Comparison of the Transport Characteristics of D- and L-Methionine in a Human Intestinal Epithelial Model (Caco-2) and in a Perfused Rat Intestinal Model

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Received April 20, 1994; accepted July 13, 1994

Absorption mechanisms of L- and D-methionine (MET) in an in vitro cultured human intestinal epithelial cell model (Caco-2) and an in situ perfused rat intestinal model were investigated to determine if the kinetic characteristics of absorption are comparable in these two popular absorption models. The results indicate that the transport of L- and D-MET were concentration-dependent in both model systems, and displayed comparable K_m values. The K_m value for L-MET is 1.34 mM in the Caco-2 model and 3.6 mM in the perfused rat intestinal model, while the K_m value for D-MET is 1.79 mM in the Caco-2 model and 2.87 mM in the perfused rat intestinal model. Although the J_{max} values were not comparable because of significant methodology differences, the J_{max} values for L-MET were always higher than that for D-MET. In addition, transport of L- and D-MET across the Caco-2 cell monolayers were also inhibited by 10 mM Phe and Lys while MeAIB, Pro and Glu were generally ineffective. Similar results were also observed with these inhibitors in the perfused rat intestinal model with the exception that a combination of Pro and Glu stimulated the uptake of L-MET. In conclusion, the transport characteristics of L- and D-MET are comparable in both model systems.

KEY WORDS: D-methionine; L-methionine; Caco-2; rat; small intestine; absorption; mechanism.

INTRODUCTION

The determination of absorption mechanism and the kinetic parameters that describe the absorption process are important because absorption affects the overall performance or bioavailability of the drugs, which in turn affects their therapeutic outcomes. Although carrier-mediated transport is responsible for the absorption of a small percentages of drugs, it is an important pathway for drug absorption because many drugs are transported via this route, including several amino acid analogs such as L-dopa, L-α-methyldopa, baclofen, and gabapentin (1–4). Because it is difficult to determine the absorption mechanism in vivo, in vitro models are commonly used.

The Caco-2 cell culture model and the in situ perfused rat intestinal model are two of the most popular methods for determining drug absorption mechanisms. Although each method has advantages and disadvantages when compared with the other one, the overall characteristics of the two model systems appear to be complimentary. For example, the Caco-2 cell culture model, which can be used to monitor uptake, efflux and transport independently, lacks mucus and the regulatory control that is associated with an intact blood supply. On the other hand, the perfused rat intestinal model has some advantages associated with intestinal perfusion in situ (e.g., relatively intact blood supply and mucus layer), but it cannot be used to conveniently determine whether the drug is transported intact. The objective of this study is to determine if these systems are indeed comparable and complimentary to each other, by characterizing the absorption kinetics of D- and L-methionine (MET). The goal is to increase our understanding of kinetics of carrier-mediated absorption in the forementioned model systems, which should strengthen the merits of utilizing these models for future absorption studies.

In order to generate comparable kinetic parameters, similar kinetic processes had to be studied, and similar experimental conditions (e.g., buffer composition and temperature) had to be used. Uptake by the rat intestine and transport across the cell monolayer can be considered as two kinetically equivalent events barring significant membrane binding and metabolism during penetration. Therefore, similar K_m values were expected for carrier-mediated transport if the kinetic processes of absorption through the intestinal epithelium were similar in the two model systems. However, similar J_{max} or maximum rates of transport were not expected because of methodology and species differences. This difference in J_{max} is not critical in the estimation of transport rates across different species, if the rank order of K_m values are preserved and there are only a finite number of carriers in a given species (or absorption mechanisms do not change). Therefore, the determination of absorption characteristics using in vitro models may be useful in choosing an oral drug candidate from a series of compounds for optimal absorption.

Two popular absorption models were chosen for this comparative study because very few comparison studies have ever been done, and the only published study focuses on the transport of a series of D-amino acid peptides transported by passive diffusion mechanism (5). The present study investigates the transport of the amino acid MET, a substrate shown to be transported by a carrier-mediated mechanism in many species. The Caco-2 cell culture model (6,7), an in vitro human intestinal epithelial model, has transport carriers for nutrients such as amino acids, peptides, nucleosides, bile acids, and sugars (8–12). However, there have been no studies comparing the transport of different isoforms of an amino acid in this cell culture model or contrasting the absorption of nutrients in the Caco-2 model and an animal model. The perfused rat intestinal model, which factors out the contribution of unstirred water layer to the overall transport process (13,14), is a popular absorption model with a large data base relating rat intestinal wall permeabilities (P_w) to percent drug absorption in humans (15,16).

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0724-8741/94/1200-1771$07.00/0

MATERIALS AND METHODS

Materials

[3H]-MET was purchased from Dupont-NEN (Boston, MA). [^{14}C]-Mannitol was purchased from Moravek (Brea, CA). Cell culture supplies, including media, serum, trypsin and N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) were purchased from JRH Biosciences (Lenexa, KS). Hank's balanced salt solution (HBSS, powder form), L-MET, D-MET, Phe, Lys, His, MeAIB, Pro, and Glu were obtained from Sigma Chem. Co. (St. Louis, MO) or ICN Biochemicals (Costa Meca, CA). All other materials (typically analytical grade or better) were used as received.

Cell Culture

The Caco-2 cells, originated from ATCC (Rockville, MD) at passage 18, were kindly provided by Dr. Ronald T. Borchardt of The University of Kansas. Cells used in the present study were from passage 64-84. The cells were plated and grown according to established procedures (5,6). Briefly, the cells are maintained in a culture flask and passed (1:3) every three days. The cells are seeded at 100,000 per Snapwell™ (diameter = 12 mm), and grown in media containing 10% fetal bovine serum. The cell monolayers were ready to use in approximately two weeks. The quality of the cell monolayers was controlled by measuring the transepithelial electrical resistance (TEER) and the leakage of a marker compound that is only transported paracellularly, such as ^{14}(C)-mannitol. The normal TEER values ranged from 350 to 600 ohms · cm^2. The normal leakage of a marker compound was less than or equal to 0.13%/hr/cm^2 (or 3.68×10^{-7} cm/sec).

Cell Culture Experiments

Transport experiments were performed in pH 7.4 HBSS supplemented with 10 mM HEPES, 25 mM glucose, and 25 mM of sodium bicarbonate. The experiments are performed using a side-by-side diffusion chamber (Precision Instrument, Redwood City, CA) with the same HBSS buffer at the both sides. This diffusion device, which is stirred with 95% O_2/5% CO_2, offers advantages to the commonly used stagnant devices in that it significantly reduces the thickness of the unstirred water layer. After the cell insert is mounted, the solution containing the compound of interest is loaded onto the apical (AP) side of the monolayer, and the appearance of the compound in the basolateral (BL) media is followed by either HPLC or liquid scintillation spectrophotometry. A similar protocol can be used to perform other experiments such as inhibition experiments by loading an inhibitor and the amino acid at the AP side of the cell monolayer and follow the appearance of MET at the basolateral side.

Rat Intestinal Surgery

The use of rats were approved by Washington State University Animal Care and use Committee. The experiments are performed on male Sprague-Dawley rats, 220–320 gram, 60–80 days old. The rats are fasted overnight before each experiment. Anesthesia is induced by an intramuscular injection of urethane (1.5 g/kg). After the anesthesia is achieved, the rats are kept on a warm heating blanket and under a heating lamp to maintain their normal body temperature. The surgical procedures were the same as previously described (13) except that the inlet cannulate is insulated in a water-filled tube and kept warm by a 37°C circulating water bath.

Perfused Intestinal Experiments

When studying the uptake of MET, a modified Hank's balanced salt solution (HBSS) supplemented with D-glucose (19 mM), penicillin (16 μM) and streptomycin (8 μM) was used. The antibiotics were used to decrease the consumption of MET, presumably by bacteria, because the absence of these antibiotics resulted in erratic P_w values, which were calculated from amount of MET remaining in the perfusate. Addition of antibiotics did not affect the mean P_w values significantly, but decreased the variation of the data (not shown). The jejunal segments were perfused with HBSS containing an amino acid at a pre-determined concentration using an infusion pump for about 20–40 min or until the effluent was clear. Four to six samples were collected each 10 min afterwards. The flow rate was 0.382 ml/min and intestinal length was varied to maintain a concentration ratio of outlet over inlet to approximately 0.9. In general, steady-state disappearance of substrates from the perfusate was usually achieved in 30 min after the perfusion started and maintained throughout the experimental period. After perfusion, the length of the intestine was measure by wetting it with normal saline and carefully laying it flat without stretching. The outlet concentration of perfusate may be determined either by HPLC or by liquid scintillation spectrophotometry or by both. The data are analyzed according to method presented later under "Data Analysis."

Sample Analysis

Radiolabeled amino acid (^{3}H-MET) was mainly analyzed using liquid scintillation spectrophotometry. In the case where we needed to identify that the labeled compound was L-MET (not one of its metabolites) or in the case where we needed to analyze D-MET, an HPLC method with post-column derivatization with ortho-phthaldehyde (OPA) was used. Studies using HPLC to measure L-MET generated results that were comparable to each other (the difference was typically less than 15%, not shown). The HPLC conditions are listed as follows: mobile phase, NaAc-HAc buffer (0.005 M, pH 5.75) 92.5% and methanol 7.5%; mobile phase flow rate, 1.0 ml/min; OPA solution, 6.25 g boric acid, 250 mg OPA, 0.5 ml mercaptoethanol, and 250 ml H_2O, pH adjusted to pH 10.4 using 50% (w/v) KOH; OPA solution flow rate, 0.5 ml/min; column, C-18 25 cm × 4.1 mm, 5 μm; internal standard, citrulline; retention time, 4.6 min for MET and 3.5 min for citrulline. A fluorescence detector set at an excitation wavelength of 240 nm and emission wavelength of 420 nm was used. The detection limit of this method is 10 pmol of MET. Protein assay was carried out using Bradford's method using bovine serum albumin as the standard (17).

Data Analysis

Cell Culture Studies. Results were expressed by plotting amount transported versus time. When the amount transported is linear with time, the initial flux or rate of transport (J) can be easily determined by linear regression. The calculated initial flux was used consistently for the purpose of presenting and comparing results, and for determining the kinetic constants of the amino acid carrier systems by nonlinear regression analysis of a Michaelis-Menten type equation (see equation 1). The nonlinear regression analysis was performed using a statistical software package (Systat, Inc., Evanston, IL). The same software was also used for other statistical analysis presented in "Results and Discussion" section.

$$\text{Rate of transport} = \frac{J_{max} * C}{K_m + C} \quad (1)$$

In equation (1), J_{max} is the maximum rate of transport, K_m is the affinity constant, and C is the concentration of substrate.

Perfused Rat Intestine Studies. The mathematical principle of this analysis method is well established and it is adapted for our use here (13,14). This method measures the steady-state uptake of MET (D or L form) from the perfusate by determining the rate of MET disappearance from the perfusate. At steady-state, the wall permeability (P_w) of the amino acid is calculated using the following equations:

$$P_w = \frac{P_{eff}}{1 - P_{eff}/P_{aq}} \quad (2)$$

Where:

$$P_{eff} = \frac{1 - C_m/C_o}{4\ Gz} \quad (3)$$

$$P_{aq} = (A(Gz)^{1/3})^{-1} \quad (4)$$

$$A = 4.5\ Gz + 1.065 \quad (5)$$

In equations (3) to (5), C_m and C_o are outlet and inlet concentrations, respectively; while Gz, or Gratz number, is the same as previously defined (13).

The P_w values are a much better representation of the intestinal membrane permeability and compounds with a P_w larger than one are generally well absorbed (>75%) (15). The P_w values will be used throughout the rest of this report for presenting the results of animal studies. They may also be used in the following equation to estimate the kinetic parameters using a Michaelis-Menten type equation:

$$Pw = \frac{J_{max}}{K_m + C_w} + P_p \quad (6)$$

Where J_{max} is the maximum flux, K_m is the affinity constant, C_w ($C_w = C_o(1 - P_{eff}/P_{aq})$ is the concentration of amino acid immediately adjacent to the intestinal wall where the absorption occurs, and P_p is the passive permeability component.

Statistical Analysis

Data were analyzed using one way ANOVA or Student T-test. A prior level of significance was set at 5% or $p < 0.05$.

RESULTS AND DISCUSSION

Cell Culture Studies

Effect of Days Post-Seeding (DPS) on Transport. The main purpose of this study was to establish a "time window" suitable for the performance of transport experiments, because earlier transport studies of other nutrients have showed that DPS affected transport (10,18). Hence, the rates of transport of L-MET and D-mannitol at different DPS were measured to seek optimal conditions where the transport of L-MET is high and transport of D-mannitol is low. Mannitol was used here as a leakage marker, and the transport of mannitol represents the extent of paracellular transport. The results indicated that the optimal conditions for our experiments were around 24 days post-seeding where the transport of L-MET was high and %transport of D-mannitol was low (Fig. 1), while the TEER values remained relatively constant (not shown). The results also indicated that the rates of MET transport was much higher ($p < 0.01$) at 22 and 25 days post-seeding when compared to that at 8–15 days post-seeding, while the difference in rates between 22 and 25 days were statistically insignificant ($p > 0.05$). The results presented in Fig. 1 were done in the same batch of cells. Results using other batches of cells indicated that the transport of L-MET and D-mannitol did not change significantly after 23 days (not shown). Based on the results of this study, all subsequent experiments were performed from 23–30 days post seeding.

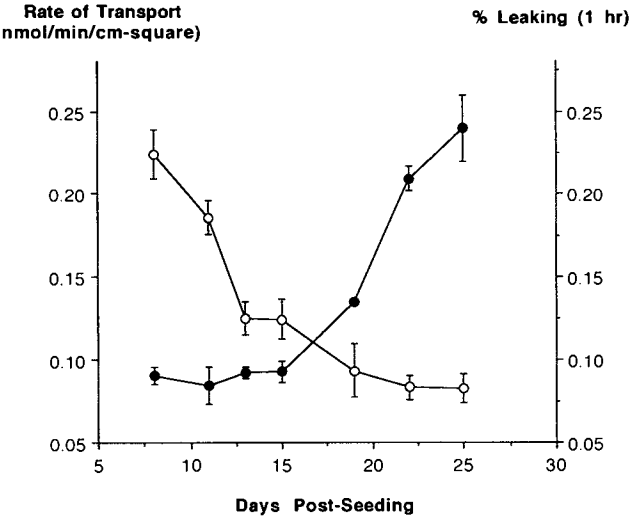

Fig. 1. Effect of post-seeding time on the transport of L-MET (solid circles) and D-mannitol (open circles) in the cell culture model. The transport experiments were performed at 37°C for 180 min. The concentration of L-MET was 1 mM. The cell passage was at 68. Cells were used between 8 and 25 days post-seeding. Each point in the graph represents the average of three determination and the error bar represents standard deviation of the mean.

Time Course of Transport. Transport of MET was studied over the time range of 0 to 120 or 180 min with 30 min sampling interval (Fig. 2). The results of the study indicated that %rate of transport (slope of the linear regression lines) of L-MET was approximately four times that of the D-MET and 12 times that of D-mannitol. Since transport was performed using the same concentration of D- and L-isoform, this results suggest that the transport of L-isoform is preferred over the D-isoform, consistent with other earlier observations (19,20). Since percent mannitol transported is not dependent on the concentration (not shown), it can be said that the transport of both isoforms are much higher than mannitol, suggesting the transcellular transport pathway significantly outweighs the paracellular pathway. In addition, the transport displayed apparent zero-order kinetic behavior, suggesting that the transport is at the steady-state, consistent with our assumption that the transport process is kinetically equivalent to the uptake process (i.e., rat intestinal uptake) at the steady state.

Vectorial Transport. AP to BL transport of D- and L-MET was shown to be faster than BL to AP transport (Fig. 3), suggesting that the distribution of the transport carriers are polarized with a preferred location at the AP side. This observation agrees with earlier results in that AP uptake of Phe, also a large neutral amino acid, is faster than BL uptake (8). It also further strengthens the hypothesis that the transport of MET is via a carrier-mediated pathway.

Effects of Concentration. To further confirm the presence of a carrier-mediated process, the rates of transport of L-MET at different concentrations were measured at both

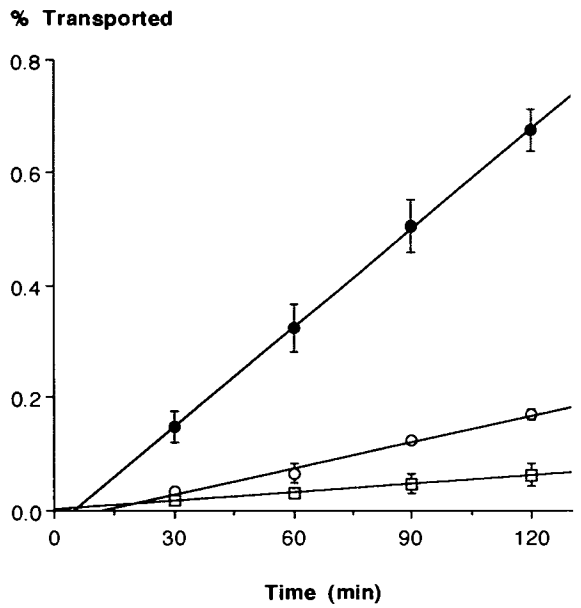

Fig. 2. Effect of time on the transport of L-MET (solid circles) and D-MET (open circles) as well as D-mannitol (open square) in the cell culture model. The transport study was performed at a concentration of 1 mM for MET and tracer amount for D-mannitol. The experiment was performed at 37°C in the diffusion chamber. D-MET was analyzed by HPLC while L-MET and mannitol by liquid scintillation. The cells used were at passage 69 and 27 days post-seeding. Each point in the graph represents the average of three determination and the error bar represents standard deviation of the mean.

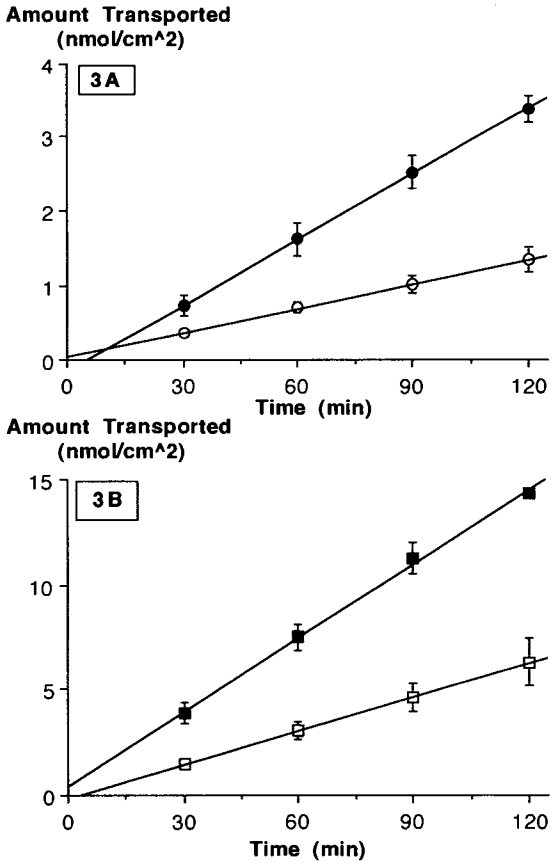

Fig. 3. Vectorial transport of MET in the cell culture model. The transport of D-MET is shown in 5A while that of L-MET is shown in 5B. Fig. 5A shows AP to BL (solid circles) and BL to AP (open circles) transcellular transport of D-MET. Fig. 5B shows AP to BL (solid squares) and BL to AP (open squares) transcellular transport of L-MET. Each point is the average of three determinations and the error bars represent standard deviation of the mean. (See text for further explanations).

37°C and 4°C. The difference between the rates of transport at 37°C and 4°C at various concentrations were calculated and used as the carrier-mediated rates of transport (Fig. 4). Using nonlinear regression analysis, K_m was determined to 1.34 mM, J_{max} 0.678 nmol/min/mg protein or 0.376 nmol/min/cm^2, and the first order rate constant 30.8 pmol/min/mg protein (calculated from 4°C experiments). The rates of transport of D-MET at different concentrations were also measured at 37°C. The kinetic parameters obtained were: K_m, 1.794 mM; J_{max}, 0.609 nmol/min/mg protein or 0.337 nmol/min/cm^2; and K (fitted first order rate constant), 14 pmol/min/mg protein/mM. Taken together with the results shown in the "Time Course of Transport," these results suggest that L-MET is better transported than D-MET, because K_m is smaller but J_{max} is larger for L-MET transport.

Effects of Competitive Inhibitors. To probe the specific carrier or carrier combinations that were involved in the transport of MET, various inhibitors were used. Transport of L-MET was significantly decreased by 10 mM of Phe (66%) and Lys (71%), but not by 10 mM of MeAIB, Glu and Pro, suggesting that the transport of L-MET was via a large neutral amino acid carrier (Table 1). The effects of these inhib-

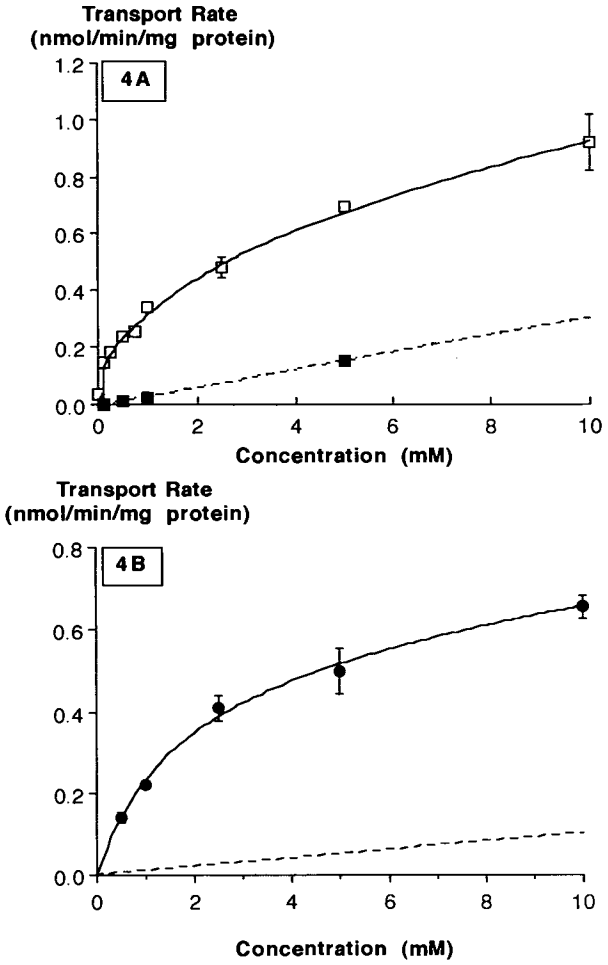

Fig. 4. Effect of concentration on the rates of transport of MET in the cell culture model. In each panel, the upper curve represents transport rates measured at 37°C, the lower curve represents the nonsaturable component of the transport, either measured or fitted. The transport of L-MET is shown in 5A while that of D-MET is shown in 5B. Each point is the average of three determinations and the error bars represent standard deviation of the mean. (See text for further explanations).

itors on the transcellular transport of D-MET were similar, consistent with the hypothesis that these two isoforms are transported via a similar set of carriers.

Perfused Rat Intestinal Studies

Concentration Effects. There is a clear effect of concentration on the steady-state uptake of L-MET (Fig. 5). A nonlinear regression analysis of the data using equation (6) generated a K_m of 3.6 mM and J_{max} of 35 mM. Uptake of D-MET also appeared to be concentration-dependent (Fig. 5). The uptake of D-MET has a K_m of 2.9 mM and J_{max} of 10.7 mM. Although there is some difference regarding J_{max} values, both forms should be well absorbed according to an earlier report linking P_w to bioavailability (15). These kinetic parameters appear to be consistent with those observed with the cell culture model; i.e., similar K_m for both isoforms but higher J_{max} for the L-isoform, indicating once again that transport of L-isoform is preferred to the D-isoform. The

Table I. Effect of Various Compounds on the Rate of Transcellular Transport of D- and L-MET

Compound	Concentration (mM)	% of Control[a] ± SD	
		L-MET	D-MET
Phe	10	34 ± 2*	17 ± 2*
MeAIB	10	72 ± 21	100 ± 13
Lys	10	29 ± 1*	31 ± 1*
Glu	10	102 ± 6	108 ± 2
Pro	10	89 ± 9	103 ± 7
D-Mannitol	10	120 ± 26	ND[b]

[a] The rates of D- (1 mM) and L-MET (0.1 mM) transport in the absence of inhibitors were 155 ± 4 and 68 ± 14 pmol/min/cm^2, respectively. Higher concentration of D-MET was used to assure adequate detection of the compound in the basolateral side. Since competition experiments were performed with different batch of cells, a set of standard experiments have to be performed. It was decided that transport of L-MET in the presence of Phe be designated as the standard experiments, and that the rate of L-MET transport under this condition be used as control value. Transport rates under other conditions are normalized against this control value. Each value is the average of three-five determinations.

[b] ND stands for results not determined.

* Statistically significant difference ($p < 0.05$) as determined by unpaired Student T-test.

preference of L-isoform over D-isoform has also been demonstrated previously for other amino acids (19–21).

Inhibitor Effects. Our earlier studies with the Caco-2 cell monolayers have indicated that several large neutral amino acids inhibited the transport of L-MET. Therefore, studies were performed to determine if these amino acids also inhibit the transport of both isoforms of the amino acid in the rat intestine. The results indicated that L-histidine

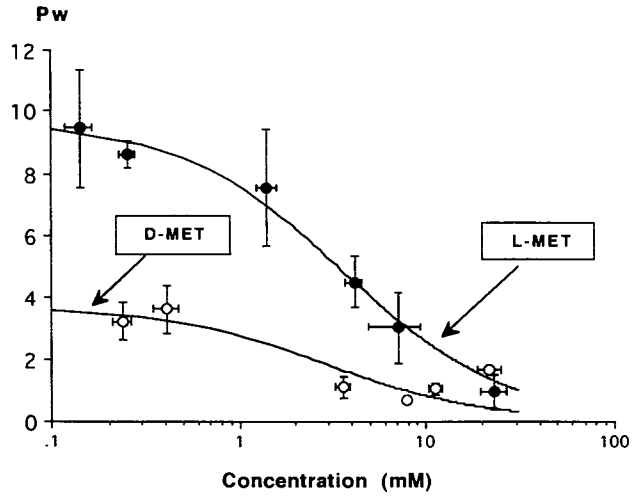

Fig. 5. Effect of intestinal wall concentration on the intestinal wall permeabilities of MET in the perfused rat intestinal model. The upper curve represents the relationship between Pw and Cw of L-MET while the lower curve represents the same relationship for D-MET. Each point is the average of three–five determinations and the error bars represent standard error of the mean (SEM). Because both Cw and Pw are calculated parameters based on experimental measurables, there are SEM associated with both Cw and Pw. (See text for further explanations).

Table II. Rat Intestinal Uptake of MET in the Presence of Various Inhibitors

Inhibitors	Concentration (mM)	%Control ± SEM[a] L-MET	D-MET
D,L-MET[b]	30	11 ± 1*	51 ± 5*
Phe	30	73 ± 18*	51 ± 24*
His	30	47 ± 12*	78 ± 4*
MeAIB	30	86 ± 26	146 ± 32
Pro + Glu	15 + 15	162 ± 7*	127 ± 28

[a] The control Pw values, which is the average ± standard error of the mean (SEM) of four determinations, are 3.58 ± 0.48 for L-isoform (1 mM) and 1.43 ± 0.11 for D-isoform (1 mM), respectively. A set of control experiments may be performed for each batch of animals and the control value may have a ± change of up to 50%.

[b] L-MET (30 mM) was used to inhibit the transport of L-isoform while D-MET was used for the D-isoform.

* Indicates a significant change when compared to the control.

(His) and L-phenylalanine (Phe) consistently inhibited the transport of MET, in either L- or D-form (Table II). The effects of several other amino acids on the transport of MET were also determined, and the results indicated that N-methyl-aminoisobutyric acid (MeAIB) did not affect the absorption of either isoform while a combination of L-proline plus L-glutamic acid (Pro + Glu) increased the absorption of L-MET but did not change the absorption of D-MET (Table II). Taken together, these result suggest both isoforms are absorbed by the large neutral amino acid carrier. However, it was unclear as to why Pro + Glu had different effects on the absorption of L versus D isoform.

In conclusion, the results of the present investigation indicates that the transport characteristics of L-MET and D-MET are similar in both model systems, and the transport of both isoforms appears to be via the same set of amino acid carriers that transport large neutral amino acids (e.g., Phe) and basic amino acids (e.g., His and Lys).

ACKNOWLEDGMENTS

Work Supported by ZinPro Corp.

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