Structure-Affinity Relationships of Substrates for the Neutral Amino Acid Transport System in Rabbit Ileum

ROBERT L. PRESTON, JOHN F. SCHAEFFER, and PETER F. CURRAN

From the Department of Physiology, Yale University School of Medicine, New Haven Connecticut 06510. Dr. Schaeffer's present address is the Biology Department, Tufts University, Medford, Massachusetts.

ABSTRACT The apparent affinities of various amino acids for the neutral amino acid transport system in rabbit ileum were determined by measuring the inhibition of L-methionine-^{14}C influx across the brush border membrane. The apparent affinity was very low for compounds lacking an α-amino group, compounds with the α-hydrogen substituted by a methyl group, D-compounds, compounds with tertiary branching in the side chain, compounds with either a positive or negative charge in the side chain, and in most cases, compounds with a hydrophilic moiety in the side chain. High apparent affinities were exhibited by compounds with unbranched carbon or carbon-sulfur side chains. Branched compounds such as valine and leucine exhibited affinities which correlate with binding of only the linear portion of the side chain. The calculated change in free energy of binding is 370 cal/mol/CH_2 group which suggests the binding region for the side chain is partially hydrophobic. The affinities of families of analogues, derivatives of cysteine, methionine, serine, alanine, valine, and phenylalanine, correlate with their calculated octanol/water partition coefficients and are also correlated with apparent structural and electronic differences between families. The data permit a preliminary description of the functional geometry of the neutral amino acid transport site. The site contains a region for binding the α-amino group, α-carboxyl group, and side chain. The regions about the α-amino group and α-hydrogen are quite sterically limited. The side chain binding region is hydrophobic in nature and appears to be shallow, binding only the linear portion of branched or ring compounds.

Many studies have been conducted in recent years on the relationships between the structure of substrates and their affinity for enzymes (1–8), and the results often permit deduction of general structural characteristics of the substrate binding site. Similar studies on amino acid transport systems can provide analogous information as indicated by the work of Christensen (9).

However, in some other cases, the small number of compounds examined and lack of detailed information about the transport system has limited the amount of information obtained concerning binding properties of the transport site (10–16). The neutral amino acid transport system in the brush border membrane of rabbit ileum has been extensively characterized (17, 18) and a detailed model developed to account for its kinetic characteristics. It thus seems an appropriate system in which to examine structure-affinity relationships. Hajjar and Curran (19) began such a study by examining the ability of certain amino acid analogs to inhibit the unidirectional influx of phenylalanine across the brush border membrane. The present study was initiated in an effort to define more completely the structure-affinity relationships and characteristics of the binding site of this transport system by examining the inhibition of methionine influx by a wide variety of amino acids. Methionine was chosen as the test amino acid because the studies of Hajjar and Curran (19) suggested that it had an unexpectedly high affinity for the transport system and did not appear to conform to the pattern observed for compounds such as valine, leucine, and phenylalanine.

METHODS

The techniques used were essentially the same as those employed by Hajjar and Curran (19). Amino acid influx from the mucosal solution into the cells across the brush border of the distal ileum of New Zealand White rabbits was measured by the technique described by Schultz et al. (18). The method involves a brief (60-s) exposure of the mucosal surface only to a test solution containing [^{14}C]amino acid and [^{3}H]inulin (to estimate residual test solution adhering to the surface). Influx of amino acid was estimated from the amount of ^{14}C taken up by the tissue after correction for the "inulin space."

The kinetic characteristics of methionine influx across the brush border were determined by measuring influx as a function of methionine concentration. The interaction of various amino acids with the transport system was then determined by examining their ability to inhibit the influx of methionine. In each experiment, control influx was measured together with influx in the presence of three different concentrations of inhibitor. Unless otherwise noted, methionine concentration was 2 mM. In all cases, a minimum of six flux determinations were made for each concentration of inhibitor and tissues obtained from at least two animals were used. Data were treated as discussed by Hajjar and Curran (19). Methionine influx, J^i, can be described by Michaelis-Menten-type kinetics so that

$$J^i = \frac{J^{i\max}[\text{Met}]}{K_t + [\text{Met}]}, \tag{1}$$

in which $J^{i\max}$ is maximal influx, K_t is the "apparent Michaelis constant," and [Met] is methionine concentration in the mucosal solution. For a competitive inhibitor, the ratio of control influx J^i_o, to influx in the presence of the inhibitor, J^i_I,

will be given by

$$\frac{J_o^i}{J_I^i} = 1 + \frac{K_t[I]}{K_I(K_t + [\text{Met}])}, \tag{2}$$

in which $[I]$ is the concentration of inhibitor in the mucosal solution and K_I is the "inhibitor constant." For strictly competitive inhibition, K_I should be equal to the Michaelis constant for the inhibitor. According to Eq. 2, J_o^i/J_I^i should be a linear function of $[I]$ and the slope of the line will provide an estimate of K_I. Slopes of the lines relating J_o^i/J_I^i to $[I]$ were determined by least squares assuming an intercept of 1.0 on the Y axis.[1]

The quantity K_I was analyzed further on the basis of the model of the amino acid transport system suggested by Curran et al. (17). According to this model, K_t is given by

$$K_t = \frac{K_1K_2}{K_2 + [\text{Na}]}, \tag{3}$$

in which K_1 is the dissociation constant for the reaction between amino acid and the transport site and K_2 is the dissociation constant for reaction of the site-amino acid complex with Na; [Na] is Na concentration in the mucosal solution. Studies using several neutral amino acids (17–19) have suggested that K_2 does not depend strongly on the particular amino acid involved and have indicated that 25 mM is a reasonable value of K_2 for neutral amino acids. Therefore to obtain an estimate of K_1 for the various inhibitors, we have assumed that K_1 for the interaction of that amino acid with the methionine transport system is also given by Eq. 3 and that $K_2 = 25$ mM;[2] K_1 was then calculated from Eq. 3 (see Hajjar and Curran [19] for further discussion). Some further tests of these assumptions will be described in the Results section. For those amino acids available only in the DL form, the effective concentration was taken as one-half the nominal value since these and previous studies (see Results and references 19, 20) indicated that affinity of D-amino acids for the transport system is relatively small.

The influx chambers were constructed so that each baseplate over which a piece of intestine was stretched was equipped with four ports permitting four influx determinations on that piece of intestine. In each such "block," measurements were made under control conditions and at three concentrations of inhibitor. For purposes of statistical analysis, each block was treated as a separate determination of K_I since interblock variance (due mainly to variable stretching) was as great as interanimal variance. In some experiments with poor inhibitors, only a single concentration was used and control and test tissues were alternated. In only a few cases were inhibitor

[1] In a few cases in which the value of control influx appeared anomalous, the data were also analyzed by plotting $1/J_I$ vs. $[I]$ in order to evaluate K_I.

[2] This assumption may be open to question for the interaction of acidic and basic amino acids with the methionine transport system. However, the apparent affinity of these compounds for the methionine system is so low that our qualitative conclusions would be unaltered if the appropraite value of K_2 varied widely from 25 mM.

concentrations above 40 mM used; no compensation was made for differences in osmolarity because previous experiments (19) had shown that results were not influenced by osmolarity changes in this range.

The normal bathing solution contained 140 mM NaCl, 10 mM $KHCO_3$, 0.4 mM, K_2HPO_4, 2.4 mM KH_2PO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, and had a pH of 7.2 when bubbled with 95% O_2–5% CO_2. Na concentration was altered by replacing NaCl with an equivalent concentration of choline chloride. All experiments were carried out in a warm room at 37°C. In the few cases in which compounds having a free −SH group were studied, 30 mM dithiothreitol was added to the solutions to prevent oxidation. Dithiothreitol was also added to the control solutions that did not contain the inhibitor to be tested even though separate control experiments indicated that this reducing agent had minimal effect on methionine influx. [^{14}C]amino acids and [^{3}H]inulin were obtained from New England Nuclear, Boston, Mass. Most of the test amino acids were obtained from Sigma Chemical Co., St. Louis, Mo. or Cyclo Chemical Corp.

RESULTS

Characteristics of Methionine Transport

The kinetics of methionine influx were examined over a concentration range of 0.1–16 mM in a series of eight experiments at a Na concentration of 140 mM. Results are shown in Fig. 1 in terms of a plot of influx, J^i, vs. J^i/[Met]. They can be described in terms of a single transport system conforming to Michaelis-Menten-type kinetics as indicated by Eq. 1. The maximal flux,

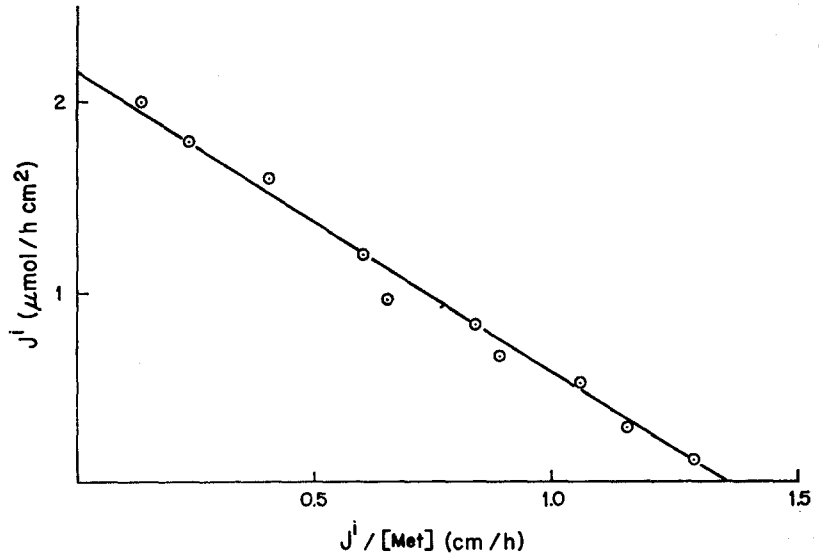

FIGURE 1. Kinetics of methionine influx across the brush border. Each point is the average of at least five determinations at methionine concentrations ranging from 0.1 to 16 mM. The line was determined by least squares.

$J^{i\max}$, given by the Y intercept of the line, is 2.2 μmol/h cm² and K_t, given by the slope, is 1.6 mM.

Two additional sets of kinetic experiments were carried out at different times using methionine concentrations in the range 2–20 mM and different Na concentrations with results summarized in Table I. These data indicate that $J^{i\max}$ does not vary significantly with Na concentration, a result obtained previously for several other amino acids (17, 19, 21). The results also provide estimates of K_1 and K_2 for methionine from Eq. 3; the values obtained are K_1 = 9.1 mM, K_2 = 23 mM from the first series and K_1 = 9.1 mM, K_2 = 25 mM from the second series. These estimates of K_2 agree well with those obtained previously for other neutral amino acids (17, 19, 21), suggesting again that this quantity does not depend very strongly on the particular amino acid. The estimate of K_1 obtained by direct measurement of methionine influx is in reasonable agreement with the value of 8.4 mM determined by Hajjar and Curran (19) from inhibition of phenylalanine influx by methionine. On the basis of this series of experiments, we have taken K_t of methionine as 1.4 mM at 140 mM Na and have used this value in Eq. 2 to estimate K_I for other compounds.

TABLE I
CHARACTERISTICS OF METHIONINE INFLUX*

[Na]	n	$J^{i\max}$	K_t
mM		*μmol/h cm²*	*mM*
140	4	3.0±0.8	1.3±0.4
0	5	2.9±0.6	9.1±2.3
140	3	4.0±0.9	1.4±0.1
47	5	4.9±0.8	3.3±0.6

* n = number of experiments. Errors are given as ±1 SEM.

In most cases, results will be expressed as the apparent affinity of the various amino acids for the transport site. This quantity is given by $1/K_1$ with units of M^{-1}. K_1 has been calculated from Eq. 3 using the observed value of K_I in place of K_t for each amino acid and assuming that K_2 = 25 mM for all amino acids tested. These values reported are referred to as apparent affinities because they are based on a particular model of the transport system that involves certain assumptions (see Discussion). Since with K_2 = 25 mM, K_1 = 6.6 K_I, or affinity = $1/K_1 = 0.152/K_I$, calculated values of affinity can be easily converted to "apparent inhibitory constants" for the various amino acids.

The calculated apparent affinities for the transport system of several methionine derivatives are shown in Fig. 2. D-methionine has an apparent affinity approximately 50-fold lower than L-methionine indicating very substantial preference for the L-isomer. Substitution of a $-CH_3$ group for the

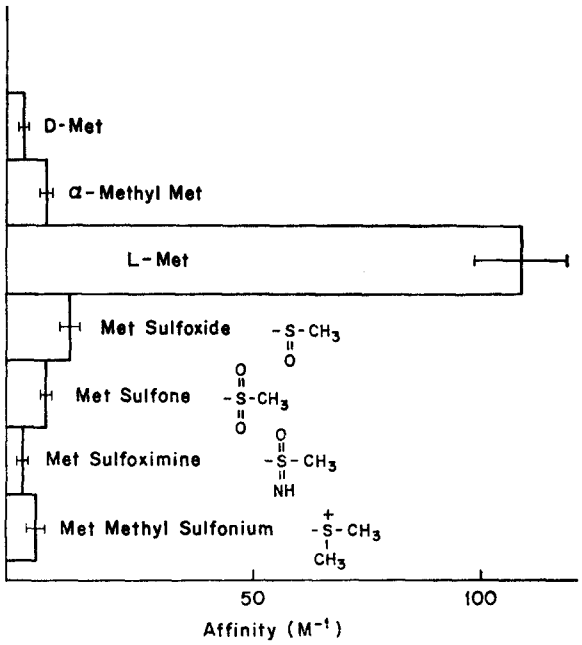

FIGURE 2. Apparent affinity of methionine and its derivatives for the transport system.

α-H atom in L-methionine causes a 10-fold decrease in affinity. Introduction of a positive charge or a relatively hydrophilic groups such as =0 and —NH into the side chain of methionine also causes a substantial decrease in apparent affinity for the transport site.

The results shown in Table II provide a further indication that amino acids with charged groups in the side chain are very poor inhibitors of methionine influx and have apparent affinities $\frac{1}{25}$–$\frac{1}{70}$ that of methionine. Within the range tested, there appears to be little effect of the position in the side chain of the charged group. This impression was confirmed in two experiments in which the effects on methionine influx of 40 mM 2,4-diaminobutyric acid, ornithine, and lysine were compared on tissue from the same animal. In 12 measurements, with each amino acid, the percent inhibitions observed were 32 ± 4 for 2,4-diaminobutyric acid, 32 ± 3 for ornithine, and 31 ± 5 for lysine. The effect of charge in the side chain is also illustrated by the fact that the γ-methyl and γ-ethyl esters of glutamic acid have a 10-fold higher affinity than glutamic acid itself. The significant increase in apparent affinity observed with the esters appears to indicate that they are not easily hydrolyzed to the acid by the tissue. In fact, their affinities are quite close to that observed for methionine sulfoxide which contains a protruding oxygen atom, on the sulfur rather than a carbon atom, in an essentially equivalent position in the side chain.

In this series of experiments, we also examined the requirement of an α-amino group for interaction with the transport site. As shown in Table III, if the amino group of amino-*n*-butyric acid is located on the β or γ carbon, affinity is reduced 20- to 30-fold compared to α-amino-*n*-butyric acid. This observation agrees reasonably well with the finding of Hajjar and Curran (19) that removal of the α-amino group from phenylalanine led to approximately a 50-fold decrease in affinity. They found an apparent affinity of 1.2 M^{-1} for phenylpropionic acid and 0.8 M^{-1} for phenyllactic acid compared with 56 M^{-1} for phenylalanine.

In an effort to obtain some insight into the possible reasons for the apparently extraordinarily high affinity of methionine for the transport system (19), we examined the behavior of norleucine which is essentially an analog of methionine with a $-CH_2-$ in place of the $-S-$ atom. As shown in Fig. 3, norleucine also had a high affinity for the system. Its apparent affinity was slightly lower than that of methionine but not actually significantly different. Since the affinity of norleucine was nearly three times higher than that estimated by Curran et al. (17) for leucine (32 M^{-1}) by direct measurement

TABLE II

EFFECT OF SIDE CHAIN CHARGE ON APPARENT AFFINITY

Amino acid	Side chain	Apparent affinity
		M^{-1}
Cysteic acid	$-CH_2SO_3^-$	3.3±0.4
Homocysteic acid	$-CH_2CH_2SO_3^-$	3.0±0.6
Aspartic acid	$-CH_2COO^-$	1.0±0.4
Glutamic acid	$-CH_2CH_2COO^-$	1.4±0.2
α-Aminoadipic acid	$-(CH_2)_3COO^-$	3.1±0.5
α-Aminopimelic acid	$-(CH_2)_4COO^-$	3.9±0.5
Glutamic acid γ-methyl ester	$-CH_2CH_2COOCH_3$	11.8±2.0
Glutamic acid γ-ethyl ester	$-CH_2CH_2COOCH_2CH_3$	11.7±2.0
2,3 Diaminopropionic acid	$-CH_2NH_3^+$	4.5±0.6
2,4 Diaminobutyric acid	$-CH_2CH_2NH_3^+$	2.7±0.3
Ornithine	$-CH_2CH_2CH_2NH_3^+$	3.0±0.3
Lysine	$-(CH_2)_4NH_3^+$	2.6±0.3
Arginine	$-(CH_2)_3NHC(NH)NH_3^+$	3.7±0.6

TABLE III

EFFECT OF THE α-AMINO GROUP ON APPARENT AFFINITY

Amino acid		Apparent affinity
		M^{-1}
α-Amino-*n*-butyric acid	$^-OOCCH(NH_3^+)CH_2CH_3$	28.0±3.2
β-Amino-*n*-butyric acid	$^-OOCCH_2CH(NH_3^+)CH_3$	0.9±0.2
γ-Amino-*n*-butyric acid	$^-OOCCH_2CH_2CH_2NH_3^+$	1.4±0.5

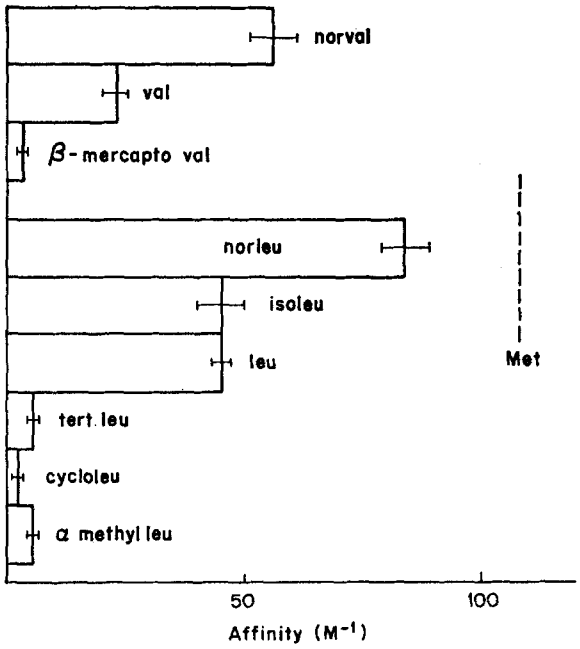

FIGURE 3. Apparent affinities of leucine and valine and their derivatives for the trans port system. The apparent affinity of methionine is shown for comparison by the dashed line.

of leucine influx, we then explored the behavior of several derivatives of leucine and valine. Results are summarized in Fig. 3. It is clear that the amino acids with straight side chains, norleucine and norvaline, have affinities 2–2.5 times greater than the corresponding compounds with branched side chains, leucine, isoleucine, and valine. These results immediately suggest that the behavior of methionine may not be anomalous. It appeared to be so in the studies of Hajjar and Curran (19) because it was the only amino acid with a straight side chain that was studied. Similar results have been obtained in studies of glycine (22, 23) and alanine (23) transport in Ehrlich ascites cells. In these cases also, amino acids with straight side chains were found to be more effective inhibitors of glycine and alanine influx than those with branched or ring side chains. The results shown in Fig. 3 also confirm the indication in Fig. 2 that substitution at the α-H position has a significant effect since both α-methylleucine and cycloleucine display very low apparent affinities for the transport site. A further indication of this effect is the observation that α-aminoisobutyric acid was found to have an extremely low affinity of 0.2 M^{-1}. In addition, extensive branching on the β-carbon atom, as exemplified by tertiary leucine and β-mercaptovaline, causes approximately a 10-fold decrease in affinity.

The results obtained for norvaline and norleucine prompted us to carry

out a more extensive study of amino acids with straight side chains, particularly those containing a sulfur atom. Three groups of amino acids were studied: (*a*) those with only carbon atoms in the side chain, (*b*) those with a sulfur atom in the γ position, and (*c*) those with a sulfur atom in the δ position. Some results are summarized in Table IV. In each series there is a progressive increase in apparent affinity with the number of methylene groups in the side chain. The presence of a sulfur atom at the δ position appears to be equivalent to a carbon atom since homocysteine and norvaline have essentially the same affinities, as do methionine and norleucine. The situation with respect to the γ sulfur atom appears to be somewhat different since

TABLE IV
APPARENT AFFINITY OF AMINO ACIDS WITH STRAIGHT SIDE CHAINS

Amino acid	Side chain	Apparent affinity
		M^{-1}
Glycine*	—H	8±1
Alanine*	—CH_3	14±2
α-Amino-*n*-butyric acid	—CH_2CH_3	28±3
Allylglycine	—$CH_2CH{=}CH_2$	39±3
Norvaline	—$CH_2CH_2CH_3$	56±5
Norleucine	—$CH_2CH_2CH_2CH_3$	84±5
Homocysteine	—CH_2CH_2SH	61±4
Methionine	—$CH_2CH_2SCH_3$	108±18
Ethionine	—$CH_2CH_2SCH_2CH_3$	143±17
Cysteine	—CH_2SH	19±2
S-methylcysteine	—CH_2SCH_3	39±2
S-ethylcysteine	—$CH_2SCH_2CH_3$	53±6
S-allylcysteine	—$CH_2SCH_2CH{=}CH_2$	77±6
S-butylcysteine	—$CH_2S(CH_2)_3CH_3$	114±6

* Affinities for glycine and alanine are those obtained by Peterson et al. (21) and Curran et al. (17) from direct measurement of influx.

cysteine, *S*-methylcysteine and *S*-ethylcysteine have lower affinities than the corresponding amino acids having a carbon atom in the γ position, α-amino-*n*-butyric acid, norvaline, and norleucine. This difference was confirmed for the pairs *S*-methylcysteine/norvaline and *S*-ethylcysteine/norleucine by carrying out experiments in which effects of both amino acids were examined on tissue from the same animal. A double bond in the side chain appears to have some effect since the affinity of allylglycine for the transport system is approximately 30% lower than that of norvaline, the comparable compound without a double bond.

Introduction of an oxygen atom into the side chain also reduced affinity since the affinity of *O*-methylserine is 30 ± 2 M^{-1}, significantly less than that of *S*-methylcysteine (38 ± 2 M^{-1}) or norvaline (56 ± 5 M^{-1}) but approxi-

mately equal to that of α-amino-*n*-butyric acid (28 ± 3 M^{-1}). In an effort to examine further possible effects of an oxygen atom (or more properly an OH group), we examined a series of amino acids with side chains containing a terminal OH with the rather interesting results shown in Fig. 4. The compound with an OH in the δ position, α-amino-δ-hydroxyvaleric acid (AHVA) has a much higher apparent affinity for the transport system than other members of the series. To check this point further, we carried out experiments in which the effects of equal concentrations homoserine, AHVA, and α-amino-ε-hydroxycaproic acid were examined in tissue from the same animal. The results are summarized in Fig. 5 in terms of methionine influx under control conditions and in the presence of 30 mM concentrations of the *L*-isomer of the three hydroxy compounds. The experiments were carried out in the presence and absence of Na. The inhibition caused by AHVA is

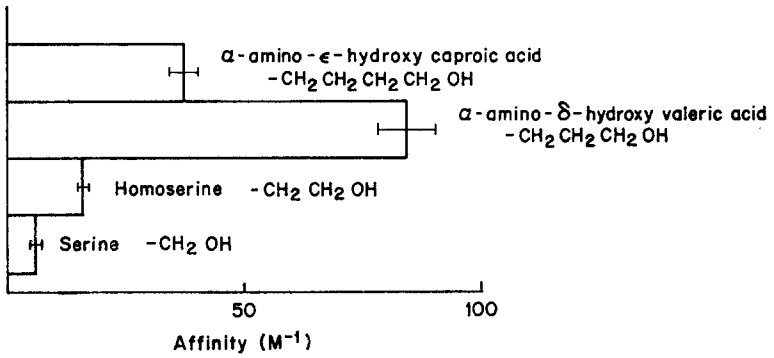

FIGURE 4. Apparent affinities of ω—OH amino acids for the transport system.

significantly greater than that caused by the other hydroxy-amino acids, confirming the observations shown in Fig. 4. Threonine, with an hydroxyl on the β-carbon has an apparent affinity of 6.1 ± 0.4 M^{-1}, the same as that of serine while homoserine, which is the straight chain analog of threonine has an affinity of 15.4 ± 1.1 M^{-1}. Thus the effect of branching in this case seems quite similar to that observed for branching in norleucine derivatives.

A possible explanation of some of the observed effects of variation in apparent affinity with the nature of the side chain could involve changes in the Na sensitivity of the interaction between amino acid and transport site. For example, the high relative affinity of AHVA or the higher affinity of norleucine compared to leucine could reside in a higher affinity for Na ion of the amino acid-transport site complex (17). Although the results obtained with methionine itself (a "normal" Na affinity as indicated by a calculated K_2 of 25 mM) argue against such an explanation, further tests seemed worthwhile particularly in view of Christensen's suggestion that a properly positioned OH group enhanced the Na affinity of the ASC amino acid transport

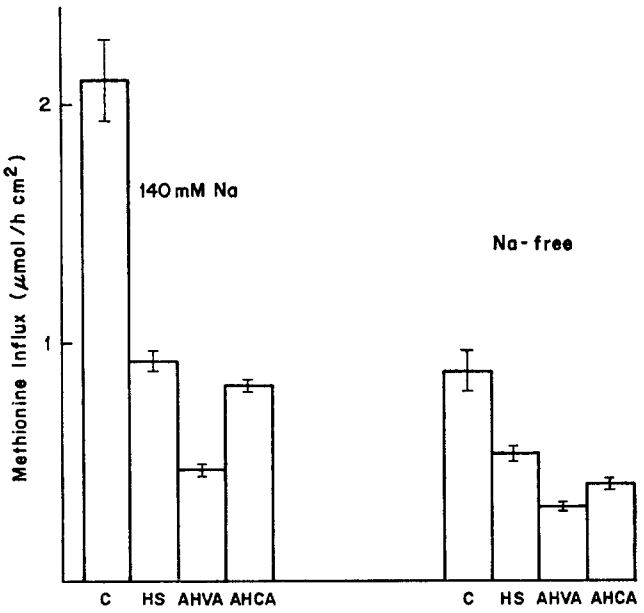

FIGURE 5. Effect of ω—OH amino acids (40 mM) on methionine influx; C, control; HS, homoserine; AHVA, α-amino-δ-hydroxyvaleric acid; AHCA, α-amino-ε-hydroxycaproic acid.

system in Ehrlich ascites cells and certain red blood cells (24, 25). We therefor examined the inhibitory effects on methionine influx of α-amino-*n*-butyric acid, norleucine, and AHVA at different Na concentrations. The results are summarized in Table V in terms of K_I. The observed values agree reasonably well with those calculated assuming that K_I is given by Eq. 3 and that K_2 = 25 mM, the approximate value determined for several other amino acids. These results may also be expressed in an alternative manner by actually evaluating K_2 for the three test amino acids using Eq. 3 with $K_I = K_t$; the resulting values are 32 mM for α-amino-*n*-butyric acid, 28 mM for norleucine, and 26 mM for AHVA. Thus, the behavior of these compounds does not appear to involve any major changes in Na sensitivity of the transport system.

Another explanation for some of the observed affinity patterns might be the presence of multiple transport systems for various neutral amino acids in rabbit intestine. For example, if phenylalanine and methionine were transported by different systems there would be no need to ask why their apparent affinities are so different. We have therefore carried out some additional experiments to examine this point by studying the ability of various amino acids to inhibit the influxes of each other and compared the calculated K_I values with values of K_t obtained by direct measurement of influx. Results are summarized in Table VI; some results of prior studies in

this laboratory are also included. In most cases, the agreement between K_t and K_I is reasonable and these results do not pose a compelling argument for postulating more than one transport system for these neutral amino acids.

Another approach to this problem was suggested by the analysis of Inui and Christensen (26). If two compounds share a single transport system, a plot of $1/1\text{-}R$ (where $R = J_I/J_0$) vs. the reciprocal of the inhibitor concentration should yield a straight line with an intercept of 1.0. Fig. 6 shows such plots for methionine as an inhibitor of phenylalanine influx and vice versa. The results indicate that each amino acid is capable of completely inhibiting the influx of the other, an observation that is consistent with, but does not

TABLE V

EFFECT OF SODIUM CONCENTRATION ON K_I

Amino acid	[Na]	K_I observed	K_I calculated
α-Amino-*n*-butyric acid	140	5.4±0.6	—
	70	8.9±1.4	9.4
	35	12.3±1.1	14.9
	0	37.3±8.1	35.7
Norleucine	140	1.8±0.1	—
	35	4.4±0.5	5.0
	0	12.7±1.4	12.0
α-Amino-δ-hydroxy valeric acid	140	1.8±0.2	—
	35	5.0±0.5	5.0
	0	11.0±1.0	12.0

TABLE VI

COMPARISON OF K_t AND K_I

Test amino acid	K_t	K_I*
	mM	*mM*
Alanine	9.1±1.0	16.0 (methionine)
		12.7 (phenylalanine)
Valine	5.0±1.0	6.8 (methionine)
		3.9 (alanine)
Leucine	4.2±0.9	3.4 (methionine)
		3.9 (alanine)
Methionine	1.4±0.2	1.2 (alanine)
		2.7 (leucine)
		1.3 (phenylalanine)
Phenylalanine	2.7±0.7	3.3 (alanine)
		3.5 (methionine)
	17.6±1.2‡	14.5‡ (methionine)

* K_I is determined from the ability of the test amino acid to inhibit the influx of the amino acid given in parentheses.

‡ Determined in Na-free solution. All other values were determined at a Na concentration of 140 mM.

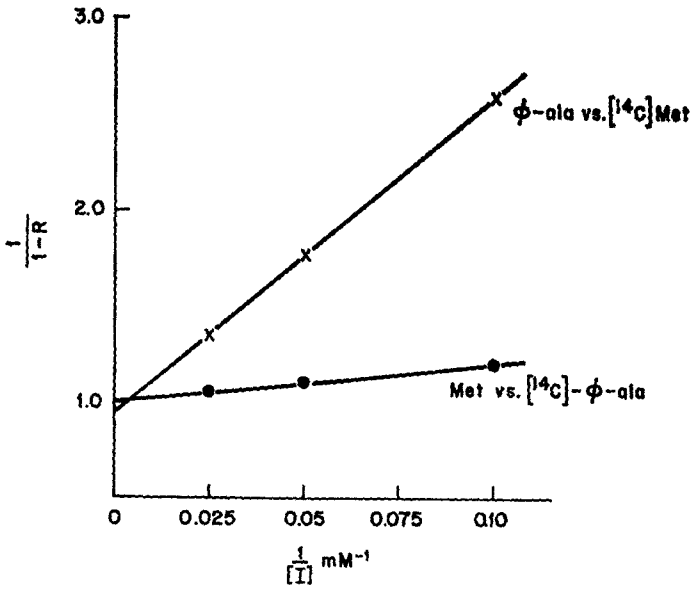

FIGURE 6. Inhibition of methionine influx by phenylalanine and phenylalanine influx by methionine as a function of the reciprocal of concentration of the inhibitor. $R = J_I^i/J_o^i$. Each point is the average of six flux determinations.

prove, that these two amino acids utilize a single transport system in rabbit ileum.

DISCUSSION

The present results should give additional insight into the nature of the transport site for neutral amino acids in the brush border membrane of rabbit ileum providing they are not complicated by the presence of multiple transport systems (27) or by the influence of unstirred layers (28). We must, therefore, begin by considering these possibilities. The data presented in Fig. 1 indicate a single transport system for methionine. Alternatively, if there are two or more transport systems, they must have very similar kinetic properties since the form of plot used (J vs. J/C) is particularly sensitive to the presence of more than one system. The results presented in Table VI and Fig. 6 also indicate that this transport system is shared by other neutral amino acids. This conclusion does not appear to agree with the report of Schultz and Markscheid-Kaspi (27) on the ability of alanine and phenylalanine to inhibit the influxes of each other across the brush border of rabbit ileum. They found in each case that K_I was twice as large as K_t and suggested that this discrepancy could occur if the inhibitory amino acid could bind to the transport site of the other amino acid but could not be translocated across the membrane. They thus proposed separate transport systems for these two amino acids. The data in Table VI indicate that there are differences between K_I

and K_t in our hands also and the differences may be as large as a factor of 2. However, the pattern is not sufficiently consistent to permit a firm conclusion regarding the importance of multiple transport systems for neutral amino acids in the tissue. (The results of Peterson et al. [21] indicate the presence in rabbit ileum of a transport system for imino acids that is also shared by glycine, but the data in Fig. 1 show that this system does not play a role in methionine influx.) In addition, all the results reported here are based on the ability of various amino acids to inhibit methionine influx. They, therefore, provide an estimate of the interaction of these compounds with the presumptive single transport system responsible for methionine influx (Fig. 1) and can be interpreted in terms of apparent affinities for this transport site.

Questions have recently been raised regarding possible influence of unstirred layers on estimates of "apparent Michaelis constants" in studies of intestinal transport processes (28, 29). Since the present work depends on the validity of these estimates, at least in relative terms, consideration of the point seems relevant. The data in Fig. 1 provide some information about possible influence of unstirred layers in our experimental system. According to Winne (29, Eq. 4) influx across the brush border should be given by the following

$$J^i = \frac{D}{\delta}[0.5(K_t + C_o + J^m\delta/D) - (0.25(K_t - C_o + J^m\delta/D)^2 + C_oK_t)^{1/2}], \quad (4)$$

in which D is the diffusion coefficient of the substrate in an unstirred layer of effective thickness δ and C_o is concentration of substrate in the stirred bulk phase. We have assumed that K_t = 1.5 mM and J^m = 4 μmol/h cm² and used Eq. 4 to calculate J^i over a concentration range of 0.1–20 mM for several values of D/δ. Results are shown in Fig. 7. Clearly, a marked downward curvature of the line relating J^i to J^i/C_o occurs for D/δ < 10 cm/h. Similar though less striking curvature is found if J^m is taken as 2 μmol/h cm². In either case, the data in Fig. 1 appear to indicate that in our system D/δ must be greater than 4 cm/h and is probably near 10 cm/h.[3] If D is taken as 9.6 × 10^{-6} cm²/s (the value given by Longsworth [30] for norleucine corrected to 37° C), δ would be less than 85 μm and probably near 35 μm.

Several other items of information are consistent with this conclusion. First, Frizzell and Schultz (31) have found that in the flux system used here, the inulin "space" is constant after 12 s of exposure of the mucosal surface to bathing solution. This observation indicates that inulin has essentially equilibrated through the unstirred layer in 12 s or less (see also Sallee et al. [32])

[3] It is perhaps appropriate to ask whether a combination of two transport systems plus an unstirred layer effect could result in a linear relationship between J^i and J^i/[Met] as shown in Fig. 1. Inspection of the equations describing such a situation, which are cubic in J^I suggest that such an effect is quite unlikely, and would certainly require an extremely fortuitous combination of transport parameters and unstirred layer thickness.

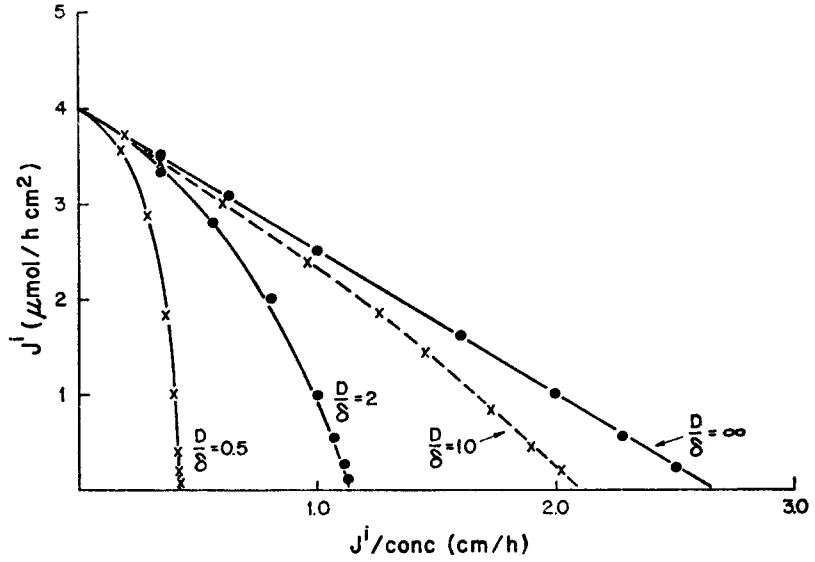

FIGURE 7. Influence of an unstirred layer on the kinetics of amino acid influx expressed in terms of influx vs. influx/external concentration. Fluxes were calculated from equation 4 using J^m = 4 μmol/h cm^2 and K_t = 1.5 mM. Values of D/δ are expressed in centimeters per hour.

and hence the half-time ($\tau_{1/2}$) for equilibration must be 1–2 s. For such a system, $\tau_{1/2}$ is given by (33, 34)[4]

$$\tau_{1/2} = 0.38\ \delta^2/D. \qquad (5)$$

Since the diffusion coefficient of inulin is 2.5 × 10^{-6} cm^2/s (35), $\tau_{1/2}$ of 1–2 s would yield values of δ of 25–35 μm, consistent with D/δ = 10 cm/h for an amino acid like norleucine (see above). Second, Rose and Schultz (36) have found that the change in potential difference across the brush border membrane caused by adding glucose to the mucosal solution is complete in approximately 4–5 s in a system less well stirred than the one used to measure influx. The half-time for response is, therefore, the order of 1 s and since at 37° C the diffusion coefficient of glucose is 9.0 × 10^{-6} cm^2/s, (35), Eq. 5 would yield δ = 48 μm.

Finally, by means of successive approximations, Eq. 4 can be used to calculate the results of a hypothetical experiment in which the influx of a test amino acid with K_t = 1.5 and J^m = 4 is inhibited by another amino acid with K_I = 1.5 taking account of the influence of the unstirred layer on both amino acids. The calculation was made assuming that the inhibitor was

[4] This equation provides only a rough approximation for effective thickness of the unstirred layer because it applies strictly only when the membrane surface is a flat sheet. It is used because no rigorous formal treatment exists for a villous surface.

present at 5, 10, and 20 mM and that experiments were carried out at three concentrations of test amino acid, 0.5, 2, and 3 mM. For relatively thick unstirred layers the calculated value of K_I depends rather strongly on the concentration of test amino acid. If $D/\delta = 4$ cm/h, the ratios of the calculated K_I's at 0.5:2:3 mM test amino acid are 1.00:0.77:0.73, while $D/\delta =$ 10 cm/h, the ratios are 1.00:0.91:0.87. To check this point, we carried out experiments at 0.5, 2, and 3 mM methionine using ethionine as inhibitor since this situation conforms fairly well to that assumed for the above calculation (see Table IV). The observed ratios of K_I for ethionine were 1.00: 0.98:1.08. These results also agree with the conclusion that $D/\delta > 4$ cm/h for our experimental conditions.

As a final point, we can make similar calculations to provide an estimate of the effect of an unstirred layer under the usual conditions of a methionine concentration of 2 mM and inhibitor concentrations of 5, 10, and 20 mM. Table VII summarizes the results of these calculations by comparing the assumed "true" with the "calculated" K_I obtained from a hypothetical experiment. For $4 < D/\delta < 10$, the maximum error in estimating K_I will be the order of 10% for high affinity inhibitors and less for ones of lower affinity. Estimates of the relative K_I's for various inhibitors should thus be reasonably accurate, and we have made no corrections for effects of unstirred layers.

In view of these considerations, the present data should provide reasonable estimates of the apparent affinities of a variety of amino acids for the site involved in methionine transport and apparently the transport of other neutral amino acids. Although results are expressed in terms of affinities, it is important to stress again that they are apparent affinities evaluated from a

TABLE VII

EFFECT OF UNSTIRRED LAYERS ON K_I

D/δ	K_I		%Δ*
	True	Calculated	
cm/h	*mM*		
2	1.50	2.14	42.7
	5.00	6.41	28.2
	10.00	11.54	15.4
4	1.50	1.76	17.3
	5.00	5.56	11.2
	10.00	10.70	7.0
10	1.50	1.60	6.7
	5.00	5.24	4.8
	10.00	10.30	3.0

* $\%\Delta = \dfrac{K_{I(\text{calc})} - K_{I(\text{true})}}{K_{I(\text{true})}} \times 100$

particular model of the transport system. One major feature of this model is the assumption that the rate-limiting step in transport is translocation across the membrane so that the reaction between amino acid and transport site at the membrane surface is essentially at equilibrium. If this assumption is incorrect, the calculated affinities will obviously be in error although they should still be related to the true affinities.

Previous data (17, 19, 21), as well as some reported here (Table V), suggest that variations in affinity of the neutral amino acids for the transport site probably cannot be ascribed to markedly different sensitivities to Na. Thus it seems appropriate to consider other explanations for the observed differences in terms of characteristics of the interaction between site and amino acid. The data in Table III confirm the importance of the α-amino group for binding since displacement of this group to the β or γ position in aminobutyric acid leads to a substantial decrease in affinity. Identical results were obtained previously by Daniels et al. (37) in studies on inhibition of methionine transport by rat intestine and a similar conclusion was reached by Hajjar and Curran (19) by examining behavior of analogs of phenylalanine that lacked an amino group. Hajjar and Curran also found that the portion of this transport site interacting with the α-amino group must be quite restricted in size since *N*-methyl phenylalanine had a very low apparent affinity. The results shown in Figs. 2 and 3 suggest a similar conclusion for the position occupied by the α-hydrogen atom since α-methylmethionine, α-methylleucine, and cycloleucine have apparent affinities 12- to 15-fold lower than the corresponding parent compounds and α-aminoisobutyric acid displays a very low affinity. Daniels et al. (37) obtained similar results for α-aminoisobutyric acid in rat intestine.

The results also show clearly (Fig. 2 and Table II) that introduction of a charged group into the side chain of an amino acid causes a very substantial decrease in apparent affinity for the transport site. The effect is essentially independent of the position of the charge on the side chain and of whether the charge is positive or negative. This behavior suggests that the interaction between the side chain and the transport site is mainly hydrophobic in nature. The data in Table IV also support such a conclusion since they indicate a progressive increase in apparent affinity with the number of methylene groups in the side chain. Similar results have been obtained in a number of previous studies of amino acid transport systems (23, 37–39). For example, Oxender and Christensen (23) found that the ability of other amino acids to inhibit leucine influx into Ehrlich ascites cells increased progressively with length of the side chain. Our results also indicate that a sulfur atom in the side chain is essentially equivalent to a $-CH_2-$ group, a concept in agreement with the calculations of Nemethy and Scheraga (40) on hydrophobic interactions of amino acid side chains. This behavior is illustrated more

clearly in Fig. 8 in which ln affinity is plotted against the number of carbon and sulfur atoms in the side chain. The compounds without a sulfur atom, (with the exception of allylglycine), or with sulfur in the δ position fall close to a straight line. The first three members of the series with sulfur in the γ position describe a line of similar slope displaced to the right by 0.4–0.5 ln units. The higher members of this series, *S*-allylcysteine and *S*-butylcysteine, clearly fall well below the lines described by the amino acids with straight side chains as do compounds with ring side chains, phenylalanine, and *S*-benzylcysteine. The slopes of the lines in Fig. 8 indicate that addition of a

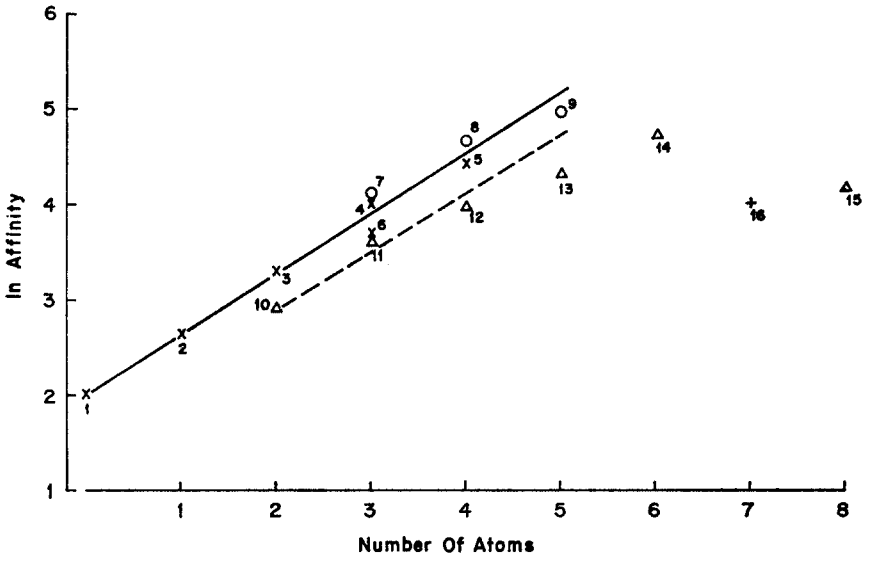

FIGURE 8. Relationship between the natural logarithm of the apparent affinity and the number of carbon and sulfur atoms in the side chain. 1, glycine; 2, alanine; 3, α-amino-*n*-butyric acid; 4, norvaline; 5, norleucine; 6, allyglycine; 7, homocysteine; 8, methionine; 9, ethionine; 10, cysteine; 11, *S*-methylcysteine, 12, *S*-ethylcysteine; 13, *S*-allylcysteine; 14, *S*-butylcysteine; 15, *S*-benzylcysteine; 16, phenylalanine.

methylene group to the side chain increases the free energy of binding by approximately 370 cal/mol.

Since the present data suggest that the interaction of the side chain of an amino acid with the transport site is mainly hydrophobic in nature, it would be of interest to examine the relationship between the apparent affinity and some specific measure of hydrophobic character such as partition between an aqueous and a nonaqueous phase. Unfortunately, direct measurements on amino acids are quite limited according to the extensive data compiled by Leo et al. (41). However, their careful study makes it possible to calculate with reasonable accuracy the water/octanol partition coefficients (P) for the compounds studied here. To do this, we have taken the value for alanine of

log P_{ala} = −2.94 given by Leo et al. (41, Table XVII)[5] and have used the information in their Tables XI and XVI for contributions of the various groups making up the side chains of the amino acids studied. The results obtained for side chains containing only carbon atoms are shown in Fig. 9. There is clearly a good linear relationship between log $(1/K_1)$ and log P that can be described by the expression

$$\log (1/K_1) = 0.54\ (\pm\ 0.03)\ \log P + 2.65\ (\pm 0.06).$$

The correlation coefficient is 0.996. It is of interest to note that the point for allylglycine falls on the line in this case but not in Fig. 8.

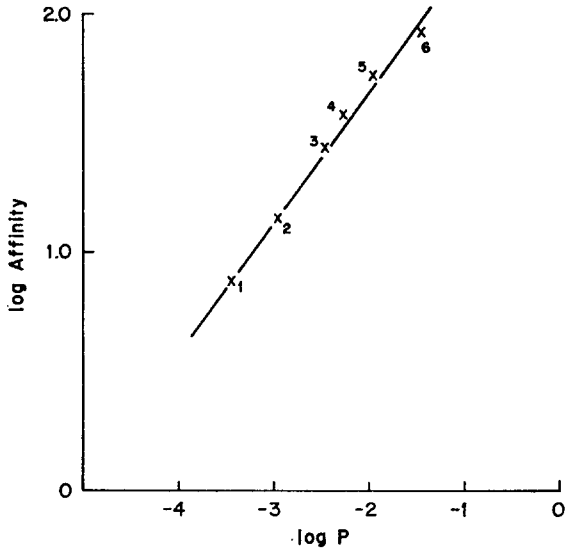

FIGURE 9. Relationship between log affinity and log of the calculated octanol/water partition coefficient P. 1, glycine; 2, alanine; 3, α-amino-*n*-butyric acid; 4, allylglycine; 5, norvaline; 6, norleucine. The line was determined by least squares.

A number of other compounds we have tested are shown in the same manner in Fig. 10. Various groups of amino acids describe a series of lines parallel to the line in Fig. 9 but displaced from it. The derivatives of cysteine with a sulfur in the γ position (including *S*-allylcysteine) lie on a line approximately 0.2 log units to the left of the line in Fig. 9. Thus, the sulfur atom confers a slight enhancement of affinity that cannot be accounted for on the basis of the calculated partition coefficient. *S*-butylcysteine has an apparent affinity substantially below that expected on the basis of these considerations. This effect could be due to the fact that the binding site cannot easily accom-

[5] This value was measured directly by Church and Hansch and is cited by Leo et al. (41) as an unpublished observation.

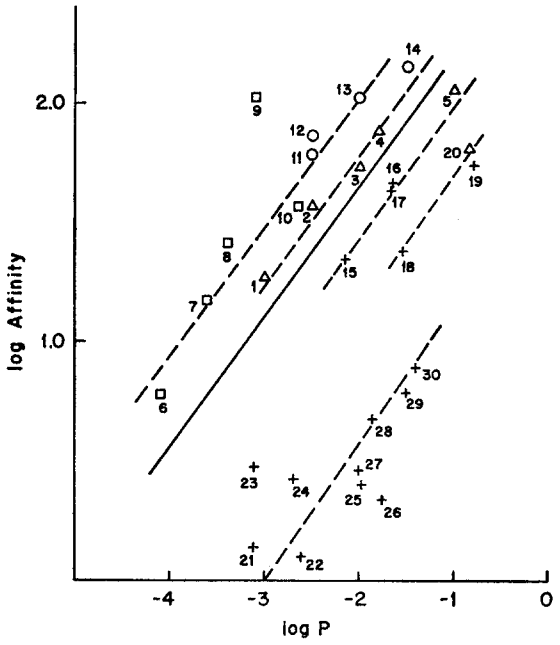

FIGURE 10. Relationship between log affinity and log of the calculated octanol/water partition coefficient. The solid line is that shown in Fig. 9 for amino acids with straight side chains containing only carbon atoms. 1, cysteine; 2, *S*-methylcysteine; 3, *S*-ethylcystine; 4, *S*-allylcysteine; 5, *S*-butylcysteine; 6, serine; 7, homoserine; 8, *O*-methylserine; 9, α-amino-δ-hydroxyvaleric acid; 10, α-amino-ε-hydroxycaproic acid; 11, homocysteine; 12, trifluoromethionine; 13, methionine; 14, ethionine; 15, valine; 16, leucine; 17, isoleucine; 18, β-2-thienylalanine; 19, phenylalanine; 20, *S*-benzylcysteine; 21, glutamic acid; 22, aspartic acid; 23, ornithine; 24, lysine; 25, β-phenylserine; 26, cycloleucine; 27, D-methionine; 28, tertiary leucine; 29, methionine methylsulfonium chloride; 30, α-methylleucine.

modate a side chain in this size or that folding of the chain prevents full interaction with the site.

Another line is described by compounds having a sulfur atom in the γ position in the side chain and those containing a terminal OH (with the exception of AHVA whose unexpectedly high affinity has been noted above). This line lies approximately 0.6 log units to the left of the one described by amino acids whose side chains contain only carbon. The reason for the significant increase in affinity caused by shifting the sulfur atom from the γ to the δ position (which is also apparent in Fig. 8) is not entirely clear. However, inspection of space-filling models of these amino acids suggests a possible explanation. In the γ position, the relatively large sulfur atom may lie very close to either the α-H or the α-amino group. Since previous (19) and present results indicate that the transport site must have quite limited space to accommodate these two groups, some steric hindrance at these

positions as a result of the proximity of the *S* atom may lead to a small but significant decrease in affinity.

Valine, leucine, and isoleucine, with branched side chains, are displaced to the right of the "control" line by 0.4 log units. This displacement is approximately equivalent to removal of one methylene group, 0.5 log units (39), and the effect suggests that branching of the side chain in these amino acids effectively prevents one of the carbon atoms from interacting with the hydrophobic region of the transport site. The behavior of the three amino acids with rings in the side chain is similar. They lie on a line displaced by 1 log unit from the control line and thus behave as though only four of the five or six carbon atoms in the ring interact with the site. These effects suggest a shallow hydrophobic region in the transport site that cannot fully accommodate either branched or ring side chains. Alternatively the relatively bulky ring side chains may cause conformational changes in the transport site that lead to a reduction in affinity. The slope of all these lines, approximately 0.5, is consistent with the concept of a shallow region. According to Leo et al. (41), a slope of less than unity in such a plot might indicate a surface interaction in which the side chain is not completely engulfed into a hydrophobic region. Such behavior might be expected for a transport site or "carrier" in which interaction presumably occurs at the interface between membrane and solution.

Finally, several amino acids with very low apparent affinities scatter around a line of similar slope displaced by approximately 2 log units from the control line. These include compounds with charged groups in the side chain and with substitutions on the α hydrogen. Although these chains must interact very poorly with the hydrophobic region, there is still some tendency for a relationship between affinity and calculated partion coefficient.

Several aspects of these results are similar to those obtained with various enzymes. For example, Anderson et al. (1) examined the ability of a series of *N*-alkylnicotinamide chlorides to inhibit yeast alcohol dehydrogenase and found, for the series C_4-C_{12}, that log $1/K_1$ was a linear function of the number of carbon atoms and that the slope of the line indicated a contribution to binding of 370 cal/mol/—CH_2— group. Schrimmer et al. (8) obtained similar results in studies on inhibition of L-cysteine sulfoxide lyase by *S*-alkyl-L-cysteines; the increment in free energy of binding per —CH_2— group was 403 cal/mol. Berezin et al. (2) have observed a close relationship between the ability of a variety of compounds to inhibit chymotrypsin and their octanol/water partition coefficients. However, for a series of alkyl alcohols, they found that each —CH_2— group contributed 700 cal/mol to the free energy of binding. Schrier and Scheraga (42), in studies of the effect of alcohols on the thermal transition of ribonuclease, found that branched chain compounds behaved as if they were one carbon shorter suggesting involvement of a

hydrophobic region of limited shape in which the branched carbon could not fit. A similar situation appears to apply for the side chains of valine, leucine, and isoleucine in the present study.

There are, however, some aspects of the results that do not fit clearly into the pattern of hydrophobic interaction. A primary example involves those compounds with a terminal —OH in the side chain. They have relatively low apparent affinities as might be expected but, as indicated in Fig. 10 they have higher affinities than expected on the basis of calculated partition coefficients. Actually, the same is true for amino acids containing sulfur in the side chain. One possibility is that the calculated *P*'s are too low. Another possibility involves the fact that both —OH and —S— groups in the side chain shift the pK of the $-NH_3^+$ group to a value approximately 0.5 units lower than normal and also alter the pK of the COO^- group slightly (43); these effects might alter amino acid binding in the region of the $-NH_3^+$ or COO^- group.

The terminal —OH might also hydrogen bond to neighboring groups in the site which would tend to compensate to some extent for the decrease in hydrophobic interaction which is expected on the basis of partition coefficient. Alternatively, the results obtained for the hydroxy compounds could be explained if the terminal —OH group led to a configuration of the side chain that prevented one $-CH_2-$ group from interacting with the hydrophobic site. Thus serine has an apparent affinity approximately equivalent to that of glycine and homoserine is approximately equivalent to alanine. However, none of these considerations provide an adequate explanation for the anomalously high affinity observed for AHVA. The results of Christensen et al. (24, 25) indicate that an apparently related effect observed in other cell systems involves changes in Na sensitivity of the transport system. The results presented in Table V suggest that such an explanation does not apply for rabbit ileum since the Na sensitivity for AHVA appears to be normal. Thus, the enhanced affinity seems to be related specifically to the binding of the side chain to its site and until we understand the nature of this important effect we cannot fully understand the nature of this site. Finally, Schultz and Markscheid-Kaspi (27) have pointed out that variations in K_I could also arise from differences in the ability of various amino acids to the translocated across the membrane once they have interacted with the transport site. In this case, the observed patterns of apparent affinity might also involve factors related to the translocation step. Nonetheless the consistency of the patterns suggest rather strongly that they are determined mainly by the ability of the various compounds to interact with the primary amino acid binding site.

With the exception of the points noted above, the results lead to a preliminary picture of the binding site of the neutral amino acid transport system in rabbit ileum. There is a site of limited size with which the α-NH_3^+

group interacts. The mode of binding is presumably ionic but we have no direct evidence on this point. There is also a site binding the COO^- group. In this case, the binding may not be primarily ionic since the results of Frizzell and Schultz (31) suggest that binding may not be much altered by lowering the pH of the mucosal solution to 2.5. There is a site of very limited capacity occupied by the α-H atom. Finally the side chain is bound in a hydrophobic region of limited depth (since the side chain does not appear to be fully transferred to a hydrophobic environment). This region also appears to be of limited length and can apparently accommodate a straight chain of four to five carbon atoms. This conclusion is suggested by the observation that *S*-butylcysteine (with a six-atom side chain) has a lower affinity than expected, one consistent with only five of the atoms interacting with the site, and by the observation that the γ-methyl and γ-ethyl esters of glutamic acid have essentially the same affinities. The exact nature of this hydrophobic region remains unknown but the results of Hajjar and Curran (19) on para-substituted derivatives of phenylalanine indicate that it is influenced by changes in the electronic configuration of the benzene ring.

We are indebted to Miss Anne Lamont and Mrs Gracie. Jones for valuable technical assistance. This work was supported by U. S. Public Health Service Research Grants from the National Institute of Arthritis, Metabolism, and Digestive Disease (AM-12028) and the National Institute of Allergy and Infectious Diseases (AI-09277).

Received for publication 26 February 1974.

REFERENCES

1. ANDERSON, B. M., M. L. REYNOLDS, and C. D. ANDERSON. 1965. Hydrophobic interactions of inhibitors with yeast alcohol dehydrogenase. *Biochim. Biophys. Acta.* **99**:46.
2. BEREZIN, I. V., A. V. LEVASHOV, and K. MARTINEK. 1970. On the modes of interaction between competitive inhibitors and the α-chymotrypsin active centre. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **7**:20.
3. HANSCH, C., and E. COATS. 1970. α-Chymotrypsin: A case study of substituent constants and regression analysis in enzymic structure-activity relationships. *J. Pharm. Sci.* **59**:731.
4. LOMBARDINI, J. B., A. W. COULTER, and P. TALALAY. 1970. Analogues of methionine as substrates and inhibitors of methionine adenosyltransferase reaction. Deductions concerning the conformation of methionine. *Mol. Pharmacol.* **6**:481.
5. MARSHALL, T. H., and A. AKGÜN. 1971. The specificity of porcine elastase and α-chymotrypsin. Effect of fatty acid chain length in a homologous series of nitrophenyl esters. *J. Biol. Chem.* **246**:6019.
6. MEISTER, A. 1968. The specificity of glutamine synthetase and its relationship to substrate conformation at the active site. *Adv. Enzymol. Relat. Areas Mol. Biol.* **31**:183.
7. SANTI, D. V., and P. V. Danenberg. 1971. Phenylalanyl transfer ribonucleic acid synthetase from *Escherichia coli*. Analysis of the phenylalanine binding site. *Biochemistry.* **10**:4813.
8. SCHRIMMER, S., C. A. RYAN, and F. F. WONG. 1964. Specificity of L-cysteine sulfoxide lyase and partially competitive inhibition by *S*-alkyl-L-cysteines. *J. Biol. Chem.* **239**:777.
9. CHRISTENSEN, H. N. 1973. On the meaning of effects of substrate structure on biological transport. *Bioenergetics*, **4**:31.
10. BENKO, P. V., T. C. WOOD, and I. H. SEGEL. 1967. Specificity and regulation of methionine transport in filamentous fungi. *Arch. Biochem. Biophys.* **122**:783.

11. HERZBERG, G. R., H. SHEERIN, and J. LERNER. 1971. Cationic amino acid transport in chicken small intestine. *Comp. Biochem. Physiol.* **40**:229. (Abstr.).
12. LERNER, J., and D. S. MILLER. 1972. Specificity limits of L-leucine transport in chicken small intestine. *Experientia (Basel).* **28**:1312.
13. LIN, E. C. C., H. HAGIHARA, and T. H. WILSON. 1962. Specificity of the transport system for neutral amino acids in hamster intestine. *Am. J. Physiol.* **202**:919.
14. LOMBARDI, F. J., and H. R. KABACK. 1972. Mechanisms of active transport in isolated bacterial membrane vesicles. VIII. The transport of amino acids by membranes prepared from *Escherichia coli*. *J. Biol. Chem.* **247**:7844.
15. READ, C. P., A. H. ROTHMAN, and J. E. SIMMONS, JR. 1963. Studies on membrane transport, with special reference to parasite-host integration. *Ann. N. Y. Acad. Sci.* **113**:154.
16. SPENCER, R. P., J. WEINSTEIN, A. SUSSMAN, Y. M. BOW, and M. A. MARKULIS. 1962. Effect of structural analogues on intestinal accumulation of glycine. *Am. J. Physiol.* **203**: 634.
17. CURRAN, P. F., S. G. SCHULTZ, R. A. CHEZ, and R. E. FUISZ. 1967. Kinetic relations of the Na-amino acid interaction at the mucosal border of intestine. *J. Gen. Physiol.* **50**: 1261.
18. SCHULTZ, S. G., P. F. CURRAN, R. A. CHEZ, and R. E. FUISZ. 1967. Alanine and sodium fluxes across the mucosal border of rabbit ileum. *J. Gen. Physiol.* **50**:1241.
19. HAJJAR, J. J., and P. F. CURRAN. 1970. Characteristics of the amino acid transport system in the mucosal border of rabbit ileum. *J. Gen. Physiol.* **56**:673.
20. SCHULTZ, S. G., L. YU-TU, and C. K. STRECKER. 1972. Influx of neutral amino acids across the brush border of rabbit ileum. Stereospecificity and the role of the α-amino and α-carboxylate groups. *Biochim. Biophys. Acta.* **288**:367.
21. PETERSON, S. C., A. M. GOLDNER, and P. F. CURRAN. 1970. Glycine transport in rabbit ileum. *Am. J. Physiol.* **219**:1027.
22. PAINE, C. M., AND E. HEINZ. 1960. The structural specificity of the glycine transport system of Ehrlich carcinoma cells. *J. Biol. Chem.* **235**:1080.
23. OXENDER, D. L., and H. N. CHRISTENSEN. 1963. Distinct mediating systems for the transport of neutral amino acids by the Ehrlich cell. *J. Biol. Chem.* **238**:3686.
24. CHRISTENSEN, H. N., E. L. THOMAS, and M. E. HANDLOGTEN. 1969. Features of amino acid structure enhancing or obstructing cosubstrate reactivity of Na^+ in transport. *Biochim. Biophys. Acta.* **193**:228.
25. THOMAS, E. L., and H. N. CHRISTENSEN. 1971. Nature of the cosubstrate action of Na^+ and neutral amino acids in a transport system. *J. Biol. Chem.* **246**:1682.
26. INUI, Y., and H. N. CHRISTENSEN. 1966. Discrimination of single transport systems. The Na^+-sensitive transport of neutral amino acids in the Ehrlich cell. *J. Gen. Physiol.* **50**:203.
27. SCHULTZ, S. G., and L. Markscheid-Kaspi. 1971. Competitive interactions between L-alanine and L-phenylalanine in rabbit ileum. *Biochem. Biophys. Acta.* **241**:857.
28. DIETSCHY. J. M., V. L. SALLEE, and F. A. WILSON. 1971. Unstirred water layers and absorption across the intestinal mucosa. *Gastroenterology.* **61**:932.
29. WINNE, D. 1973. Unstirred layer, source of biased Michaelis constant in membrane transport. *Biochim. Biophys. Acta.* **298**:27.
30. LONGSWORTH, L. G. 1953. Diffusion measurements, at 25°, of aqueous solutions of amino acids, peptides, and sugars. *J. Am. Chem. Soc.* **75**:5705.
31. FRIZZELL, R. A., and S. G. SCHULTZ. 1970. Effects of monovalent cations on the sodium-alanine interaction in rabbit ileum. Implication of anionic groups in sodium binding. *J. Gen. Physiol.* **56**:462.
32. SALLEE, V. L., F. A. WILSON, and J. M. DIETSCHY. 1972. Determination of unidirectional uptake rates for lipid across the intestinal brush border. *J. Lipid Res.* **13**:184
33. DAINTY, J. and C. R. HOUSE. 1966. Unstirred layers in frog skin. *J. Physiol. (Lond.).* **182**: 66.
34. DIAMOND, J. M. 1966. A rapid method for determining voltage-concentration relations across membranes. *J. Physiol. (Lond.).* **183**:83.
35. LANMAN, R. C., J. A. BURTON, and L. S. SCHANKER. 1971. Diffusion coefficients of some ^{14}C-labeled saccharides of biological interest. *Life Sci.* **10**:803.

36. ROSE, R. C., and S. G. SCHULTZ. 1971. Studies on the electrical potential profile across rabbit ileum. Effects of sugars and amino acids on transmural and transmucosal electrical potential differences. *J. Gen. Physiol.* **57**:639.
37. DANIELS, V. G., A. G. DAWSON, H. NEWEY, and D. H. SMYTH. 1969. Effect of carbon chain length and amino group position on neutral amino acid transport systems in rat small intestine. *Biochem. Biophys. Acta.* **173**:575.
38. JACQUEZ, J. A. 1963. Carrier-amino acid stoichiometry in amino acid transport in Ehrlich ascites cells. *Biochim. Biophys. Acta.* **71**:15.
39. MATTHEWS, D. M., and L. LASTER. 1965. Kinetics of intestinal active transport of five neutral amino acids. *Am. J. Physiol.* **208**:593.
40. NEMETHY, G., and H. A. SCHERAGA. 1962. The structure of water and hydrophobic bonding in proteins III. The thermodynamic properties of hydrophobic bonds in proteins. *J. Phys. Chem.* **66**:1773.
41. LEO, A., C. HANSCH, and D. ELKINS. 1971. Partition coefficients and their uses. *Chem. Rev.* **71**:525.
42. SHRIER, E. E., and H. A. SCHERAGA. 1962. The effect of aqueous alcohol solutions on the thermal transition of ribonuclease. *Biochim. Biophys. Acta.* **64**:406.
43. PERRIN, D. D. 1965. Dissociation constants of organic bases in aqueous solution. Butterworth & Co. (Publishers) Ltd., London.