Transporter-mediated Uptake by PepT1: A Comparison and Evaluation of 4 Methods

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### Abstract

**Introduction**—To quantify transmembrane transport of dipeptides by PepT1, passive uptake (non-PepT1 mediated) must be subtracted from total (measured) uptake. Three methods have been described to estimate passive uptake: perform experiments at cold temperatures, inhibit target dipeptide uptake with greater concentration of a second dipeptide, or use modified Michaelis-Menten kinetics. We hypothesized that performing uptake experiments at pH (8.0) would estimate passive uptake accurately, because PepT1 requires a proton gradient. Our aim was to determine the most accurate method to estimate passive uptake.

**Methods**—Caco-2 cells were incubated with various concentrations of glycyl-sarcosine (gly-sar) at pH 6.0 and at 37 °C to measure total uptake. Passive uptake was estimated: 1) by incubating Caco-2 cells with varying concentrations of gly-sar at 4 °C, 2) in the presence of 50 mM glycyl-leucine, 3) in solution at pH 8.0, or 4) using modified Michaelis-Menten kinetics. PepT1-mediated uptake was calculated by subtracting passive uptake from total uptake.  $K_m$ ,  $V_{max}$ , and % gly-sar transported by PepT1 were calculated and compared.

**Results**— $K_m$ ,  $V_{max}$ , and % gly-sar transported by PepT1 varied from 0.7-2.4 mM, 8.4-21.0 nmol/mg protein/10 min, and 69%-87%, respectively. Uptakes calculated with cold, 50 mM gly-leu and using modified Michaelis-Menten kinetics were similar but differed significantly from uptake at pH 8.0 ( $p < 0.001$ ).

**Conclusions**—Estimating passive uptake at pH 8.0 does not appear to be accurate. Measuring uptake at cold temperatures or in the presence of a greater concentration of a second dipeptide, and confirming results with modified Michaelis-Menten kinetics is recommended.

### Keywords

peptide; PepT1; Caco-2; dipeptide transport; passive dipeptide transport; transporter-mediated dipeptide transport

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## INTRODUCTION

In the study of intestinal nutrient absorption and transporter physiology, uptake of a specific substance by the transporter of interest can be difficult to measure accurately. For many substances, especially hexoses and short peptides, there is a transporter-mediated, saturable component (active) and a non-transporter-mediated, nonsaturable component of absorption (passive) (1, 2). The saturable component is determined by the kinetic parameters of the membrane transporter and represents the amount of absorption that is mediated by the transporter, either as an active, energy-requiring process or as a “facilitated” transport process. Curves corresponding to simple, saturable, transporter-mediated uptake exhibit asymptotic behavior and are described accurately by Michaelis-Menten kinetics (1, 3, 4).

The nonsaturable or “passive” component of absorption is directly proportional to the concentration of the substance and increases linearly without plateau as the extracellular concentration of a substance increases (1, 2, 5). In laboratory experiments, this nonsaturable component can be attributed not only to what is absorbed in a nontransporter-mediated manner (paracellular flow, solvent drag, etc.) across the epithelial lining but also to the amount of substance of interest that is *adsorbed* onto the tissue being tested or adherent to the tissue in which the assay is being performed. Therefore, it is imperative to account for passive uptake in order to study accurately the physiology of the transporter without the “noise” of passive uptake. Diagrammatic representations of nonsaturable or “passive” absorption are usually straight lines with an origin at zero that exhibit a linear increase in absorption as the concentration increases.

In most experiments, total uptake is the value that is measured, and the passive component is subtracted from this value resulting in the calculated, “transporter-mediated” component of absorption (transporter-mediated = total – passive) (1, 2, 4-6). To these transporter-mediated values, biologic formulas such as the Michaelis-Menten equation can be applied and important kinetic descriptors (i.e.  $K_m$  and  $V_{max}$ ) determined.

In our laboratory we have studied previously the hexose transporters SGLT1 and GLUT2. Our method of determining specifically the active transport component of uptake exploits the stereospecificity of these transporters. We use [ $^{14}C$ ] $_D$ -glucose to determine total uptake and [ $^3H$ ] $_L$ -glucose, which is not transported by the stereospecific transporters SGLT1 and GLUT2, to determine passive uptake. Subtracting passive uptake from total uptake allows accurate calculation of the amount of glucose actually transported by SGLT1 and GLUT2 (7-11).

As we have expanded the scope of our laboratory to study peptide absorption, we found that determining the transporter-mediated proportion of peptide transport is not as straightforward. PepT1 (human gene SLC15A1) is the putative transporter of the bulk of dietary protein/nitrogen in the form of all 400 di- and all 8,000 tripeptides from the intestinal lumen across the apical membrane into the enterocyte (12, 13). PepT1 is also responsible for the absorption of many peptidomimetic drugs, such as angiotensin-converting enzyme inhibitors (14, 15),  $\beta$ -lactam antibiotics (16), and antivirals such as oseltamivir (17), acyclovir, valganciclovir (18), and azidothymidine (AZT) (19).

The wide variety of compounds transported by this protein presents a unique challenge to its study. Unlike the glucose transport system of SGLT1 and GLUT2, stereospecificity cannot be exploited, and thus, differentially radio-labeled stereoisomers cannot be used. Reports in the literature are not unanimous and employ typically one of three methods to correct total uptake for passive uptake in the study of PepT1-mediated transport: 1) perform the experiment at near-freezing temperatures in order to “inactivate” the transporter (20, 21), 2) inhibit competitively the uptake of the compound of interest with a markedly greater

concentration of a second substrate for which the transporter has a greater affinity (lesser  $K_m$ ) (2, 6, 15, 22-24), and (3) use nonlinear regression analysis of total uptake with modified Michaelis-Menten kinetics (1, 6, 25-28). Because PepT1 relies on a proton gradient and functions most efficiently at a pH of 6.0, we hypothesized that passive uptake might also be estimated by increasing the pH of test solutions to a pH of 8.0 (29).

To our knowledge, there has not been a direct, rigorous evaluation and comparison of these four methods to estimate passive uptake of peptides. We devised a study using Caco-2 cells and the model dipeptide glycyl-sarcosine (gly-sar). The Caco-2 cell line is a well-characterized, human-derived, cell culture model of intestinal epithelium known to express PepT1; Caco-2 cells are used frequently in studies of nutrient absorption and pharmacokinetics (1-3, 6, 14, 15, 20-22, 24-28, 30-32). Gly-Sar is a model, hydrolysis-resistant dipeptide not found in nature and is used frequently as a substrate to study PepT1-mediated transport (13). The  $K_m$  of PepT1 for gly-sar has been reported to be 0.35-3.8 mM depending on experimental conditions (3-6, 30-34). Additionally, in transfection studies comparing PepT1 and non-PepT1 containing cells, PepT1 is responsible for approximately 80% of the absorbed gly-sar at concentrations less than its  $K_m$  (1, 4, 5, 29).

The aim of our study was to determine the best method to estimate passive uptake of dipeptides and thereby calculate accurately the PepT1-mediated transport in Caco-2 cells. An appropriate method per our criteria would produce a linear passive curve without a plateau that would correct total uptake resulting in an uptake curve consistent with a transporter-mediated process. Our hypothesis was that all four methods would be equivalent and yield similar results.

## METHODS

### Materials

Caco-2 cells were purchased from American Type Culture Collection at passage 18 (Manassas, VA), while the 24-well cell culture plates were from Corning Life Sciences (Lowell, MA) and T-75 culture flasks were from BD Biosciences (San Jose, CA). HEPES, MES, glycylsarcosine (gly-sar), and glycyl-leucine (gly-leu) were purchased from Sigma (St. Louis, MO). Hank's balanced salt solution with calcium and magnesium (HBSS), minimum essential medium nonessential amino acids (NAA) (100x), and sodium pyruvate (100 mM) were purchased from Mediatech Cellgro Inc. (Manassas, VA). Phosphate-buffered saline (PBS), Dulbecco's modified Eagle medium (DMEM), and penicillin 10,000 units/ml-streptomycin 10,000 µg/ml (P-S) were purchased from Invitrogen Corp. (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from PAA laboratories (Dartmouth, MA) and [<sup>14</sup>C]Glycyl-sarcosine ([<sup>14</sup>C]gly-sar) from Moravек Biochemicals (Brea, CA). BCA Protein Assay Kit (#23225) was purchased from Thermo Fisher Scientific Inc. (Rockford, IL). Solvable™ and Opti-Fluor were obtained from PerkinElmer (Waltham, MA) and high density polyethylene liquid scintillation vials (7 ml) from Research Products International Corp. (Mount Prospect, IL). Liquid scintillation counting was performed using a Beckman LS6000SC (Beckman Coulter Inc., Brea, CA).

### Cell Culture

Caco-2 cells were maintained in DMEM (4.5 g/L D-glucose, 4 mM L-glutamine) supplemented with 20% v/v FBS, 1% sodium pyruvate, 1% NAA, and 1% P-S. Cells were cultured in T-75 flasks at 37°C in 95% O<sub>2</sub>/5% CO<sub>2</sub> and 90% humidity and were replated in a 24 well plate when they reached >80% confluence. The culture medium was changed every 2-3 days as needed.

## Uptake Studies

Caco-2 cells were plated at a density of  $1 \times 10^5$  cells/cm<sup>2</sup> in 24-well plates. After confluence was reached (~3 days), cells were maintained for 14 days to polarize under the conditions described previously. We performed scanning electron microscopy and showed that at 14 days, the cells had differentiated by forming tight junctions and microvilli at the apical membrane of the cells (unpublished data). Uptake studies were performed in triplicate on the same day and in the same passage and were repeated in triplicate (n=9 independent monolayers) on different days and in different passages. Passages 28-32 were used in this study. Media was always changed the day prior to the experiment.

Control monolayers (to measure total uptake) were washed gently with 500  $\mu$ l of HBBS buffered with 10 mM HEPES and adjusted to pH 7.4 (HBSS 7.4) after which they were incubated in an additional 500  $\mu$ l HBSS 7.4 for 10 min. The HBSS 7.4 was then aspirated, and 300  $\mu$ l of HBSS buffered with 10 mM MES and adjusted to pH 6.0 containing 0.02, 0.1, 0.5, 1, 2.5, 5, 7.5, or 10 mM gly-sar labeled with 125 nCi/ml [<sup>14</sup>C]gly-sar was instilled. Monolayers were incubated with these test solutions for 10 min. Uptake of gly-sar was stopped by aspirating the test solution and rinsing and aspirating gently three times with 500  $\mu$ l of ice-cold PBS (pH 7.4). All solutions and incubations were maintained at 37 °C unless noted otherwise. Cells were solubilized overnight with 300  $\mu$ l/well of Solvable™; 200  $\mu$ l was used for liquid scintillation counting in rigid polyethylene vials to which 4.5 ml of Opti-Fluor was added, and 10  $\mu$ l  $\times$  2 was used for determining total protein/well in duplicate using the BCA Test Kit according to the manufacturer's directions.

Passive uptake was determined at cold (~4 °C) temperature, in the presence of 50 mM gly-leu and in solution at pH 8.0. To test uptake at cold temperatures, monolayers were treated similar to control monolayers, except all solutions were ice-cold, and incubations took place on ice. To test uptake in the presence of a competitive inhibitor, 50 mM gly-leu was added to each gly-sar containing solution. Gly-Leu was chosen, because it is a hydrolysis-resistant dipeptide used frequently in PepT1 transport studies; moreover, gly-leu has a  $K_m$  less than the  $K_m$  of gly-sar (0.08 mM vs. 1.9 mM) (29). The gly-leu concentration of 50 mM was selected to saturate PepT1, so that it preferentially became saturated with gly-leu, and all [<sup>14</sup>C]gly-sar detected after lysing the cells was attributed to passive uptake. To test uptake at pH 8.0, the same procedure as for controls was followed except that the HBSS containing gly-sar was buffered at pH 8.0 with 10 mM HEPES (HBSS 8.0).

## Data Analysis

Values for transporter-mediated uptake at each gly-sar concentration were determined by subtracting passive uptake (pH 8.0, 50 mM gly-leu, or cold temperature) from total (control) uptake. Using nonlinear regression (GraphPad Prism 4.03, GraphPad Software Inc., San Diego, CA), the best fit curve of the Michaelis-Menten equation was determined for transporter-mediated uptake values using equation 1:

$$V_o = (V_{max} [S]) / (K_m + [S]) \quad \text{Equation 1.}$$

where  $V_o$  is the initial uptake velocity,  $V_{max}$  is the maximal uptake velocity at saturating substrate concentrations,  $K_m$  is a constant analogous to the Michaelis-Menten constant, and  $S$  is the substrate concentration.

To determine transporter-mediated and passive components of total uptake using only nonlinear regression, prior to subtracting passive uptake, the best fit curve for total uptake values was determined using a modified Michaelis-Menten equation reported previously (1, 6, 25-28):

$$V_o = (V_{max} [S]) / (K_m + [S]) + K_d [S] \quad \text{Equation 2.}$$

where  $V_o$ ,  $V_{max}$ ,  $K_m$ , and  $S$  are as described above, and  $K_d$  is the rate constant for the nonsaturable (passive) uptake. To display graphically the line for passive uptake and transporter-mediated uptake using this model,  $K_d$  was multiplied by the tested gly-sar concentrations. The resulting product was subtracted from the values of total absorption resulting in values for transporter-mediated transport. These values were then plotted using the traditional Michaelis-Menten equation and nonlinear regression. The  $K_m$  and  $V_{max}$  values reported, however, are those calculated using the modified Michaelis-Menten equation applied to total uptake.

First order polynomial nonlinear regression was used to fit the lines for passive uptake using the equation:

$$y = m(x) + b \quad \text{Equation 3.}$$

where  $y$  is reaction velocity,  $x$  is substrate concentration,  $m$  is the slope of the line, and  $b$  is the  $y$ -intercept;  $b$  was not constrained to zero in order to fit the line most accurately (and to account for adsorption).

### Statistical Analysis

Matched pairs ANOVA using the Tukey post hoc test (GraphPad Prism 4.03) was used to determine if differences in passive and transporter-mediated values existed between the various methods. All results are expressed as mean  $\pm$  standard error of the mean (SEM) unless noted otherwise.

## RESULTS

As expected, the total uptake of gly-sar, uncorrected for passive uptake, demonstrated a saturable component and nonsaturable component based on its appearance – an initial sharp increase in uptake followed by a gentle, constant, positive slope (fig 1). Analysis of total uptake by the Michaelis-Menton equation, without any correction for passive uptake, resulted in a  $K_m$  and  $V_{max}$  of  $5.1 \pm 1.4$  mM and  $41.4 \pm 5.1$  nmol/mg protein/10 min, respectively.

Each method of estimation of passive uptake of gly-sar showed appropriately a directly proportional, linear, concentration-dependent increase in uptake that did not plateau or become asymptotic (fig 1). The  $y$ -intercept and slopes ranged from 0.14-0.41 nmol/mg protein/10 min and 1.1-2.1 nmol/mg protein/10 min/mM, respectively (table 1). Values for passive uptake of gly-sar as estimated by testing gly-sar absorption at pH 8.0 were substantially greater than the values estimated at cold temperatures or using 50 mM gly-leu ( $p < 0.05$  respectively). There were no differences in the values for passive uptake of gly-sar estimated at cold temperature compared to 50 mM gly-leu ( $p > 0.05$ ) (table 2). Subtracting passive uptake from total uptake resulted in values of transporter-mediated uptake that were greater when corrected by the cold assay technique and by the 50 mM gly-leu technique than for values of transporter-mediated uptake as determined by correction for passive uptake estimated at pH 8.0 ( $p < 0.001$  respectively) (fig 2 and table 2). Values for  $K_m$  and  $V_{max}$  ranged from 0.7-2.4 mM and 8.4-21.0 nmol/mg protein/10 min respectively (table 3).

Analyzing total uptake by the modified Michaelis-Menten equation resulted in values for  $K_m$ ,  $V_{max}$ , and  $K_d$  of  $1.0 \pm 0.8$  mM,  $12.2 \pm 5.3$  nmol/mg protein/10 min, and  $1.8 \pm 0.5$  nmol/mg

protein\10 min\mM, respectively (table 3). In order to separate graphically the transporter-mediated and passive components of uptake,  $K_d$  was multiplied by the tested concentrations of gly-sar to estimate passive uptake. Estimated values of passive uptake were subtracted from total uptake to determine transporter-mediated uptake which was graphed using the Michaelis-Menton equation (fig 3). This approach resulted in a curve of transporter-mediated uptake that lies between but was not significantly different than transporter-mediated curves corrected for passive uptake using pH 8.0, cold temperature, and 50 mM gly-leu ( $p>0.05$  respectively) (fig 2 and table 2).

When the amount of gly-sar estimated to be transported by PepT1 was analyzed for each method over the concentration range of 0.02-1 mM, the method using pH 8.0 to determine passive uptake estimated 69% of the gly-sar taken up was transported by PepT1, while the methods of 50 mM gly-leu, cold temperature, and the modified Michaelis-Menton equation resulted in estimates that were remarkably consistent – 85%, 87%, and 83%, respectively (table 3).

## Discussion

In our previous *in vitro* study of dipeptide uptake by PepT1 in whole tissue, we used the method of Matthews *et al.* to correct total uptake for passive uptake in order to study transporter-mediated uptake. With this method, passive uptake at lesser concentrations is estimated from total uptake at much greater (10-100x) concentrations of gly-sar. As substrate concentration increases, so does passive uptake, and once the transporter is saturated, the linear increase in total uptake is attributed only to passive uptake independent of a membrane transport protein, i.e. passive diffusion and mucosal adherence<sup>(33-35)</sup>. When we applied this method to our cell culture work, however, we suspected this method to be less reliable, yielding poor results and prompting us to seek a better technique. A thorough search of the literature yielded the methods that we tested against one another in this experiment. To our knowledge, this is the first study comparing the three methods (cold temperature, markedly greater concentration of a different dipeptide, and modified Michaelis-Menten kinetics) used most commonly to correct total uptake of peptides by passive uptake to estimate the specific PepT1-mediated uptake.

All methods for estimating passive uptake were reproducible. Cold temperature, a markedly greater concentration of a different dipeptide, the modified Michaelis-Menten equation appear to be relatively equivalent and differ significantly from using an increased pH to quench the proton gradient-dependent, transporter-mediated uptake of PepT1. A pH of 8.0 was chosen based on previous studies demonstrating that PepT1 functions most efficiently at pH 6.0 – a pH at which a proton gradient from the extracellular environment into the cell is present. Our assumption was that at pH 8.0 (1/100 of the proton gradient at pH 6.0), the proton gradient to transport dipeptides would be abolished. We were concerned that at more alkaline pHs, issues of cell membrane stability would be present, altering more variables in our experimental setup than transport of dipeptides by PepT1. Additionally, it is possible that at pH 8.0, passive diffusion of dipeptides is increased due to alteration of several characteristics of the cell membrane which might account for the increase in passive absorption of dipeptides that we observed in our experiments (figure 1). Also possible is that at pH 8.0, sufficient protons were present to create a small gradient into the cell allowing for PepT1 to continue transporting gly-sar into the cell. This possibility could explain the increased absorption at pH 8.0 compared to the other methods tested.

A relative advantage of using modified Michaelis-Menten kinetics when compared to the other methods is that it is quicker to compute and does not require “extra” experiments to be conducted or additional solutions to be prepared. Because of the consistency of our results

with three of the four tested methods, we maintain that cold temperature, a markedly greater concentration of a different dipeptide, and the modified Michaelis-Menten equation are more accurate than correction using a pH 8.0 based on this inter-method variation. Although it is possible that the  $K_m$  and  $V_{max}$  estimated for PepT1 using the three equivalent methods are incorrect and those estimated using the increased pH method are more accurate, such a scenario seems unlikely, because the other three methods gave very similar and congruent values of passive uptake of gly-sar. Our data could be criticized, because we do not present evidence confirming or disproving that other transporters are or are not involved. We and others have confirmed the presence of PepT1 in the Caco-2 cell line (3, 22, 24-26, 28). We believe, however, that our estimation of  $K_m$  and  $V_{max}$  of PepT1 for gly-sar is consistent with previously reported values under different conditions. While  $K_m$  is relatively easy to compare between studies,  $V_{max}$  is more difficult because of the variety of durations of incubation that have been evaluated and the manner in which it is reported (i.e. per min, per 10 min, per min after 10 min incubation, etc.).  $K_m$  may also vary depending on a wide variety of experimental factors, including modifications to PepT1 with tags (myc, hemagglutinin, green fluorescent protein etc.), transfection into a non-PepT1 expressing cell, cloning, or other alterations. The most convincing evidence from the results of our study using a cold temperature, a markedly greater concentration of a second dipeptide, and/or the modified Michaelis-Menten equation to correct total uptake for passive uptake is the estimate that ~80% of gly-sar is taken up by a transporter-mediated process at concentrations of gly-sar  $\leq 1$  mM (table 3). This value is similar to and consistent with PepT1 knockout and transfection studies comparing dipeptide uptake in PepT1 and non-PepT1 containing cells/tissues. (1, 4, 5, 29).

This information should allow investigators studying PepT1 to make an informed, evidence-based decision about methods required to correct for passive uptake. This topic is of considerable importance in the study of epithelial transport when trying to determine potential methods to augment peptide absorption under various disease states, such as short bowel syndrome, mucosal disease, or other disorders of absorption. As for other membrane transport proteins, PepT1 is believed to be regulated in part by translocation to the apical membrane from preformed intracellular stores of cytoplasmic PepT1. To study this important mechanism, accurate measurements of transporter-mediated uptake are imperative.

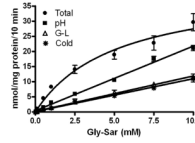
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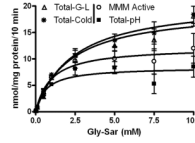
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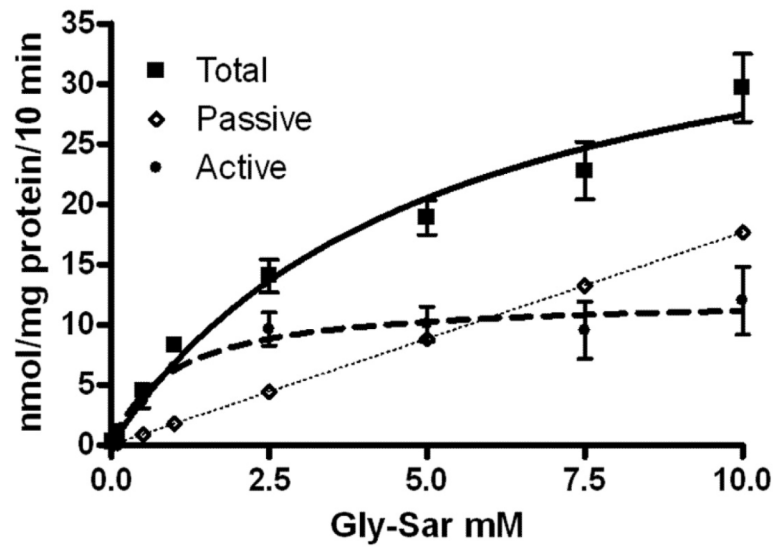
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**Figure 1.** Total and passive uptake (pH 8.0, 50 mM gly-leu, and 4 °C) of gly-sar into Caco-2 cells. Uptake values are presented as mean  $\pm$  the standard error of the mean (n=9).



**Figure 2.** PepT1-mediated uptake of gly-sar into Caco-2 cells. Uptake values are presented as mean  $\pm$  the standard error of the mean (n=9). MMM is Modified Michaelis-Menten Equation



**Figure 3.** Graphic representation of the passive and active components of absorption using the modified Michaelis-Menten equation (eq 2). Uptake values are presented as mean  $\pm$  the standard error of the mean (n=9).

**Table 1**

## Methods of Estimation of Passive Uptake

Method	Slope (SEM)	y-intercept (SEM)
pH=8.0	2.1 (0.1)	0.41 (0.23)
Gly-Leu	1.2 (0.1)	0.19 (0.23)
Cold	1.1 (0.1)	0.14 (0.26)

Line parameters for passive uptake determined by nonlinear regression using the equation  $y=m(x) + b$ .

**Table 2**

Comparison of Curves Estimating Transporter-mediated and Passive Uptake

	Comparison of Transporter-mediated Curves p	Comparison of Passive Uptake Curves p
pH vs. Gly-Leu	< 0.01	< 0.05
pH vs. Cold	< 0.01	< 0.05
pH vs. Modified Michaelis-Menten Equation	> 0.05	
Gly-Leu vs. Cold	> 0.05	> 0.05
Gly-Leu vs. Modified Michaelis-Menten Equation	> 0.05	
Cold vs. Modified Michaelis-Menten Equation	> 0.05	

p-values for the comparisons of active and passive uptake curves using different methods to estimate passive uptake.

**Table 3**

Kinetic Parameters of Transporter-mediated Uptake

Method of Correction*	Uncorrected	pH	Gly-Leu	Cold	Michaelis-Menten Equation
$K_m$ (mM)	5.1 ± 1.4	0.7 ± 0.3	2.2 ± 0.6	2.4 ± 0.6	1.0 ± 0.8
$K_m$ 95% CI	2.4-7.8	0.02-1.3	1.0-3.4	1.1-3.6	0.1-1.8
$V_{max}$ (nmol/mg protein/10min)	41.4 ± 5.1	8.4 ± 0.9	19.6 ± 1.7	21.0 ± 1.8	12.2 ± 5.3
$V_{max}$ 95% CI	31.2-51.5	6.6-10.2	16.1-23.1	17.4-24.6	9.5-14.9
$K_d$ (nmol/mg protein\10 min\ mM)					1.8 ± 0.5
Average % gly-sar transported by PepT1 (0.02-1 mM)		69	85	87	83

\* Kinetic characteristics of PepT1-mediated gly-sar uptake using different methods to correct total uptake for passive uptake (n=9). \*  $\bar{x} \pm SEM$  when appropriate