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Transport characteristics of L-glutamate in human jejunal brush-border membrane vesicles

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Previous work using human jejunal brush-border membrane vesicles has demonstrated the existence of a distinct transport system in man for acidic amino acids. This system is energized by an inwardly directed Na^+ gradient and an outwardly directed K^+ gradient. These studies further characterize the transport of L-glutamate in the human jejunal brush-border membrane vesicles. Efflux studies were performed by loading the brush-border membrane vesicles with radiolabeled L-glutamate and sodium chloride. Extravesicular K^+ accelerated the efflux of L-glutamate when compared to extravesicular Na^+ or choline, indicating that potassium serves to recycle the carrier. Unlabeled extravesicular L-glutamate (but not D-glutamate) also enhanced the efflux of radiolabeled L-glutamate demonstrating that there is a bidirectional similarity to the transport system. The effect of pH on the transport system was also investigated by varying the intravesicular and extravesicular pH from 5.5 to 9. A pH environment of 6.5 produced the highest initial uptake rates as well as the greatest overshoots for transport of L-glutamate into brush-border membrane vesicles. The imposition of an inwardly directed pH gradient (5.5 outside, 7.5 inside) accelerated both the influx and efflux of L-glutamate. These results demonstrate that the L-glutamate carrier system in human jejunum appears to have similar energizing characteristics in either direction across the brush-border membrane. In addition, the system operates at an optimal pH of 6.5 and protonation of the system may enhance its mobility.

Introduction

We have previously shown that organ donor intestine can be used to obtain brush-border membrane vesicles for intestinal transport studies in man [1]. These brush-border membrane vesicles can be used to study detailed transport processes of intestinal absorption in man which could not be done previously due to the limitations of in vivo perfusion studies. Using these vesicles, we demonstrated a distinct transport system for acidic amino acids in the human intestine [2,3]. These studies demonstrated that the dicarboxylic amino acid carrier symported acidic amino acids with sodium and antiported these acidic amino acids with potassium. Since the process was electroneutral,

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we proposed a simple stoichiometry of two Na^+ and one glutamate transported inward for the exchange of one K^+. This system could be inhibited by other acidic amino acids but not by neutral or basic amino acids. These studies in man were similar to the earlier studies in rabbit intestine [4], and rat intestine [5,6]. The present studies were carried out to further characterize this acidic amino acid carrier in man by studying the transport characteristics of L-glutamate.

Materials and Methods

Preparation of brush-border membrane vesicles. Human organ donor intestine was obtained and processed as previously described [2]. Briefly, the human intestine was obtained immediately after organ donation and intestinal mucosa was scraped after being thoroughly rinsed with 0.9% NaCl. The mucosa was then frozen at $-70°C$. On the day of experimentation, the mucosa was thawed and brush-border membranes were purified by the calcium chloride precipitation method of Schmitz et al. [7], and brush-border membrane vesicles were obtained by the method of Hopfer et al. [8], as described previously [9].

Transport studies. The purified brush-border membrane vesicles were resuspended to a final protein concentration of approx. 10 mg/ml. For efflux studies the vesicles were loaded prior to the final resuspension with radiolabeled L-glutamate and allowed to equilibrate for one hour. Otherwise, all other substances contained in the final resuspension buffers were used prior to the first set of centrifugations assuring that the concentrations desired in the intravesicular medium were correct. Transport studies were initiated by diluting the vesicles into an incubation medium approximately ten times the vesicular volume. At various time points, 50-μl aliquots of the reaction mixture were transferred to 1 ml of cold stop solution, filtered and washed as described previously [9]. The filters were then processed for scintillation counting. For efflux studies, zero-time points were obtained by transferring vesicles directly to 1 ml of cold stop solution in order to obtain the initial amount of L-[^{14}C]glutamic acid present in the vesicles. The experiments presented here were repeated at least three times and were always performed in duplicates. Using different membrane preparations, qualitatively identical results were obtained for the same experiments but in view of the significant variations in equilibrium uptake values, only results of typical experiments are shown. The variations and duplicates for the experiments shown were always less than $\pm 5\%$ of the mean value. Protein was assayed by the method of Lowry et al. [10] using bovine serum albumin as standard. Purification of the brush-border membrane fraction was assayed by sucrase enrichment by measuring the sucrase activity according to the method of Dahlqvist et al. [11].

L-[U-^{14}C]Glutamic acid was obtained from New England Nuclear as was the D-[1(n)-^{3}H]mannitol. The membrane filters (pore size 0.45 μm) were obtained from Sartorius. All other chemicals were obtained from Sigma and were of the highest purity.

Results

The brush-border membrane vesicles formed for these experiments exhibited typical sodium-dependent D-glucose uptake resulting in an overshoot of at least 10 times the equilibrium value obtained at 60 min, demonstrating that these were functional membrane vesicles. Also, the assay of sucrase enrichment showed that there was an approximate 22-fold enhancement of sucrase activity when comparing the purified brush-border membrane fraction to the crude homogenate.

Effect of extravesicular cations on L-glutamate efflux. The results of the effects of various cations on the efflux of L-glutamate from the membrane vesicles is demonstrated in Fig. 1. The vesicles were loaded with sodium as the intravesicular cation to be transported in symport with the labeled glutamate. Choline was used as a non-transported extravesicular cation to demonstrate the baseline efflux under these conditions. As Fig. 1 demonstrates, extravesicular Na^+ tended to increase the efflux of the glutamate slightly over baseline. However, when K^+ was substituted as the extravesicular cation, L-glutamate efflux was greatly enhanced. This demonstrates that an opposing K^+ gradient stimulates the transmembrane movement of glutamate in a manner similar to our previous influx studies [2,3].

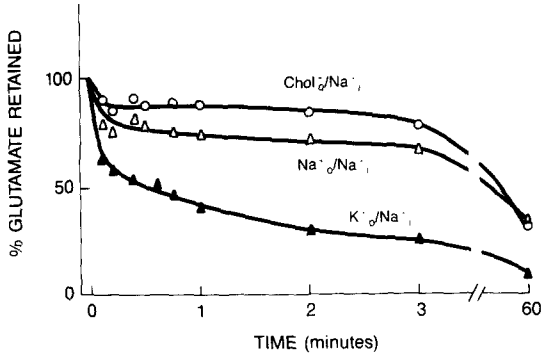

Fig. 1. Efflux of L-[^{14}C]glutamic acid from human jejunal brush-border membrane vesicles. Brush-border membrane vesicles were preloaded with 100 mM NaCl, 50 mM choline chloride, 10 mM Tris-Hepes buffer (pH 7.5), 0.1 mM $MgSO_4$ and 50 μM L-[^{14}C]glutamic acid. Efflux studies were performed by diluting vesicles ten times into an incubation medium containing 10 mM Tris-Hepes buffer (pH 7.5), 0.1 mM $MgSO_4$, and either 150 mM choline chloride (circles), 50 mM choline chloride and 100 mm NaCl (open triangles) or 50 mM choline chloride and 100 mM KCl (closed triangles). Results are expressed as percentage of L-[^{14}C]glutamic acid remaining in the vesicles as compared to zero time.

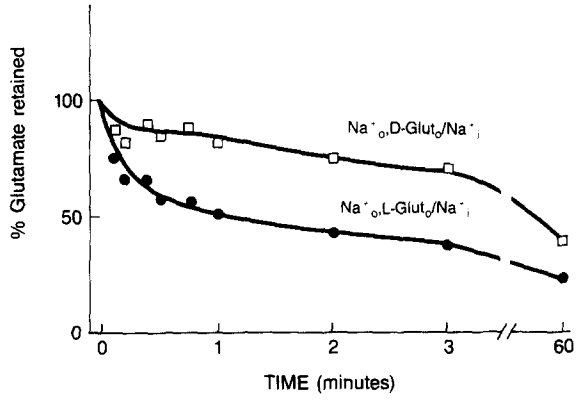

Fig. 2. Transstimulation of L-[^{14}C]glutamic acid efflux from brush-border membrane vesicles by extravesicular L-glutamic acid. Vesicles were preloaded with 100 mM NaCl, 50 mM choline chloride, 10 mM Tris-Hepes (pH 7.5) buffer, 0.1 mM $MgSO_4$ and 50 μM L-[^{14}C]glutamic acid. Efflux studies were performed by diluting vesicles ten times into incubation media containing 10 mM Tris-Hepes buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM choline chloride, 50 mM NaCl and either 100 μM D-glutamic acid (squares) or 100 μM L-glutamic acid (circles). Results are expressed as percentage of L-[^{14}C]glutamic acid remaining in the vesicles as compared to zero time.

Effect of extravesicular D- or L-glutamate on L-glutamate efflux. Fig. 2 demonstrates the effects of L- and D-glutamate in the extravesicular medium on the efflux of labeled L-glutamate from the membrane vesicles. For these studies, the cation was Na^+ with no concentration gradient. Since D-glutamate has been previously shown by us to be not transported by this system [2,3], this curve demonstrates the baseline efflux (not stimulated efflux) of labeled L-glutamate from the vesicles. Fig. 2 demonstrates that there is transtimulation by extravesicular unlabeled L-glutamate of the efflux of labeled L-glutamate from the vesicles. These studies are consistent with previous influx studies which demonstrated that unlabeled L-glutamate indeed inhibited the uptake of labeled L-glutamate demonstrating a carrier system rather than merely passive diffusion. The efflux studies presented in Figs. 1 and 2 together demonstrate that the carrier system for acidic amino acids exhibits similar transport characteristics in either direction across the brush-border membrane.

pH environment studies. Berteloot [4] has stated in his model for the acidic amino acid carrier system in rabbit intestine that protonation of the carrier may serve to enhance its mobility. Therefore, we next investigated the effect of differing pH environments on the transport of L-glutamate by the system. Fig. 3 demonstrates the results of various pH environments on the system. The top curve demonstrates that the initial rates of uptake of L-glutamate as measured by 0.25 min is greatest at a pH environment of 6.5, indicating that a certain degree of protonation of the carrier is beneficial. Likewise, the bottom curve demonstrates that the peak to equilibrium uptake ratio as measured by the peak overshoot (usually occurring at 2 min) as compared to the 60 min equilibrium value is also greatest in a pH environment of 6.5. These data demonstrate that limited protonation enhances the transport of L-glutamate by the dicarboxylic amino acid carrier system. Since protons may enhance mobility by competing for K^+ or Na^+, then total replacement of these cations by protons may actually decrease the efficiency of the transport system as demonstrated by declining transport in the pH 5.5 to 6.0 range.

Effect of pH gradients. The effects of a pH gradient on the influx of L-glutamate was next studied after it was observed that the pH environ-

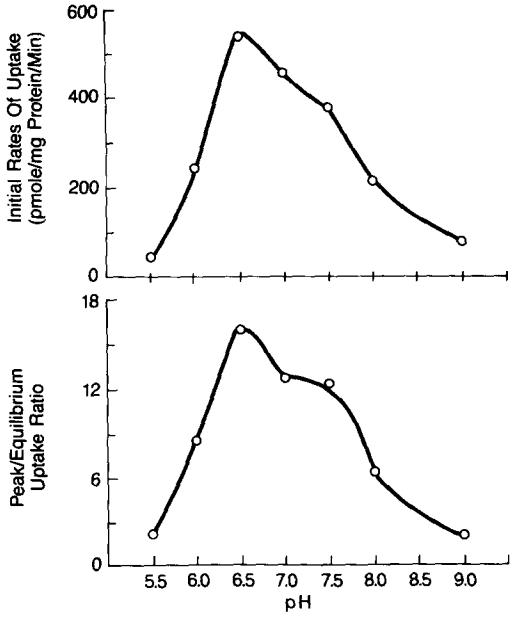

Fig. 3. Effect of pH environment on uptake of L-[^{14}C]glutamic acid into brush-border membrane vesicles. Vesicles were preloaded with 200 mM KCl, 0.1 mM $MgSO_4$ and differing combinations of 10 mM Tris-Hepes or Mes buffers to vary intravesicular pH from 5.5 to 9.0. Uptakes were performed by diluting vesicles ten times into incubation media of identical pH containing 100 mM NaCl, 100 mM KCl, 0.1 mM $MgSO_4$, 50 μM L-[^{14}C]glutamic acid and differing combinations of 10 mM Tris-Hepes or Mes to vary pH from 5.5 to 9.0. Top graph shows initial rates of uptake (performed at 0.25 minutes) normalized to a one minute uptake. Bottom graph exhibits the peak (greatest uptake points always occurring between 1.5 and 2 min) to equilibrium (60 min uptake) ratio.

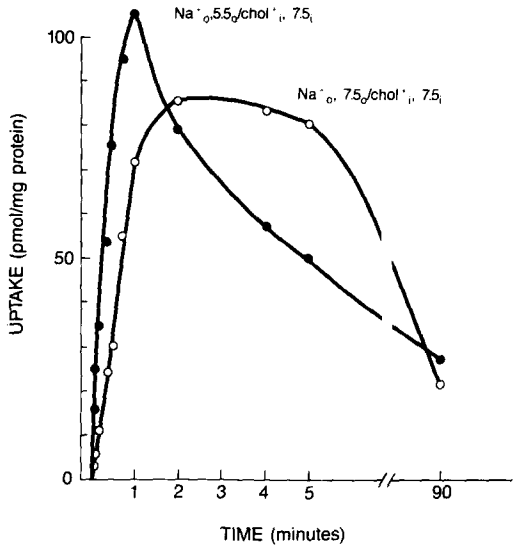

Fig. 4. Effect of inwardly directed pH gradient on Na^+ stimulated uptake of L-[^{14}C]glutamic acid into brush-border membrane vesicles. Vesicles were preloaded with 150 mM choline chloride, 50 mM Tris-Hepes (pH 7.5), 0.1 mM $MgSO_4$. Uptakes were measured by diluting vesicles ten times into incubation media containing 100 mM NaCl, 50 mM choline chloride, 0.1 mM $MgSO_4$, 50 μM L-[^{14}C]glutamic acid and either 50 mM Tris-Hepes (pH 7.5, open circles) or 50 mM Tris-Mes (pH 5.5, closed circles).

ment had a definite effect on transport by the acidic amino acid carrier system. Fig. 4 demonstrates the effect of an inwardly directed pH gradient on the Na^+-dependent uptke of L-glutamate. The intravesicular cation was choline in order to slow down the total mobility of the carrier. Fig. 4 demonstrates that an inwardly directed proton gradient enhances both the influx and subsequent efflux of L-glutamate under only an inward Na^+ gradient (but without an outwardly directed potassium gradient). This is demonstrated by both an increase in the magnitude of the overshoot as well as a decreased width of the overshoot as compared to the overshoot obtained with no pH gradient. Fig. 5 demonstrates the effect of an inwardly directed pH gradient with a simultaneous inwardly directed Na^+ gradient and an outwardly directed K^+ gradient. As opposed to Fig. 4, this graph demonstrates a marked attenuation of the overshoot peak for glutamic acid uptake under a pH gradient. However, the peak uptakes are greatly increased under outward potassium gradients as compared to those in Fig. 4. When compared to no pH gradient, this phenomenon can be explained in two ways: either the actual uptake of L-glutamate is decreased as evidenced by a decreased overshoot, or the subsequent efflux is so greatly enhanced that the full overshoot cannot be measured because the net influx is markedly decreased by the greatly enhanced efflux. Note that the early time points (< 1 min) in Fig. 5 and other similar experiments (results not shown) are consistently enhanced under pH gradient conditions. When this data is taken with that shown in Fig. 4 (since an opposing K gradient has been demonstrated to enhance recycling of the carrier system and increased uptake), this most likely demonstrates that the attenuated overshoot is due to markedly increased efflux from the vesicles

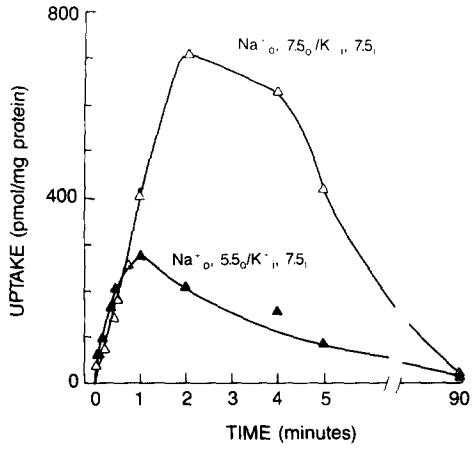

Fig. 5. Effect of inwardly directed pH gradient on Na^+- and K^+-stimulated L-[^{14}C]glutamic acid into brush-border membrane vesicles. Vesicles were preloaded with 150 mM KCl, 50 mM Tris-Hepes (pH 7.5), 0.1 mM $MgSO_4$. Uptakes were measured by diluting vesicles ten times into incubation media containing 100 mM NaCl, 50 mM choline chloride, 0.1 mM $MgSO_4$, 50 μM L-[^{14}C]glutamic acid, and either 50 mM Tris-Hepes (pH 7.5, open triangles) or 50 mM Tris-Mes (pH 5.5, closed triangles).

This theory of rapid efflux was tested by measuring the efflux of radiolabeled L-glutamate under pH gradient conditions. Fig. 6 shows the effects of an inward pH gradient on the efflux of

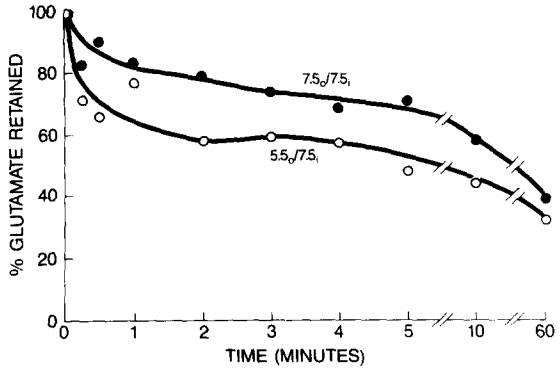

Fig. 6. Effect of inward pH gradient on efflux of L-[^{14}C]glutamate. Vesicles were preloaded with 100 mM KCl, 50 mM choline chloride, 50 mM Tris-Hepes (pH 7.5) buffer and 50 μM L-[^{14}C]glutamic acid. Efflux studies were performed by diluting vesicles ten times into incubation medium containing 100 mM NaCl, 50 mM choline chloride, 0.5 mM glutamic acid and either 50 mM Tris-Hepes (pH 7.5, closed circles) or 50 mM Tris-Mes (pH 5.5, open circles) buffer. Results are expressed as percentage of L-[^{14}C]glutamic acid remaining in the vesicles as compared to zero time.

L-[^{14}C]glutamate under inward Na^+ and outward K^+ gradient conditions. Unlabeled L-glutamate was present in the extravesicular medium to duplicate simultaneous L-glutamate influx. Fig. 6 clearly shows increased efflux with an inward pH gradient.

Discussion

Acidic amino acids are actively absorbed from the human intestine [12]. Because they are the preferred metabolic substrate of the enterocyte [13] and therefore rapidly metabolized [14], in vivo perfusion studies in man proved difficult to demonstrate the existence of a transport system specific for dicarboxylic amino acids. Two case reports of acidic amino aciduria in children pointed to the possible existence of a distinct transport carrier [15,16]. Intestinal brush-border membrane vesicles enable the study of nutrient uptake without interference of intracellular metabolism. Using brush-border membrane vesicles prepared from organ donor intestine, we investigated the human acidic amino acid transport system and found it is specific for acidic amino acids, somewhat stereospecific and capable of secondary active transport [2]. The energy for this uphill accumulation of L-glutamate is supplied by an inwardly directed Na^+ gradient as well as an outwardly directed K^+ gradient. Imposition of both gradients results in a more than additive effect on transport. Intravesicular K^+ did not alter the apparent K_m (affinity) of the transport system but increased the apparent V_{max}. This transport system functions efficiently in the intact human intestine because of the maintenance of these sodium and potassium gradients by the basolaterally located ($Na^+ + K^+$)-ATPase.

The current study further investigated the characteristics of this dicarboxylic amino acid trasport system. To assess possible transport (not molecular) symmetry in the carrier system, efflux studies were performed. These studies confirm a bidirectional similarity of the transport system as efflux of L-glutamate from the vesicle was enhanced by simultaneous outwardly directed Na^+ gradients and inwardly directed K^+ gradients. These results are similar to those of Fukuhura and Turner [17] using rabbit renal brush-border membrane vesicles. Likewise, the relative stereospecificity of the sys-

tem was bidirectionally similar since there was transstimulation of labeled L-glutamate efflux by unlabeled L-glutamate in the incubation medium as compared to unlabeled D-glutamate. A difference between the efflux and influx studies is shown in Fig. 1, where the presence of extravesicular Na^+ enhanced the efflux of L-glutamate. Previous influx studies had demonstrated that an inward Na^+ gradient (not merely the presence of Na^+) accelerated the transport of L-glutamate into the vesicles. In Fig. 1, however, the glutamate efflux is enhanced by the presence of Na^+ without a gradient. A likely explanation is the relatively small intravesicular volume when compared to the volume of the incubation medium. With Na^+-loaded vesicles placed into a choline incubation medium, the Na^+ will be very quickly dissipated into the incubation medium. Thus, intravesicular Na^+ has disappeared leaving only diffusion of labeled L-glutamate to account for the subsequent efflux. With the presence of extravesicular Na^+ at an equimolar concentration to the intravesicular media, the continued presence of Na^+ inside the vesicle is assured, allowing sodium coupled efflux of L-glutamate to proceed. The imposition of an inwardly directed K^+ gradient enables much faster efflux as demonstrated in Fig. 1. Thus, the above experiments when coupled with previous influx experiments [2] demonstrate bidirectionally similar energization requirements of this transport system.

Berteloot [4] had previously demonstrated that H^+ plays a role in the efficient function of the rabbit intestine acidic amino acid carrier system. He theorized that protonation enhanced carrier mobility and this protonation was only beneficial to transport system function if it was extavesicular. He found the rabbit carrier functioned most effectively in a non-gradient, nonacidic pH milieu of 7.0 to 7.5. In contrast, we found that the human carrier was most efficient at a pH of 6.5. The initial rates and the peak equilibrium uptake ratios were highest at pH 6.5, indicating that a slightly acidic environment maximizes the initial efficiency and the driving force for uptake. Because maximal transport occurs in an acidic, nongradient environment of pH 6.5, this points toward effective protonation of the carrier rather than the possibility that pH gradients enhance uptake via glutamate anion/OH^- exchange. Similar beneficial effects of protonation were seen in renal brush-border membrane vesicles [18–20] and in *Escherichia coli* cytoplasmic membrane vesicles [21,22]. However, Weiss et al. [23] demonstrated optimal glutamate uptake into rat renal BBMV at pH 7.4.

To further investigate this proton effect, uptake studies were performed with an inwardly directed proton gradient (pH 5.5 outside, 7.5 inside). An inwardly directed proton gradient increased the early uptakes of L-glutamate resulting in a larger overshoot. However, the efflux of L-glutamate from the vesicles was also enhanced indicating that protons improve the mobility of the carrier. With the simultaneous imposition of inwardly directed H^+ and Na^+ gradients and an outward K^+ gradient, there was overshoot attenuation compared to no H^+ gradient. The most likely explanation is that with a Na^+ gradient alone, an inwardly directed H^+ gradient enhances influx and efflux. However, the imposition of all three gradients makes the system so efficient that efflux is occurring at a greatly enhanced rate, resulting in paradoxical overshoot attenuation. This is shown by enhanced uptake rates at the early time points until the attenuation of the overshoot occurs (Fig. 5) and shown also by enhanced efflux under the simultaneous imposition of sodium, potassium and proton gradients (Fig. 6). This fast efflux would not occur in vivo due to rapid intracellular metabolism; the system would continue to function efficiently even in the milieu of an acid microclimate occurring in the extracellular brush-border region [24]. One other explanation is the imposition of an inwardly directed H^+ gradient activates an H^+/K^+ exchanger and quickly dissipates the intravesicular K^+ gradient, but this is unlikely because of the initial stimulation.

In summary, these studies on the acidic amino acid transport system in man demonstrate that this system is bidirectionally similar across the brush border membrane. There is a definite pH effect on the system as evidenced by peak function occurring at pH 6.5 and enhancement of influx and efflux with an extravesicular H^+ gradient. It is unknown whether this H^+ effect acts via protonation of the carrier itself, by conversion of the glutamate anion to glutamic acid or by the symport of a proton and a glutamate anion on two

different carrier sites. Further studies will better define the role of H^+ in this system. Studies are necessary to determine the existence of a K^+/H^+ exchanger and to assess competition between Na^+, K^+ and H^+ as regards the transport system. Vesicle studies using potential and pH sensitive dyes may clarify these issues.

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