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A COMMON STEP IN THE INTESTINAL ABSORPTION MECHANISMS OF D- AND L-METHIONINE*

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SUMMARY

1. The intestinal absorption of D- and L-methionine in the chicken has been shown to occur by attachment to a common L-preferring site presumably on the mucosal epithelial membrane. The possibility of separate binding sites with overlapping specificities for these isomers has been ruled out by a kinetic test.

2. An equation for uptake of sugars at the steady state in bacteria was used to study uptake in intestinal segments.

3. Affinity constants for inhibitors (K_i) were determined from plots of reciprocal per cent inhibition *versus* reciprocal inhibitor concentration.

4. Neutral amino acids of the L-configuration had high affinity for the methionine transport site, while neutral D-amino acids, except for D-methionine had very low affinity.

5. L-Lysine, L-histidine and L-aspartic acid had very poor affinity for this site, while D-aspartic acid had no affinity. D-Histidine caused stimulation of L-methionine uptake.

INTRODUCTION

The intestinal absorption of both D- and L-methionine has been found to follow saturation kinetics[1] which presumably indicates that chemically mediated processes are involved in which substrate attaches to a membrane receptor site. However, D-methionine has been shown to have lower affinity than its enantiomer for mediated transport[1,2]. PAINE, NEWMAN AND TAYLOR[3] observed that chickens bearing Thiry-Vella fistulas absorbed L-methionine at a rate 3-fold that of the D-isomer. In their studies, the D-isomer did not become inverted during absorption as determined by a D-amino acid oxidase assay of blood samples. Furthermore it has been reported that both isomers are transported against concentration gradients in hamster and chicken intestines, although the L-isomer develops the larger gradient[2,4].

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In the work to be reported below, D- and L- neutral and polar amino acids were tested as inhibitors of D- and L-methionine transport. Neutral amino acids of the L-configuration and D-methionine possessed the greatest relative affinity for the methionine transport site. The results further clarify the nature of the transport processes for these isomers by offering evidence for a common membrane receptor site in their absorption mechanisms.

THEORETICAL TREATMENT

A rigorous test has been proposed by AHMED AND SCHOLEFIELD[5] which can be used to show that the uptake of D- and L-methionine occurs by attachment of these isomers to a common site on the mucosal epithelial membrane. Three conditions must be fulfilled:

(1) L-Methionine must be a competitive inhibitor of D-methionine transport, and D-methionine must be a competitive inhibitor of L-methionine transport.

(2) The apparent Michaelis constant, K, for the transport of L-methionine must equal its K_i when acting as an inhibitor of D-methionine transport, and the same argument must apply when D-methionine is the substrate for transport or when it is the inhibitor of L-methionine transport.

(3) A third amino acid must have the same K_i when used as an inhibitor on either D- or L-methionine.

In the present work, the apparent Michaelis constant, called by COHEN AND MONOD[6] the dissociation constant of the tissue–substrate complex, was found using the following steady-state expression of these authors:

$$S_2 = Y\left(\frac{S_1}{S_1 + K}\right) \tag{1}$$

where S_2 = tissue accumulation of substrate at the steady state; Y = maximal accumulation of substrate under conditions of saturating external concentration; S_1 = medium concentration of substrate.

Since classical laws of competition for a common site are obeyed in uptake studies done at the steady state[6], fully competitive inhibition may be described by:

$$\frac{1}{\%I} = \frac{K_i}{100}\left(1 + \frac{S_1}{K}\right)\frac{1}{I} + \frac{1}{100} \tag{2}$$

where K_i = dissociation constant of the inhibitor–receptor site complex; I = inhibitor concentration; $\%I$ = per cent inhibition of substrate uptake.

In the RESULTS AND DISCUSSION given below, the K_i values for inhibition of substrate uptake were found graphically by application of Eqn. 2.

MATERIAL AND METHODS

12–18-week-old White Leghorn female chickens were fasted 24 h prior to sacrifice and then killed by decapitation. A modified tissue accumulation method was used in all studies[7]. A portion of intestine 15 cm on either side of the yolk stalk was removed[4] and kept in cold, previously oxygenated (5% CO_2–95% O_2) physiological saline containing 0.3% glucose for not over 8 min during the preparative steps

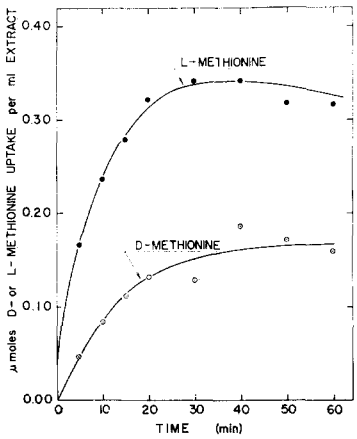
before incubation. The intestine was then cut into 3-cm segments each weighing about 0.5 g. Each segment was cut lengthwise and blotted dry. Five even-numbered or odd-numbered segments of tissue were incubated at 37° with shaking in an atmosphere of 5% CO_2–95% O_2 with 25-ml portions of Krebs–Henseleit buffer which contained the test amino acid in concentrations ranging from 0.1 mM to 2 mM and labeled with ^{14}C. Glucose (0.3%) was added to the test solutions as an energy source. Concentrations of amino acids used as inhibitors ranged from 1 mM to 20 mM. The amino acids were of the highest purity commercially available and were used without further purification. L-[*Me*-^{14}C]Methionine and D-[*Me*-^{14}C]methionine were purchased from Calbiochem (Los Angeles, Calif.).

After incubation for 1 h, the segments were blotted, weighed, and then shaken overnight in a portion of aqueous ethanol solution (95% ethanol–water, 1:1, v/v) using 10 ml of solution per g of tissue. The extracts were centrifuged at 18 400 × *g* for 12 min. [^{14}C]Methionine was measured using a 1-ml aliquot added to 10 ml of scintillation solution composed of 0.025% 1,4-bis-(5 phenyloxazolyl-2)benzene, 1% 2,5-diphenyloxazole, and 10% naphthalene, all in 1,4-dioxane[8]. Amino acid standards consisted of 0.5 ml distilled water and 0.5 ml Krebs–Henseleit buffer containing the ^{14}C-labeled amino acid added to 10 ml of scintillation solution. The ^{14}C-labeled amino acids, both extracts and standards, were determined in an ambient-temperature Nuclear–Chicago scintillation detector, which gave 75±5% efficiency using [^{14}C]-toluene as an internal standard. The uptake of labeled amino acid was expressed in terms of μmoles accumulated per ml aqueous ethanol extract, the uptake then representing a constant fraction of the concentration accumulated. Because substrate uptake was found to be a function of segment location in the intestinal tract, in the inhibition studies segments were kept in the order of their appearance in the intact animal without distinguishing between orad and caudad ends of the 30-cm portion, and per cent inhibition of uptake was calculated from the fraction uptake S_2'/S_2 where S_2' = concentration of substrate accumulated by even-numbered segments incubated in the presence of inhibitor; S_2 = concentration of substrate accumulated by odd-numbered segments incubated without inhibitor.

RESULTS AND DISCUSSION

The time course of uptake for both isomers (Fig. 1) is similar in that their steady states are reached after about 30 min incubation. No appreciable change in accumulation occurs after this time, so one may terminate incubation thereafter without stringent regard to time. In these experiments, 60 min incubation time was used. Perhaps the most important advantage in using a steady-state treatment is the avoidance of measuring the initial velocity which is highly dependent on the time of termination of incubation.

In determining K values for the methionine isomers, substrate concentrations ranging from 0.1 mM to 2 mM were used. Fig. 2 shows the reciprocal uptake to be a linear function of the reciprocal substrate concentration, which indicates that the kinetics are followed reasonably well, and that non-mediated uptake probably is not a factor influencing total accumulation over the concentration range employed; otherwise the graphs would be curvilinear due to the effect of diffusion at the higher external substrate concentrations[9].



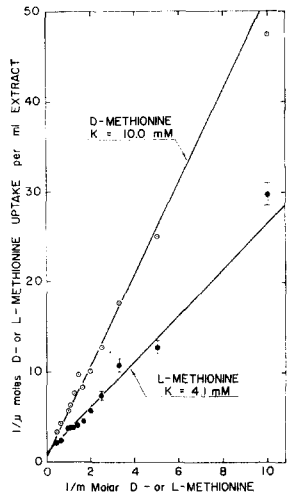


Fig. 1. Time course of D- and L-methionine accumulation. Labeled amino acids were incubated in Krebs–Henseleit buffer as described in text. Each value for the uptake of 1 mM L-methionine represents one determination of the combined uptake of five alternate intestinal segments from one animal. For D-methionine uptake each graphed value represents one determination of the combined uptake of ten alternate segments from two animals.

Fig. 2. Double reciprocal plot of the steady-state D- or L-methionine accumulation as a function of medium concentration. Incubation of labeled amino acids was in Krebs–Henseleit buffer as described in text. Each graphed value represents the average of five determinations on a total of 25 intestinal segments from three animals. Typical variability is shown by the standard error of the mean.

The K values for D- and L-methionine were found to be 10.0 mM and 4.1 mM, respectively. As noted earlier, differences in affinity of D- and L-methionine for intestinal transport systems have been reported for the rat[1] and hamster[2], in each case the L-isomer showing greater affinity. From the K values stated here and from the fact that the steady-state concentration for the L-isomer, 0.34 μmole/ml of extract, is twice that of its enantiomer, 0.17 μmole/ml of extract, it appears that the amount of each isomer accumulated at steady state from 1 mM solutions is proportional to its affinity for a receptor site.

D-Methionine gave a K_i value of 14.1 mM (Table I) when used as an inhibitor of L-methionine transport. Its own affinity for transport (K = 10.0 mM) was thus approximately equal to its affinity for the L-methionine transport site. Although the y intercept in a plot of the reciprocal per cent inhibition of L-methionine uptake *versus* the reciprocal D-methionine concentration was 0.0125, this value was judged to be not significantly different from 0.01, the intercept value of Eqn. 2. CHAKRABARTI[4], employing the everted-sac method in the chicken, found that the inhibition of L-methionine uptake by the D-isomer was of the competitive type and *vice versa*. In our work, L-methionine (Table II) was determined to be an inhibitor of the D-isomer with a K_i of 4.5 mM, which was obtained from the reciprocal inhibition plot. Hence, its K value (4.1 mM) agreed well with its K_i for D-methionine inhibition. The information elaborated here thus satisfies criteria 1 and 2 of AHMED AND SCHOLEFIELD[5].

L-Cystine proved to be an interesting inhibitor. Despite the fact that its

TABLE I

PER CENT INHIBITION AND K_i VALUES FOR INHIBITORS OF L-METHIONINE UPTAKE

Experimental conditions are given in the text. Values for per cent inhibition of L-methionine uptake were found from the fraction uptake (see text) using five pairs of segments from one animal. The number of animals used is given in parentheses.

Inhibitor	*Inhibitor concentration (mM)*	*L-Methionine concentration (mM)*	*Inhibition of L-methionine uptake (% ± S.E.)*	K_i* *(mM)*
D-Methionine	2.0	1.0	13.5 ± 1.5 (4)	14.1
	3.5		15.8 ± 1.3 (5)	
	8.0		28.4 ± 2.6 (3)	
	20.0		46.1 ± 1.7 (3)	
L-Cystine	1.0	1.0	8.1 ± 1.8 (3)	10.1
	2.2		11.9 ± 1.4 (5)	
	3.1		21.4 ± 2.6 (5)	
	6.2		35.1 ± 1.8 (5)	
L-Ethionine	1.0	0.1	23.4 ± 2.1 (5)	3.5
	1.3		24.4 ± 1.6 (5)	
	2.0		36.4 ± 2.3 (5)	
	3.0		45.6 ± 2.4 (5)	
S-Methyl-L-cysteine	1.0	0.1	16.2 ± 1.2 (5)	4.3
	2.0		31.8 ± 2.5 (5)	
	3.0		40.0 ± 1.1 (5)	
	4.0		49.9 ± 0.7 (5)	
Glycine	1.0	0.1	2.1 ± 1.5 (5)	40
	2.0		3.6 ± 2.7 (5)	
	3.0		3.6 ± 2.2 (5)	
	4.0		9.0 ± 2.4 (5)	
	5.0		9.8 ± 3.9 (5)	
	6.0		8.0 ± 1.9 (5)	

* $K_i = \dfrac{\text{slope} \times 100\,K}{S_1 + K}$ in a plot of $1/\%I$ *versus* $1/I$.

solubility in Krebs–Henseleit buffer is very low (about 1 mM), it was possible to obtain linear plots of $1/\%I$ *versus* $1/I$ for L-cystine concentrations as high as 10 mM. The explanation of this behavior lies in the observation that concentrations of L-cystine well above 1 mM were apparently solubilized in the presence of intestinal tissue. This solubilization effect may be due to the presence of bile salts which have been shown to be capable of solubilizing certain water-soluble materials[10]. It is conceivable that sufficient bile had been adsorbed to the tissue segments to create an emulsion. Because chicken intestine is relatively high in fat content, fatty acids released by tissue hydrolysis may have aided substantially in this effect.

Using the graphical method applied above, one finds the K_i values of L-cystine for the inhibition of D- and L-methionine uptake to be 8.4 mM and 10.1 mM, respectively (Tables I and II). These values are approximately equal within experimental error, thus satisfying criterion 3 of AHMED AND SCHOLEFIELD[5] and indicating that L-cystine had competed with both methionine isomers for a common site. L-Cystine had less affinity for this site than did L-methionine. LIN, HAGIHIRA AND WILSON[2] noted that whereas affinity of a series of amino acids increased as the alkyl side-chain increased in size, the presence of a charge in the side-chain abolished

TABLE II

PER CENT INHIBITION AND K_i VALUES FOR INHIBITORS OF D-METHIONINE UPTAKE

Conditions were identical with those given in Table I.

Inhibitor	*Inhibitor concentration (mM)*	D-*Methionine concentration (mM)*	*Inhibition of* D-*methionine uptake (%±S.E.)*	K_i* *(mM)*
L Methionine	2.0	1.0	28.6 ± 1.2 (5)	4.5
	8.0		61.4 ± 0.8 (5)	
	20.0		75.8 ± 1.0 (5)	
L-Cystine	1.0	1.0	9.0 ± 0.3 (5)	8.4
	4.0		30.8 ± 2.8 (5)	
	10.0		49.0 ± 2.8 (5)	
L-Ethionine	1.0	0.1	28.6 ± 1.5 (5)	2.7
	2.0		43.7 ± 3.0 (5)	
	3.0		50.1 ± 4.5 (5)	
	4.0		58.4 ± 2.0 (5)	
S-Methyl-L-cysteine	1.0	0.1	17.8 ± 2.8 (5)	3.1
	2.0		42.4 ± 1.2 (5)	
	3.0		50.5 ± 1.0 (5)	
	4.0		56.1 ± 2.6 (5)	
Glycine	5.0	1.0	15.5 ± 10.0 (5)	40
	6.0		11.3 ± 6.7 (5)	
	10.0		19.3 ± 3.8 (5)	

* $K_i = \dfrac{\text{slope} \times 100\, K}{S_1 + K}$ in a plot of $1/\%I$ *versus* $1/I$.

affinity. Contrary to their findings, the information given here suggests that the side-chain charge in cystine decreased but did not abolish its affinity. In fact, L-cystine had about the same affinity as did D-methionine for the common site involved in D- and L-methionine absorption, although its K_i was two to three times larger than the K_i values for the neutral amino acids tested below (see Tables I and II).

The inhibitions by L-ethionine and *S*-methyl-L-cysteine were also tested. In each case their K_i values for the inhibitions of D- and L-methionine uptake agreed fairly closely. Since L-ethionine and *S*-methyl-L-cysteine are homologues of L-methionine, it was expected that their affinity constants would serve as checks on the K and K_i values for L-methionine. Tables I and II show these homologues to have similar affinity constants. In contrast, the affinities of L-cystine, possessing a charged side-chain, D-methionine, which has the D-configuration about its α carbon, and glycine, which lacks a side-chain, were appreciably higher than those of the neutral amino acids mentioned.

Glycine gave K_i values somewhat above 40 mM. Wide variability in per cent inhibition of D- and L-methionine uptake was observed but no attempt is made to explain it. BEGIN AND SCHOLEFIELD[11] have concluded that glycine is transported *via* a different site in mouse pancreas than is L-methionine, and the poor affinity of glycine for the methionine transport site in the chicken may be indicative of a separate transport site for this compound.

Table III gives a comparison of K_i values of both D- and L-amino acids on the transport of L-methionine. Values of both D- and L-amino acids already men-

TABLE III

COMPARISON OF AFFINITY CONSTANTS OF D- AND L-AMINO ACIDS FOR THE METHIONINE TRANSPORT SITE

Experimental conditions given in the text.

	K_i *of amino acid on* L-*methionine transport (mM)*
L-*Amino acids*	
L-Methionine	4.1 (K)
L-Cystine	11.0*, 10.1**
L-Ethionine	3.6*, 3.5**
S-Methyl-L-cysteine	4.3*, 4.3**
L-Phenylalanine	8.4***
L-Lysine	26***
L-Histidine	32***
L-Aspartic acid	197***
L-Valine	5.8***
D-*Amino acids*	
D-Methionine	14.1**, 20.0*
D-Ethionine	65***
D-Histidine (20 mM)	stimulates 0.1 mM L-methionine uptake by 7.6 ± 1.7%
D-Aspartic acid (20 mM)	no inhibition
D-Valine	176***
D-Phenylalanine	83***

* K_i determined graphically from a plot of $1/\%I$ *versus* $1/I$. From Eqn. 2, $K_i \cong I$ at 50% inhibition.

** K_i determined graphically from a plot of $1/\%I$ *versus* $1/I$. From Eqn. 2, K_i = [slope × 100 K]/($S_1 + K$).

*** K_i calculated from the following expression (from Eqn. 2): $K_i = [K(I)(100-\%I)]/[(\%I)(K + S_1)]$, with S_1 = 0.1 mM; K = 4.1 mM; and I = 20 mM. $\%I$ was determined from the fraction uptake (see text) using 25 pairs of segments from five animals. The variability in $\%I$ was of the same order of magnitude as found in Tables I and II.

tioned were determined graphically from plots of $1/\%I$ *versus* $1/I$ by two methods given in Table III. Except for the K_i values of D-methionine, 14.1 and 20.0 mM, the methods give similar values. K_i values for other amino acids in the tables were determined from the per cent inhibition of 0.1 mM L-methionine uptake by a 20 mM level of inhibitor. These data are in support of the results of NATHANS, TAPLEY AND ROSS[12] found for the inhibition of monoiodotyrosine by a series of D- and L-amino acids. The neutral L-amino acids tested all possess high affinity for the methionine transport site. Their affinities are all approximately equal and less than 10 mM. L-Cystine behaved like an uncharged species, and one may suppose that its charged terminus, because of the length of the chain, does not enter into side-chain interactions with the neutral amino acid carrier for methionine. Of the L-amino acids with charged or polar side-chains, L-lysine and L-histidine had relatively low affinities with about the same order of magnitude, and L-aspartic acid possessed extremely low affinity. Presumably a negatively charged side-chain is incompatible with carrier affinity. D-Methionine had especially high affinity compared to the other D-amino acids tested. On the other hand, D-ethionine unexpectedly had much lower affinity than D-methionine despite similar structures. D-Valine and D-phenylalanine inhibited L-methionine weakly, and consequently gave very large K_i values. Presumably the

configuration about the α carbon exceeded in importance the neutral side-chain for carrier specificity toward these materials. D-Aspartic acid had no affinity for the methionine transport site. D-Histidine which has the unfavorable α configuration *plus* a polar side-chain, causes stimulation of L-methionine uptake. Perhaps the stimulatory effect arises from a facilitation of the rate of methionine transport by a carrier modification (allosteric interaction) brought about by irreversible binding of D-histidine to a site not involved in methionine transport. ALVARADO[13] has given evidence for allosteric interactions occurring between binding sites from experimental finding on the partially competitive inhibition of cycloleucine transport by D-galactose and L-arginine.

The attachment of D- and L-methionine to membrane receptor sites can occur in four ways. (1) Two types of receptor site may exist; one would have absolute specificity for L-isomers and the other would have absolute specificity for D-isomers. This model is ruled out on the basis of experimental findings showing competitive inhibition between D- and L-methionine[4]. (2) A common site for the absorption of both isomers may exist which does not possess stereospecificity. This model is rejected because of the difference in affinity shown by D- and L-methionine for transport[1-4]. (3) One site may exist which is L-preferring (or D-preferring). An L-preferring site is compatible with our experimental findings. It predicts that L-methionine should have greater affinity for transport than should D-methionine, and that competition should be observed between these isomers. Their K values for transport should equal their K_i values for inhibition of each other. A third amino acid, either D- or L-configuration, should give equal K_i values for inhibition of D- or of L-methionine. (4) Two sites may exist, one which is L-preferring, the other D-preferring, and each site would have overlapping specificities. In this model, one should expect D- (or L-) methionine to give a low K for transport but a high K_i for the inhibition of L- (or D-) methionine. However, experimental results given in this paper do not support such a model.

It is concluded, therefore, that one site exists on the mucosal epithelial membrane which provides for the absorption of both D- and L-methionine. This site has been shown to be L-preferring.

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