L-Threonine Transport in Pig Jejunal Brush Border Membrane Vesicles

FUNCTIONAL CHARACTERIZATION OF THE UNIQUE SYSTEM B IN THE INTESTINAL EPITHELIUM

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Uptake and inhibitory kinetics of [3H]L-threonine were evaluated in preparations of pig jejunal brush border membrane vesicles. Uptake of [3H]L-threonine under 0-trans, Na⁺ gradient, and 0-trans, Na⁺-free conditions was best described by high affinity transport ($K_m < 0.1$ mM) plus a nonsaturable component. The maximal velocity of transport was 3-fold greater under Na⁺ gradient conditions. $100$ mM concentrations of all of the dipolar amino acids and 2-aminoisobicyclo[2.2.1]heptane-2-carboxylic acid caused complete inhibition of [3H]L-threonine transport under Na⁺ gradient and Na⁺-free conditions. Imino acids, anionic amino acids, cationic amino acids, and methylaminoisobutyric acid caused significant partial inhibition of L-threonine uptake. Inhibitor concentration profiles for proline and lysine were consistent with low affinity competitive inhibition. The $K_i$ values of alanine and phenylalanine approximated 0.2 and 0.5 mM, respectively, under both Na⁺ gradient and Na⁺-free conditions. These data indicate that the transport system available for L-threonine in the intestinal brush border membrane (system B) is functionally distinct from other amino acid transport systems. Comparison of kinetics parameters in the presence and absence of a Na⁺ gradient suggests that both partially and fully loaded forms of the carrier can function to translocate substrate and that Na⁺ serves to accelerate L-threonine transport by a mechanism that does not involve enhanced substrate binding.

Transport systems within the brush border membrane of the small intestinal epithelial cell serve to catalyze the absorption of the amino acid products of protein digestion. In the plasma membrane of nonpolarized mammalian cells and in the basolateral membrane of epithelial cells amino acid transport pathways have been characterized on the basis of substrate specificity, cation dependence, electroneutrality, pH dependence, and regulatory phenomena. Dipolar amino acids have been shown to be transported by Na⁺-dependent systems A, ASC, N, Gly, β-system, and B⁰⁺⁺ and Na⁺-independent systems L, T, and B⁰⁺⁺ (reviewed in Refs. 1–3). A consistent finding of this work in nonepithelial cells is that systems A, ASC, and L are found in the majority of metabolically active cell types and are often referred to as “ubiquitous” transport systems. System A transports short chain linear dipolar amino acids, excludes phenylalanine, isoleucine, and valine as substrates, and can transport MeAIB with high affinity (4, 5). System ASC has a high affinity for linear dipolar amino acids, such as alanine, serine, cysteine, and threonine, and does not transport MeAIB, phenylalanine, or histidine (5–7). Na⁺- independent system L is a broad specificity transport system with a high affinity for leucine, phenylalanine, and methionine and a low affinity for the short chain amino acids glycine, alanine, and serine (5, 8). System L transports BCH with high affinity and excludes proline and MeAIB as substrates (5, 8, 9). Considerable substrate overlap between dipolar amino acid transport systems has been demonstrated often within the same membrane. For example L-threonine is transported by all three systems in Chinese hamster ovary cells (5).

The pathway(s) of dipolar amino acids transport in the intestinal brush border membrane are less well characterized than in nonepithelial cell types. Stevens et al. (10) described a multiplicity of dipolar amino acid pathways in rabbit jejunal brush border membrane vesicles consisting of a broad specificity Na⁺- dependent “neutral brush border” system that did not transport MeAIB, a Na⁺- dependent, phenylalanine-prefering system, a Na⁺- dependent “imino” system, and a Na⁺- independent system with a substrate specificity similar to that of system L as characterized in other cell types. Recently Maenz et al. (11) found that L-glutamic acid could be transported by the high affinity anionic amino acid transport system XAG and by a second low affinity system in rabbit jejunal brush border membrane vesicles. Flux of L-glutamic acid through the low affinity system was found to increase with acidification of the media from pH 8 to 6 and could be blocked by excess phenylalanine. The property of pH-dependent conversion of substrate specificity to the uptake of anionic amino acids has been demonstrated for system ASC in several nonepithelial cell types (12, 13). This finding implies that either system ASC is present in the intestinal brush border or that another brush border membrane dipolar amino acid transport system has the same functional property of pH-dependent conversion to the uptake of anionic amino acids. In his review article of 1990 Christensen (1) speculated that the broad specificity amino acid transport system B⁰⁺⁺ characterized in oocytes and blastocytes (2, 14, 15) may be present in the brush border membrane of intestinal and renal epithelial cells. Recently Stevens (16) has proposed that the neutral brush border system be renamed as system B to reflect the

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similarity in substrate specificity to system B. In addition to the broad specificity system(s) found in the intestinal brush border a novel broad specificity system (termed system G) able to recognize dipolar as well anionic and cationic amino acids has been found in the brush border membrane of the kidney epithelial cell line MDCK (17).

In this study we utilized a preparation of purified pig jejunal brush border membrane vesicles with a protocol of measuring rapid uptake kinetics of L-threonine to begin to clarify and characterize the pathways of dipolar amino acid transport in the intestinal brush border membrane.

**EXPERIMENTAL PROCEDURES**

**Preparation and Storage of Jejunal Brush Border Vesicles—**Brush border membrane vesicles (BBV) were prepared from pig jejunal mucosal scrapings using the MgCl₂ precipitation procedure (18) with some modifications. For each batch preparation of BBV, three pigs of 15-kg weight were induced and maintained under halothane anesthesia. Four feet of proximal jejunum were surgically removed from each pig and rinsed immediately with ice-cold saline. The intestinal mucosa was scraped off with a spatula, and the scrapings were pooled and frozen at -70 °C. The next day the scrapings were thawed, weighed, and resuspended with homogenate media (50 mM mannitol, 2 mM Tris-HCl, pH 7.4) at a ratio of 1 ml of homogenate media per g of mucosal scrapings. The scrapings were homogenized for 60 s at setting 3 with a polytron homogenizer (Brinkman Instruments) and centrifuged for 15 min at 2,000 X g. MgCl₂ was added to the supernatant (S1) to give a final concentration of 10 mM, and the supernatant was stirred on ice for 15 min and then centrifuged for 15 min at 2,400 X g to pellet the precipitant. The resultant supernatant was centrifuged for 30 min at 19,000 X g to generate the crude BBV pellets. The supernatant was poured off, and a total of 6 ml of resuspension media (300 mM mannitol, 50 mM Hepes-Tris, pH 7.0) were added to the centrifuge tubes. The pellets were resuspended by repeated passage through a 26-gauge needle, pooled, divided into 0.25-ml aliquots, and frozen in liquid N₂ until the day of use. For a given experiment a suitable number of aliquots of crude BBV were thawed in 70 ml of the resuspension media required for the particular experiment and homogenized in a glass-Teflon homogenizer (6 strokes) prior to centrifugation for 15 min at 500 X g. The resultant supernatant was centrifuged for 30 min at 39,000 X g to generate the final BBV pellets. The final pellets were resuspended with a 26-gauge needle, and a suitable volume of resuspension media was pooled, assayed for protein content, and diluted to give a vesicle concentration of 16 mg protein/ml. The BBV were then divided into 20-μl aliquots and frozen in liquid N₂ under vacuum of assay.

**Uptake Assays—**Initial rates of [³H]--threonine uptake were assayed “by hand” or with an automated fast sampling apparatus constructed by the engineering shops at the University of Saskatchewan following the principles of a similar apparatus first developed at the University of Montreal (19).

In the by hand procedure an aliquot of frozen BBV was thawed, and a series of single time point uptakes were performed. For each uptake 5 μl of BBV were rapidly mixed with 120 μl of uptake media. At the conclusion of the uptake time period 1 ml of ice-cold stop solution (5 mM NaCl, 50 mM Hepes-Tris, pH 7.0, 304 mM mannitol, 0.05 mM HgCl₂) was injected into the mixture. The stopped mixture was filtered through 0.45-μm cellulose acetate filters, the filters were washed twice with 5 ml of ice-cold stop solution, and the [³H]-labeled substrate content of the filters was determined by liquid scintillation counting. “Zero time” BBV substrate content was determined by injecting 5 μl of BBV into a mixture of 1 ml of ice-cold stop solution plus 120 μl of uptake media. The mixture was filtered and the filters were washed and assayed for [³H]-labeled substrate content.

The automated fast sampling apparatus was constructed to test the validity of the by hand technique of measuring true initial rates of substrate uptake under steady-state conditions and, of greater significance, to provide an experimental tool that would allow for the precise measurements of steady-state and pre-steady-state uptakes that are impossible to achieve by hand and are necessary for the testing of more sophisticated models of transporter function. Multiple time point assays were performed by loading the uptake media into the uptake chamber of the apparatus and then loading a positive displacement pipette containing an aliquot of vesicles into the chamber. Uptake was initiated by injecting the vesicles into the media and at each sampling time pulse of air pressure was automatically imposed over the mixture such that 45 μl were expelled via a needle opening at the base of the chamber. Samples were collected in a series of sample wells which had been preloaded with 1 ml of ice-cold stop solution, and the stopped mixture was filtered through 0.65-μm cellulose acetate filters. The filters were washed five times with 1 ml of 0.3 M sucrose to remove stop solution before analysis.

**Protein and Enzyme Assays—**The purity and recovery of the brush border membrane fraction was determined by marker enzyme assays. Alkaline phosphatase was assayed according to Parkinson et al. (20) and NADPH cytochrome c reductase was assayed following the procedure of Sottocasa et al. (21). Protein was determined using the Sigma microprotein diagnostic kit with human serum albumin as the standard.

**Data Analysis—**Initial rates of [³H]--threonine uptake were calculated as the slope of the regression line obtained for vesicle substrate content assayed at multiple time points within the first 2 s from initiation of uptake. The kinetic parameters of substrate uptake or inhibition were determined by nonlinear regression analysis of the nontransformed data using the P.Fit curve fitting program (Biosoft) as described by Maio and Berteloot (22) and the kinetic parameters of each transport model fit to the data are reported in the figures.

**Chemicals—**[³H]--threonine (19 Ci/mmol) was purchased from Amersham Corp. Unlabeled amino acids, BCH, and MeAIB were purchased from Sigma.

**RESULTS**

**Purity and Stability of Pig Jejunal BBV—**The purity of the vesicle preparation was evaluated using marker enzymes known to be localized in specific membranes of the cell. Comparison of specific activities in the homogenate and the final vesicle preparation revealed a 21-fold enrichment in specific activity of alkaline phosphatase while the specific activity of NADPH cytochrome c reductase in the vesicles was 0.3 times the specific activity found in the homogenate. These results indicate that the final preparation was highly enriched in brush border membrane with no evidence of significant contamination by basolateral membrane or cell organelles.

An important criteria in fitting models of substrate uptake to data obtained with membrane vesicles is that the preparation is stable and maintains a consistent uptake rate for any given set of conditions over the course of the experiment. Recently, Maenz et al. (23) demonstrated that standard preparations of rabbit and rat intestinal BBV will lose substantial substrate uptake capacity within 6 h from the time of preparation when maintained on ice or at room temperature. Dividing the preparation into aliquots suitable for individual uptake assays and freezing the aliquots in liquid N₂ until the time of assay resulted in stable uptake rates (23). We chose to follow the protocol of freezing aliquots of the BBV in liquid N₂ until the time of assay to avoid any possible loss of activity during the course of the experiments. By using this procedure the specific activity of substrate uptake was found to be the same in aliquots of BBV assayed immediately after preparation and in aliquots which had been frozen in liquid N₂ for 6 h from the time of preparation. In addition, the large batch preparation of partially purified vesicles showed no evidence of any loss of substrate uptake capacity even after 6 months of storage in liquid N₂.

**Steady-state Initial Rates Measurements—**Kinetic evaluation of transporter function using preparations of purified membrane vesicles are often limited by the crude methods employed in measuring uptake rates under steady-state conditions. The standard by hand procedure involves a single time point assay taken within the first few seconds from initiation of the reaction. Initial rates are subsequently cal-

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2 The fast sampling apparatus was constructed with the kind permission and cooperation of Dr. Alfred Berteloot of the Membrane Transport Research Group at the University of Montreal.
culated using an assumed zero time intercept and assuming that uptake rates are linear over the time span of the procedure. As such, the procedure does not directly measure true initial rates and could potentially contain systematic errors in defining steady state uptake kinetics. These systematic errors could introduce serious artifacts when attempting to fit transport models to the data (22).

In this study we employed a multiple time assay procedure and directly measured true initial rates as the slope of the linear portion of the uptake. The initial experiments were performed by hand with sampling at 0, 1, and 2 s from initiation of uptake. More recently we have made use of an automated fast sampling apparatus, constructed by the engineering shops at the University of Saskatchewan and based on the principles of an existing apparatus at the University of Montreal (19). The apparatus will perform precise and rapid multiple sampling of a vesicle uptake media mixture and thereby provide a direct and accurate measure of true initial rates of substrate uptake.

Fig. 1 shows initial rates of [3H]L-threonine uptake across purified pig jejunal brush border membrane vesicles obtained using the multiple time point assay performed by hand (A) or with the fast sampling apparatus (B). Uptake rates were linear over the 2-s time period from initiation with a positive zero time intercept. The intercept is assumed to represent "background" substrate association with the filters and/or a rapid association of substrate to the vesicle membrane that is resistant to the washing procedure. In either case the time course of this background binding is within the time scale of the first measurement of vesicle substrate content. True initial rates of substrate uptake were calculated as the slope of the regression line obtained from multiple time point assays of vesicle substrate content. It is worthy of note that indirect initial rate calculations based on a single time point assay with a presumed zero time intercept would have resulted in erroneously high estimates of steady state uptake rates. The intercept and the linear time course for substrate uptake obtained with the fast sampling apparatus is comparable to the results obtained by hand with sampling at 0, 1, and 2 s from initiation. As such, the results obtained with the apparatus serve to validate the earlier results obtained using the by hand technique. The advantage of the fast sampling apparatus is in the precision of the measurements of initial rates which allows for greater discrimination in model fitting to the data.

Kinetics of [3H]L-Threonine Uptake in the Presence of a Na+ Gradient—Fig. 2 shows a direct plot of initial rates of 0.5 μM [3H]L-threonine uptake in the presence of an inwardly directed Na+ gradient with various concentrations of unlabeled L-threonine incorporated in the uptake media. A model for L-threonine uptake of high affinity transport plus an unsaturatable diffusion component converged to the data within the parameters of the computer program. More complex uptake models such as multiple transport pathways with widely separate Km values did not converge. The data suggest that L-threonine transport across the vesicles in the presence of a Na+ gradient occurs by a high affinity system(s) with a Km of less than 0.1 mM and a Vmax approximating 14 pmol/mg/s with no evidence of a second low affinity route. The inset plot in Fig. 2 shows an Eadie-Hofstee transformation of the transport-mediated component of total uptake rates at nonsaturating concentrations of unlabeled L-threonine. The transformed data approximate a linear relationship between transport rate versus transport rate/substrate concentration and provide no visual evidence of multiple pathways with widely separate Km values.

Inhibitor Specificity under Na+ Gradient Conditions—Fig. 3 shows the effect of 100 mM concentrations of various amino acids and analogs on the initial rate of 0.5 μM [3H]L-threonine uptake in the presence of a Na+ gradient. All of the amino acids and analogs caused a significant inhibition of uptake in comparison to the mannitol control condition. Incorporation of 100 mM unlabeled L-threonine in the uptake media would cause complete inhibition of the transport-mediated component of total [3H]L-threonine uptake (Fig. 2). As such, 100 mM concentrations of all of the dipolar amino acids and BCH caused complete inhibition of [3H]L-threonine transport in the vesicles. MeAIB and the cationic amino acids lysine and arginine caused a slight but significant inhibition that approximated 25% of total [3H]L-threonine transport. The imino acids proline and OH-proline and the anionic amino acids...
uptake by excess concentration of lysine and proline could not be determined using the fast sampling apparatus. 16 gl of vesicle/uptake media mixture was automatically sampled at 0.4, 0.8, 1.2, 1.6, and 2 s from initiation of uptake. The points represent the mean and standard deviation of four replicate initial rate determinations. The bars show the mean and standard deviation obtained from four uptake rates calculated for each concentration of l-threonine. Models of substrate uptake were tested for convergence to the data. An uptake model of one transport system plus an unsaturable component with the indicated values of \( V_{\text{max}}, K_a, \) and \( C \) provided the best model fit to the data. The inset plot shows a Hofstee transformation of the transport-mediated component of substrate uptake.

**FIG. 2.** Kinetics of 0.5 \( \mu \)M \(^{[3H]}\)l-threonine uptake under 0-trans, \( Na^+ \) gradient conditions. BBV were prepared from a total of 3 ml of frozen P, using the resuspension media and procedures described in Fig. 1. Individual zero time and 1- and 2-s uptakes were performed on each aliquot of BBV using uptake media consisting of 156 mM NaCl, 200 mM mannitol, 50 mM Hepes-Tris, pH 7.0, 0.5 \( \mu \)M \(^{[3H]}\)l-threonine, 104 mM l-threonine + mannitol with the indicated concentration of l-threonine incorporated in each media. Uptake rates were calculated as the slope of the regression line obtained from vesicle substrate content at 0, 1, and 2 s from initiation. The points represent the mean and standard deviation of four replicate initial rate determinations. The bars show the mean and standard deviation obtained from four replicate initial rate determinations.

**FIG. 3.** Effect of 100 mM concentrations of amino acids and analogs on initial rates of 1.0 \( \mu \)M \(^{[3H]}\)l-threonine uptake under 0-trans, \( Na^+ \) gradient conditions. BBV were prepared from 4.5 ml of frozen P, as described in Fig. 2. Initial rates of substrate uptake were determined using the fast sampling apparatus. 16 \( \mu \)l of BBV and 384 \( \mu \)l of uptake media, consisting of 156 mM NaCl, 200 mM mannitol, 50 mM Hepes-Tris, pH 7.0, 1.0 \( \mu \)M \(^{[3H]}\)l-threonine, 304 mM indicated test agent, were loaded into the apparatus, and the vesicle/uptake media mixture was automatically sampled at 0.4, 0.8, 1.2, 1.6, and 2.0 s from initiation of uptake. Initial rates were calculated as the slope of the regression line obtained for vesicle substrate content from 0.4 to 2.0 s from initiation. The bars show the mean and standard deviation obtained from four uptake, and a common number indicates no statistically significant difference in mean uptake rates (\( p = 0.05 \)).

glutamate and aspartate were somewhat more potent partial inhibitors of \(^{[3H]}\)l-threonine transport.

**Concentration Profiles for Lysine and Proline Inhibition of \(^{3}H\)l-Threonine Uptake**—Partial inhibition of \(^{3}H\)l-threonine uptake by excess concentration of lysine and proline could represent a low affinity for inhibitor interaction with the transport system or a block of substrate uptake through a second lysine and proline sensitive system. In the case of a low affinity of the inhibitors for the transport system one would expect to find no evidence of saturation of the inhibitory effect with increasing concentrations of inhibitor in the media. A saturation of the partial inhibitory effect would be consistent with blockage of substrate flux through a second system with a high affinity for the inhibitors. Fig. 4 shows the effects of increasing concentrations of lysine and proline on the initial rate of 1.0 \( \mu \)M \(^{[3H]}\)l-threonine uptake across the vesicles. A gradual decline in uptake rates occurred over the concentration range employed in the experiment with no evidence of a saturation of the partial inhibitory effects of lysine and proline on \(^{[3H]}\)l-threonine uptake.

**Kinetics of \(^{[3H]}\)l-Threonine Uptake in the Absence of \( Na^+ \)**—Fig. 5 shows a direct plot of initial rates of 0.5 \( \mu \)M \(^{[3H]}\)l-

**FIG. 4.** Effect of varying concentrations of l-proline and l-lysine on initial rates of 1.0 \( \mu \)M \(^{[3H]}\)l-threonine uptake under 0-trans, \( Na^+ \) gradient conditions. BBV were prepared from 3.5 ml of frozen P, and initial rates of substrate uptake were determined using the fast sampling apparatus as described in Fig. 3. The uptake media contained the indicated concentration of test agent + mannitol to give a final concentration of 104 mM. The points represent the mean and standard deviation obtained from four replicate initial rate determinations.

**FIG. 5.** Kinetics of 0.5 \( \mu \)M \(^{[3H]}\)l-threonine uptake under 0-trans, \( Na^+ \)-free conditions. All procedures were the same as described for Fig. 3 except that the uptake media contained 156 mM ChCl in place of NaCl. The line represents an uptake model of one transport system plus diffusion with the indicated values of \( V_{\text{max}}, K_a, \) and \( C \), and the inset plot shows a Hofstee transformation of the transport-mediated component of substrate uptake.
threonine uptake versus log L-threonine concentration in the absence of Na\(^+\). The best model fit to the data is that of high affinity transport plus diffusion. The parameters of \(K_m\) and diffusion are comparable to those obtained in the presence of a Na\(^+\) gradient while the \(V_{max}\) approximated 5 pmol/mg/s. An Eadie-Hofstee transformation of the transport-mediated component of total uptake provided no visual evidence of more complex transport models involving multiple systems with widely separate \(K_m\) values.

Inhibitor Specificity under Na\(^+\)-free Conditions—The specificity of inhibition of \([3^H]L\)-threonine uptake under Na\(^+\)-free conditions by amino acids and analogs is shown in Fig. 6. The results are comparable to those obtained under Na\(^+\) gradient conditions in that all of the dipolar amino acids and BCH caused complete inhibition of the transport-mediated component of total substrate uptake. Under these conditions, MeAIB and the cationic amino acids lysine and arginine had no significant effects on the rate of \([3^H]L\)-threonine uptake. The imino acids, proline and OH-proline, and the anionic amino acids, glutamate and aspartate, did cause significant partial inhibition of \([3^H]L\)-threonine uptake.

Kinetics of Alanine Inhibition of \([3^H]L\)-Threonine Uptake—Fig. 7 shows the rates of \([3^H]L\)-threonine uptake in the presence of varying concentrations of L-threonine with 0, 0.05, or 0.50 mM alanine incorporated in the media under 0-trans, Na\(^+\)-gradient conditions. In the absence of alanine the best model fit to the data was that of high affinity transport system plus a nonsaturable component. Incorporating alanine in the uptake media caused an apparent increase in the \(K_m\) with no change in the maximal velocity of transport. These results are consistent with a model of competitive inhibition for substrate binding to the transport system. A model of high affinity \([3^H]L\)-threonine transport with competitive inhibition by alanine did converge to the data within the 95% confidence interval of the curve fitting program. The \(K_m\) values for alanine inhibition of \([3^H]L\)-threonine uptake under 0-trans, Na\(^+\) gradient conditions were 0.186 ± 0.014 mM and 0.202 ± 0.007 mM as determined with 0.05 mM alanine and 0.50 mM alanine, respectively. The inset graph is a double-reciprocal plot of the transport-mediated component of total threonine uptake in the presence and absence of alanine. The three regressions lines have a common intercept on the y-axis which provides visual evidence of competitive inhibition by alanine for threonine transport.

Fig. 8 shows the kinetics of alanine inhibition of \([3^H]L\)-threonine uptake under 0-trans, Na\(^+\)-free conditions. An uptake model of high affinity \([3^H]L\)-threonine transport plus an unsaturable component with competitive inhibition by alanine converged to the data. The \(K_m\) values for alanine inhibition under Na\(^+\)-free conditions were 0.198 ± 0.028 mM and 0.264 ± 0.080 as determined with 0.05 mM alanine and 0.50 mM alanine, respectively.

Kinetics of Phenylalanine Inhibition of \([3^H]L\)-Threonine Uptake—Fig. 9 shows the effects of 0.1 and 1.0 mM phenylalanine on the kinetics of \([3^H]L\)-threonine uptake under 0-trans, Na\(^+\)-gradient conditions. Uptake models of high affinity transport plus an unsaturable component with competitive inhibition by phenylalanine converged to the data. \(K_m\) values of 0.530 ± 0.036 mM and 0.546 ± 0.019 mM were obtained with 0.1 and 1.0 mM phenylalanine, respectively. The common intercept of the regression lines on the y-axis of the double-reciprocal plots of threonine transport rates provides visual support of competitive inhibition by phenylalanine.

Fig. 10 shows the effects of phenylalanine on \([3^H]L\)-threonine uptake kinetics under 0-trans, Na\(^+\)-free conditions. Uptake models of high affinity transport with competitive inhibition converged to the data with \(K_m\) values of 0.710 ± 0.151

**FIG. 7.** Kinetics of alanine inhibition of 1.0 \(\mu\)M \([3^H]L\)-threonine uptake under 0-trans, Na\(^+\)-gradient conditions. BBV were prepared from 5 ml of frozen BB at initial rates of substrate uptake were determined using the fast sampling apparatus as described in Fig. 4. The uptake media contained the indicated concentration of L-threonine and L-alanine and the points represent the initial rate obtained from a single 5-point assay of 1.0 \(\mu\)M \([3^H]L\)-threonine uptake. In the absence of alanine an uptake model of a single high affinity transport system plus an unsaturable component converged to the data. In the presence of alanine uptake models of high affinity transport plus diffusion with competitive inhibition by alanine converged to the data. The insert shows a double-reciprocal plot of the transport-mediated component of total L-threonine transport in the presence and absence of alanine.

**FIG. 6.** Effect of 100 mM concentrations of amino acids and analogs on initial rates of 1.0 \(\mu\)M \([3^H]L\)-threonine uptake under 0-trans, Na\(^+\)-free conditions. All procedures were the same as described for Fig. 3 except that the uptake media contained 156 mM ChCl in place of NaCl. The bars show the mean and standard deviation obtained from four uptakes and a common number indicates no statistically significant difference in mean uptake rates (\(p = 0.08\)).
activity of substrate uptake as has been shown to occur in rabbit and rat intestinal vesicles maintained "on-ice" from the time of preparation (23). Our approach in evaluating the kinetics of steady-state substrate uptake across the vesicles was to directly calculate true initial rates as the slope of the regression line obtained from vesicle substrate content assayed at multiple times within the first 2 s from initiation of the reaction. This procedure avoids any assumptions as to the zero time intercept and the time course of the reaction that are inherent in single time point assays of initial rates (22). The recent development of an automated fast sampling apparatus in our laboratory allows for precise and rapid sampling of a vesicle/uptake media mixture. The improved precision of initial rate determination allows for greater discrimination in fitting models of substrate uptake to the data (19, 22).

Using a protocol of measuring true initial rates of \([\text{H}]\)-threonine uptake across a stable preparation of purified pig jejunal brush border vesicles we have begun to characterize the pathway(s) available for dipolar amino acid transport in the intestinal brush border membrane. In evaluating initial rates of \([\text{H}]\)-threonine uptake with varying concentrations of unlabeled L-threonine in the media, a model of high affinity transport \((K_a < 0.1 \text{ mM})\) plus a nonsaturable component provided the best model fit to the data under 0-trans, Na+ gradient, and 0-trans, Na+-free conditions. An evaluation of transport kinetics in itself cannot be used to discriminate between one versus two or more transport systems. Substrate uptake rates obtained in vesicle systems containing multiple high affinity transport routes with similar \(K_a\) values would converge with models of uptake defined by a single transport system. These results do however tend to exclude significant transport of \([\text{H}]\)-threonine by low affinity routes in the intestinal brush border membrane.

The broad specificity of inhibition of threonine uptake by excess concentrations of amino acids and analogs suggests that the high affinity transport route for L-threonine shows marginal selectivity among amino acid substrates. All of the dipolar amino acids and BCH caused complete inhibition of transport while the anionic and cationic amino acids, the imino acids, and MeAIB caused significant partial inhibition under Na+ gradient conditions. One could speculate that threonine could function as a high affinity substrate for the anionic amino acid system (11), the "imino" system (10), and lysine-preferring, Na+-independent pathway (10) described in the brush border membrane. However, the inhibition profiles obtained with varying concentrations lysine and proline are not consistent with saturable high affinity partial inhibition of threonine flux through these alternate pathways. As such, available evidence indicates that anionic and cationic amino acids and imino acids likely function as low affinity competitive inhibitors of threonine transport. In rabbit jejunal brush border vesicles L-glutamic acid is transported by a specific high affinity system and a second low affinity phenylalanine-sensitive system (11).

In this study the \(K_a\) values obtained for \([\text{H}]\)-threonine transport were found to be remarkably similar under Na+ gradient and Na+-free conditions. This finding is compatible with either of two transport models. In the first model distinct high affinity Na+-dependent and Na+-independent transport systems function to catalyze threonine uptake in the vesicles. However in this study the kinetics of inhibition of threonine uptake by amino acids and analogs were remarkably similar under Na+ gradient and Na+-free conditions, and as such we found no functional evidence of two distinct transport systems. An alternative model is that of a single high affinity
system functioning under Na⁺ gradient and Na⁺-free conditions.

If L-threonine is transported across the intestinal brush border membrane via a single high affinity system, this transporter could operate via an ordered or a random substrate binding mechanism. A strictly ordered Na⁺-first, L-threonine transport system B does serve to catalyze threonine uptake in the intestinal brush border membrane via a single high affinity system, this transporter could operate via an ordered or a random substrate binding mechanism. A strictly ordered Na⁺-first, L-threonine transport system B does serve to catalyze threonine uptake in the intestinal brush border membrane. Recently Stevens (16) has renamed the broad specificity system G (general) described in the apical membrane of early confluent cultures of the kidney epithelial cell line MDCK as occurring through systems A, ASC, L, and the novel broad specificity system G. In this study we found no evidence for such a complex spectrum of pathways for L-threonine transport in intestinal brush border vesicles.

This study and that of Stevens et al. (10) indicate that the intestinal system B is functionally distinct from any other amino acid transport system found in other cell types. L-Threonine would appear to be an ideal substrate for the further characterization of system B in that we found no evidence of L-threonine transport by additional system such as the “Phe” and “Imino” systems of the intestinal brush border membrane (10). Our work suggests that system B has a broad specificity for dipolar amino acids and BCH and may function via a mechanism that includes provisions for translocation of both the fully and partially loaded forms of the carrier. In future more exacting kinetic evaluations of transport rates with varying concentration of Na⁺ and substrate under 0-trans and equilibrium exchange conditions will be used to further characterize the transport mechanism of system B in the intestinal brush border membrane.

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