The Use of N-Methylation to Direct the Route of Mediated Transport of Amino Acids*

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Inhibition analysis and the study of interactions for counter-transport indicate that for the Ehrlich cell (1) and for the mammalian erythrocyte (2) (and apparently also for intestinal and renal transport, as discussed elsewhere (1)), the neutral amino acids share a small number of distinct broad affinity transport systems. Recently, evidence pointing in somewhat the same direction for slices of kidney (3) and pancreas (4) has been interpreted in quite a different way. Because observed values for uptake in prolinuria (5). Hagihira et al. concluded that the system serves specifically for the several amino acids with secondary, tertiary, or quaternary amino groups.

In the course of the present work, the useful observation has been made that N-monomethylation minimizes or eliminates transport by the A (alanine-preferring) system (see p. 3601 (6) for a brief description of the several transport systems). At the same time the prior view that a single N-methyl group does not greatly decrease transport by the A (alanine-preferring) system has been established by quantitative criteria. N-Methylation accordingly has been used to suppress selectively one or the other of these two systems, thereby permitting clearer observation of the minor component of entry occurring by the other system. The results confirm the original supposition that the routes are shared in approximate correspondence to the observed inhibitory actions. At the same time a general correspondence has been shown, among amino acids entering the Ehrlich cell predominantly by one mediated pathway or the other, between the $K_m$ and $K_i$ values pertaining to each amino acid.

EXPERIMENTAL METHODS

Glycine-1-14C, L-alanine-1-14C, betaine-1-14C, L-lysine-6-14C, and taurine-1-14C were obtained from Calbiochem, and their radioactive purity confirmed by paper chromatography. N,N-Dimethylleucine was prepared by reductive methylation of taurine (11), the product being recrystallized twice from generous volumes of methanol. N-Methylphenylalanine was prepared according to Fischer and Lipschitz (12) with the assistance of Mr. Bruce Kuiken. Radioactive methyl iodide was used to prepare the 14C-labeled form. MeAIB-1-14C was prepared by the Strecker method from acetone, K14CN, and methylamine hydrochloride.

The mode of collecting and handling the Ehrlich cell has been described in prior papers (1, 13). Intervals of 1 min at 37° and pH 7.4 were used for observing the rate of uptake of glycine and alanine, of 3 min for N-methylphenylalanine, and of 5 min for betaine and taurine. These intervals restrict uptake to a small, initial portion, which occurs linearly with time. If uptake includes to a significant extent a radioactive contaminant, a change in the characteristics of uptake may be expected on changing the proportion of cells used; therefore Ehrlich cell suspensions were tested at both 4 and 10% concentrations in Krebs-Ringer bicarbonate medium. The time intervals selected were not long enough to occasion disturbing degrees of metabolic modification of the amino acids used. The added amino acids

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replaced an isosmolar quantity of NaCl in the incubation medium. Uptake was terminated by adding an equal volume of ice-cold 0.9% NaCl and at once centrifuging for 2½ min. Lower pH values were obtained with phosphate-buffered Krebs-Ringer solutions. Procedures for extraction of the cells and counting radioactive disintegrations have been described (13, 14).

**RESULTS**

The smallness of the action of proline on the uptake of leucine (3% inhibition for 1 mM leucine by 5 mM proline), despite the considerable hydrocarbon mass presented by the imino acid, was the first clue that alkylation of the amino group may eliminate all, or nearly all, affinity for the leucine-preferring system. In agreement, leucine and valine each at a 5 mM level inhibited 120

\[ N\text{-Me Val} \]

\[ \text{VN-MeVal} \]

\[ 3-4 \text{ (MeAIB), 3.3 (NMDA)} \]

\[ 3.4-4 \text{ (AIB), 3.3 (NMDA)} \]

\[ 0.9 \text{ (MeAIB), 0.7 (NMDA)} \]

\[ 1 \text{ (NMDA), 0.4 (L-valine)} \]

\[ 0.23 \text{ (L-alanine), 0.2 (NMDA)} \]

\[ 0.33 \text{ (sarcosine), 0.3 (N-methyl-phenylalanine), 0.4 (proline)} \]

\[ 4.4 \text{ (MeAIB)} \]

\[ 1.7 \text{ (MeAIB), 1.8 (glycine)} \]

\[ 0.4 \text{ (L-leucine), 0.6 (NMDA)} \]

\[ 4.2 \text{ (L-phenylalanine), 5 (NMDA)} \]

\[ 0.5 \text{ (L-phenylalanine), 0.5 (L-valine)} \]

\[ 0.5 \text{ (L-leucine), 0.5 (L-valine)} \]

\[ * \text{ See the text, footnote 2.} \]

\[ \dagger \text{ Corrected for entry of the test amino acid by a second, mediated process, as illustrated in Fig. 3.} \]

That degree of inhibition could well represent the rather weak reactivity of leucine and valine with the \( A \) system (1). Fig. 1 shows the \( N \)-methyl-\( L \)-leucine and \( N \)-methyl-\( D \)-valine have very small inhibitory actions on the uptake of valine. The unmethylated amino acids have actions on valine uptake described by a \( K_m \) for \( D \)-valine of 3 to 4 mM and a \( K_i \) for \( L \)-leucine of about 0.5 mM. With their inhibitory action on the \( L \) system largely if not entirely eliminated by \( N \)-methylation, their effects, despite the unfavorable presence of branched side chains (1), now fall conspicuously on the \( A \) system amino acids, alanine and sarcosine. The weak actions which they exert on the uptake of leucine and valine may well be exerted on the small portions of the latter amino acids presumed to be entering by the \( A \) system. One may note, however, that even at 100 mM \( \text{MeAIB} \) showed no measurable inhibition of the uptake of valine.

In contrast, for the \( A \) system the presence of a single \( N \)-methyl group tends to have only a moderate effect on affinity. The small influence of the \( N \)-monomethylation of glycine may be seen in Fig. 2. The value of \( K_m \) obtained for sarcosine by the Lineweaver-Burk plot lies between 4 and 6 mM. This value is not clearly distinguishable from that of 3 to 5 mM obtained for glycine (1). The three amino acids, alanine, sarcosine, and \( \text{MeAIB} \), were chosen to test whether the introduction of an \( N \)-methyl group shifted the amino acid to a different transport family. The results of Table I are consistent with the entry of all three by one and the same alanine-preferring mediating system. The same conclusion applies for \( L \) proline. In the case of \( \text{AIB} \), monomethylation actually increases the transport affinity, lowering the \( K_m \) from about 1 mM to about 0.4 mM.

Table I compares the \( K_m \) value of each of several amino acids with the \( K_i \) values describing its inhibitory action on the uptake.

\[ \text{TABLE I} \]

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>( K_m )</th>
<th>( K_i )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>3-5</td>
<td>3.4-4</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>0.6-0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>AIB</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>MeAIB</td>
<td>0.4</td>
<td>0.23</td>
</tr>
<tr>
<td>Sarcosine</td>
<td>5</td>
<td>4.4</td>
</tr>
<tr>
<td>L-Proline</td>
<td>1.5</td>
<td>1.7</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>3.2</td>
<td>3-4</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>0.6-0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>L-Valine</td>
<td>3-4</td>
<td>4.2</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>0.4</td>
<td>0.5</td>
</tr>
</tbody>
</table>

\[ * \text{ See the text, footnote 2.} \]

\[ \dagger \text{ Corrected for entry of the test amino acid by a second, mediated process, as illustrated in Fig. 3.} \]
of certain other amino acids believed to be transported by the same agency. The comparison reveals that these kinetic parameters for each amino acid appear generally to be similar, within the accuracy obtained in estimating them, a result that would be anticipated if these acids entered the cell and inhibited the entry of other amino acids by reacting with the same transport site.

The behavior that we are attempting to verify, namely the sharing by analogues of more than one transport system, shows itself as a complication in measuring some of the $K_i$ values of Table I. In Fig. 3 the line marked by triangles shows the rather poor linearity obtained when the reciprocal of the mediated entry rate of $^{14}$C-leucine (after correcting for the nonsaturable entry (14)) is plotted against the concentration of phenylalanine present as an inhibitor. Fig. 4 supplies a probable explanation for the curvature: Lineweaver-Burk plots of leucine entry are in general more consistent with two mediated routes of entry, than with one. In Fig. 4 a second linear section, corresponding to a $K_i$ of about 17 mM, has been drawn. Although one could instead draw a fairly satisfactory single line to represent these results, the distinct tendency toward the steeper slope at high concentrations has appeared on every repetition of the experiment with leucine. The phenomenon probably results from the entry of leucine also by the $A$ system. We have presented elsewhere such biphasic Lineweaver-Burk plots for lysine (15), $\beta$-alanine (16), and other amino acids. The quantity of leucine calculated to enter by this presumed lower affinity route has been eliminated from consideration in plotting the upper line of Fig. 3, on the tentative assumption that migration by that route is not inhibited to an appreciable extent by phenylalanine at concentrations up to 10 mM. The corrected slope corresponds to a value for $K_i$ in better agreement with the $K_m$ estimated for the high affinity entry process for leucine (Table I). The uncertainties that may be presented by this direct test for multiplicity of routes motivated us to the experiments that follow. Fig. 5 provides another illustration of the problem of resolving multiple routes of transport; in this case the need for at least two $K_i$ values to describe the inhibitory action of phenylalanine on two modes of lysine entry is obvious even in the non-reciprocal plot. Inui has proposed a direct graphic solution for one aspect of the problem of determining how much of the flux of a solute can be inhibited by an analogue, when a single $K_i$ value will describe the interaction. This method has been used for the results of Fig. 7, below. The results of Table I indicate that the inhibitory actions among amino acids for the $A$ system (or for the $L$ system) arise from their use of that system for transport; no evidence of inhibition unrelated to transport is seen. Unfortunately this demonstration is restricted to the dominant affinity of the amino acids; it is still possible that their lower affinity for a second system is not associated with actual transport by that system.

Having identified $N$-monomethylation as an effective means of

![Figure 3](image3.png)

**Fig. 3.** Plot of the reciprocal uptake rate of leucine (1 mM) against the concentration of L-phenylalanine. $\Delta$-$\Delta$, uptake rate corrected only for an apparently nonsaturable component; $O$-$O$, uptake rate corrected also for a presumed second mediated process (indicated by $K_m$ in Fig. 4) at a rate of 0.5 mmole per Kg of cell water per min.

![Figure 4](image4.png)

**Fig. 4.** Lineweaver-Burk plots of the uptake rates for $L$-leucine and $L$-valine by the Ehrlich cell. Reaction time was 1 min at 37°. The estimate for $K_m$ of 17 mM for leucine has been made after deducting entry by the component showing a $K_m$ of 0.6 mM. Because of the high affinity of leucine, this experiment is marked by a high degree of variability (unless the cells have been previously loaded with an exchanging amino acid (1)), so that the values for $V_{max}$ and $K_m$ cannot be considered accurate.

![Figure 5](image5.png)

**Fig. 5.** Plot showing that the inhibitory action of phenylalanine on lysine uptake concerns at least two processes. Two $K_i$ values, one below 1 mM and one very much higher, would be required to account for the shape of the curve. Computation of each $K_i$ value requires an evaluation of the influx of lysine at a 1 mM level by that particular route.
for minimizing migration of an amino acid by the L system we applied that procedure to L-phenylalanine, a typical substrate for the L system. We found that the inhibitory action of MeAIB is restricted to about one-fifth of the entry of 1 mM phenylalanine, the inhibition in a typical experiment being 18% at 0.5 mM, 20% at 50 mM, and 22% at 100 mM MeAIB. Although our test did not include a range of concentrations wide enough to justify calculation of a $K_I$ for MeAIB, its value lies below 0.5 mM; hence the behavior is consistent with the degree of reactivity found for MeAIB for the A system. Fig. 6 shows that when phenylalanine is methylated, a product is obtained with a substantially slower uptake. The initial rate was consistently only about one-third that for free phenylalanine. Despite its slow uptake, however, N-methylphenylalanine eventually is concentrated to a much greater degree than phenylalanine, a behavior characteristic of amino acids that enter by the A system if they cannot readily escape by the L system (1). Fig. 7 shows that the entry of N-methylphenylalanine is highly sensitive to inhibition by MeAIB ($K_I = 0.3$ mM). Furthermore, the entry retained at high levels of MeAIB is not measurably sensitive to the presence of phenylalanine or of alanine. Indeed the residual rate ($K_R = 0.12$ per min) falls within the range of our observations of the nonsaturable component of entry into the Ehrlich cell seen for many a-amino acids (1). These results indicate that the minor fraction of the entry of 1 mM phenylalanine subject to inhibition by MeAIB (or AIB; see below) very probably occurs by the A system, and that the inhibitory action of phenylalanine on entry of amino acids by the A system represents a transport-producing reaction rather than an abortive reaction with that system.

We then applied the directive influence of an N-methyl group to see whether amino acids entering mainly by the A system can be shown also to migrate by the L system. Fig. 8 shows that MeAIB does not eliminate all of the mediated uptake of 1 mM L-alanine, although it does that of sarcosine. The residual mediated component (eliminating from consideration the apparently nonsaturable migration) represents about 37% of the total. It is not subject to inhibition by taurine or by AIB. The latter observation, along with the data of Table I, shows that in this context one apparently does not succeed in narrowing the reactivity of AIB by N-methylating it; so far it appears to be just as good a model substrate for the A system as is the N-methylated derivative. Table II shows further that this residual mediated uptake of alanine is sensitive to the presence of methylalanine, and may therefore include entry by the L system, as predicted from the pattern of inhibitory actions seen for alanine (1). The residual component does not occur exclusively by the L system, however, because it is more sensitive to the presence of an excess of alanine than to the presence of

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**Fig. 6.** Contrast in the time course of the uptake of L-phenylalanine and N-methyl-L-phenylalanine. Each amino acid was 1 mM in Krebs-Ringer bicarbonate medium at pH 7.4, 37°C.

**Fig. 7.** Inhibitory action of MeAIB on the uptake of 1 mM N-methyl-[14C]-L-phenylalanine during 2 min. The curve shows the relation between the uptake rate (scale at right) and MeAIB concentration. The dot and the bar just below the curve at 10 mM MeAIB show that the addition of 2 mM alanine or phenylalanine had little further inhibitory action on the uptake. The straight line describes the relation between the reciprocal of the rate of uptake subject to inhibition (1/Vmed, scale at left) and the MeAIB concentration. It corresponds to a $K_I$ for MeAIB of 0.3 mM. The flux subject to inhibition was obtained from the intercept at infinite concentration of MeAIB from a plot of 1/(V0 - Vf) against 1/[MeAIB]. Because of this difference in the method of computation, the symbol $V_{med}$ rather than $Y$ (14) has been used.

**Fig. 8.** Contrast in the extent to which the migration of L-alanine and that of sarcosine can be blocked by the presence of MeAIB. Each test amino acid was present in the suspending medium at a 1 mM level; time was 1 min at 37°C. The uninhibited rate was 2.2 mmoles per Kg of cell water per min for alanine, and 0.84 mmole per Kg of cell water per min for sarcosine. The arrows at the right show how much of the residual uptake would be saturable, assuming for each case an uptake rate not subject to saturation of 0.1 mmole per mmole of extracellular concentration per min.
phenylalanine (Table II), whereas the reactivity of these two amino acids with the \( L \) mediator falls in the opposite order (1). Accordingly, the routes of alanine entry include an unidentified route, conceivably the incompletely described one designated as \( L^+ \) (15). Lowering the pH from 7.4 to 5.0 slowed the entry of alanine (1 mM) in the presence of 50 mM MeAIB by only about 1%. Hence the pH-sensitive portion of the entry of alanine was almost totally eliminated by the inhibitory action of MeAIB. A lack of pH sensitivity is characteristic of the \( L \) system (1).

The uptake of 1 mM lysine during 1 min was inhibited to the extent of only 10 to 20% by 5 mM MeAIB, to only 13% by 5 mM \( N \)-methylphenylalanine, and to even lesser extents by 10 mM \( N \)-methyl-DL-leucine and \( N \)-methyl-DL-valine. \( N \)-Methylation accordingly has greatly decreased the inhibitory action reported earlier for the latter three amino acids with the lysine-accepting transport activity (15). The inhibitory action of a 5-fold excess of MeAIB on the uptake of \( \alpha,\gamma \)-diaminobutyric acid, like that of AIB, increased as the concentration of diaminobutyric acid was increased from 0.3 to 10 mM, indicating that action was greater on the mode of diaminobutyric acid entry that becomes prominent at higher levels. We conclude that \( N \)-methylation extensively decreases reactivity of neutral amino acids with the so-called \( L^+ \) transport system (1), but less extensively with the system serving for diaminobutyric acid at higher concentrations.

The introduction of a second or third \( N \)-methyl group into the glycine molecule sharply decreases affinity for transport. The \( K_m \) of \( N,N \)-dimethylglycine was at least 80 mM, too high for ready evaluation. Its \( K_i \) for inhibition of betaine uptake was estimated at 60 mM. But on further \( N \)-methylation the apparent affinity increases again, as shown by the range of values for the \( K_m \) of 24 to 45 mM derived from Lineweaver-Burk plots for betaine (Fig. 9). The corresponding \( V_{\max} \) was approximately 6 mmole per Kg of cell water per min. Typical values for the \( A \) system have fallen between 5 and 6 mmole.

In evaluating the relationship between concentration and initial rate of entry the inhibitory action of MeAIB on the uptake of a 1 mM lysine (Table II), whereas the reactivity of these two amino acids with the \( L \) mediator falls in the opposite order (1). Accordingly, the routes of alanine entry include an unidentified route, conceivably the incompletely described one designated as \( L^+ \) (15). Lowering the pH from 7.4 to 5.0 slowed the entry of alanine (1 mM) in the presence of 50 mM MeAIB by only about 1%. Hence the pH-sensitive portion of the entry of alanine was almost totally eliminated by the inhibitory action of MeAIB. A lack of pH sensitivity is characteristic of the \( L \) system (1).

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Fig. 10 shows how we evaluated \( K_i \) values to describe the action of betaine on the uptake of labeled 1 mM glycine (upper curve of Fig. 9). The lower line shows the total rate of uptake, \( V \), in millimoles per Kg of cell water per min. The upper line shows the result of correcting for a supposedly nonsaturable component, \( K_D = 0.05 \) min\(^{-1}\), which, however, betaine does not actually show.

**TABLE II**

**Sensitivity of uptake of \( \alpha \)-alanine to other amino acids persisting in presence of 50 mM MeAIB**

<table>
<thead>
<tr>
<th>Amino acid added (in addition to 1 mM alanine and 50 mM MeAIB)</th>
<th>Uptake</th>
<th>Retention of uptake rate on adding the third amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.66</td>
<td>(100)</td>
</tr>
<tr>
<td>AIB, 10 mM</td>
<td>0.73</td>
<td>110</td>
</tr>
<tr>
<td>Phenylalanine, 10 mM</td>
<td>0.49</td>
<td>74</td>
</tr>
<tr>
<td>Alanine, 10 mM</td>
<td>0.34</td>
<td>51</td>
</tr>
<tr>
<td>Taurine, 10 mM</td>
<td>0.69</td>
<td>105</td>
</tr>
<tr>
<td>None</td>
<td>0.82</td>
<td>(100)</td>
</tr>
<tr>
<td>Phenylalanine, 2 mM</td>
<td>0.60</td>
<td>74</td>
</tr>
<tr>
<td>Alanine, 4 mM</td>
<td>0.39</td>
<td>48</td>
</tr>
<tr>
<td>Alanine, 4 mM, phenylalanine, 2 mM</td>
<td>0.30</td>
<td>37</td>
</tr>
<tr>
<td>Lysine, 2 mM</td>
<td>0.74</td>
<td>90</td>
</tr>
</tbody>
</table>

Fig. 9. Lineweaver-Burk plot for betaine uptake during 5 min. The lower line shows the total rate of uptake, \( V \), in millimoles per Kg of cell water per min. The upper line shows the result of correcting for a supposedly nonsaturable component, \( K_D = 0.05 \) min\(^{-1}\), which, however, betaine does not actually show.

Fig. 10. Determination of \( K_i \) values for betaine in inhibiting the uptake of glycine (upper curve) and of alanine (lower curve). \( V \) represents the uptake of the amino acid per Kg of cell water during the 1 min period of observation. In computing \( K_i \), the \( K_m \) and \( V_{\max} \) values for glycine and alanine determined by Oxender and Christensen (1) were used.
The term $V$ represents total uptake of betaine in millimoles per Kg of cell water per min. Period of observation of uptake was 5 min. The difference in initial rate indicated by the intercepts was the largest encountered.

**TABLE III**

*Test for nonidentity of transport system for betaine with that for glycine and alanine*

The values are given in millimoles per liter. The $K_i$ values have been determined in each case by the inhibition of transport, betaine by the amino acid, and the amino acid by betaine.

<table>
<thead>
<tr>
<th>Interaction studied</th>
<th>$K_i$ of the [amino acid] mm</th>
<th>$K_m$ of the [amino acid] (mm)</th>
<th>$K_i$ for betaine (mm)</th>
<th>$K_m$ for betaine (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine vs. betaine</td>
<td>0.8 1.0</td>
<td>0.6-0.8</td>
<td>40 48</td>
<td>24-45</td>
</tr>
<tr>
<td>Glycine vs. betaine</td>
<td>3-6</td>
<td>3-5</td>
<td>51 43</td>
<td>24-45</td>
</tr>
</tbody>
</table>

**Fig. 11. Determination of $K_i$ values for glycine (lower line) and alanine (upper line) in their inhibition of betaine uptake.** The term $V$ represents total uptake of betaine in millimoles per Kg of cell water per min. Period of observation of uptake was 5 min. The difference in initial rate indicated by the intercepts was the largest encountered.

**Fig. 12. Interactions between two analogues for transport by two or more mediating systems.** $B$, $X$, and $D$ are transport-mediating systems; $\beta$ and $\delta$ are solute analogues. The solid-line arrows passing through the boxes represent transport, whereas the dashed arrows represent competitive inhibition without transport. See the text for discussion.
The relation between $\alpha$-alanine and $\beta$-alanine, however, appears to represent alternative Case 2b (16), whereas between $\alpha,\alpha$-diethylglycine and phenylalanine (6) may represent the first type. These results show that none of the cases listed applies universally.

The present results show that when the uptake of phenylalanine by the $L$ system (corresponding, let us say, to the vertical arrow on the right of Fig. 12) is largely if not totally suppressed by $N$-monomethylation, the typical entry by the $A$ system, suspected for this and other amino acids using predominantly the $L$ system, stands uncovered (slanting solid arrow pointing to the left). In the other direction, suppression by the presence of excess MeAIB of the predominant migration of alanine by the $A$ system (solid vertical arrow at the left) uncovers a residual component of migration showing characteristics of the $L$ system (slanting solid arrow pointing to the right), although in that case the characterization is not unequivocal, because of a contribution by a third route. The dashed arrows of Fig. 12, to indicate mutual inhibitory actions abortive of transport, do not seem necessary in describing this case.

Before concluding that the situation is entirely different for other tissues, i.e., that inhibition by analogues is not accompanied by sharing of utilization of the systems in question, we should consider two aspects of the experiments tending to that conclusion (3, 4). The values of $K_m$ and $K_a$ were determined by observing the relations between amino acid concentration and steady state uptake by the tissue slices, rather than by direct measurement of rates of uptake. This procedure appears to depend on the assumption that efflux of amino acids is linearly dependent on concentration, whereas exocytotic processes demonstrably include mediated components (1), so that the relationship may not be linear. Secondly, the observations were not extended to extracellular concentrations higher than 10 mM, whereas we find we must frequently proceed to levels as high as 100 mM to describe the second of two shared modes of entry. There is also the danger of obtaining an artifactual apparent $K_m$ or $K_a$ value (see Fig. 4) intermediate between two insufficiently separated values, each describing a distinct mode of migration, a hazard that is of course not entirely avoided by extending the concentration range studied. Levels as high as 100 mM still permit an isotonic medium to contain a level of Na$^+$ well above that required for maximal amino acid fluxes into the Ehrlich cell, and do not appear to have led to discontinuities in kinetic plots. It should be emphasized that reactivity with a given transport system is not excluded by showing that a 5- or 10-fold excess of a potentially inhibitory analogue decreases uptake by no more than a few per cent.

When it is understood that a given amino acid may be transported by two or more mediating agencies, the delineation of a new, distinct transport agency appears more complex than it did formerly. The inhibitory actions need to be evaluated in both directions between pairs of analogues, and these actions need to be referred to a corresponding component of transport of each of them, before correspondence or noncorrespondence to the use of a given pathway can be concluded. Although the system proposed for methylamino acids for the hamster intestine has in this sense not been fully defined, nevertheless it is clear that the transport of betaine and $N$-dimethylglycine into that tissue does not occur by the agency serving largely for alanine (10), as in the Ehrlich cell.

With the above observation that in the Ehrlich cell betaine and $\beta$-alanine (16) participate only minimally in the apparently nonsaturable uptake process described previously (14, 1), we must abandon any tendency to suppose that this mode of migration of the neutral amino acids represents simple diffusion. Although no assumption of this kind was made originally, the method of correcting for this process requires that it should tend toward a 1:1 distribution ratio between the interior and the exterior phase (14). This aspect of the method of calculation is severely tested only when one selects long enough time intervals to cause the distribution ratios to rise well above unity. It may, moreover, be plausible to suppose that the process is a facilitated diffusion since no evidence of a limitation in the energy supply is obtained in tests at high solute concentrations, and since the corrections made by the method have generally eliminated deviations from linearity at high concentrations in the Lineweaver-Burk plot. Nevertheless, more precise experimental determination of the position of the steady state is now needed. It was in fact in a search for an amino acid which might be taken up predominantly by the nonsaturable route that $\alpha,\alpha$-diethylglycine (6) was studied. The $\beta$ forms of the amino acids (17) may be very useful for investigating this route.

The ability of betaine to react with the alanine-preferring system, even though substantial concentrations are required, reinforces an earlier conclusion, namely that the charged form of the amino group is probably the one that bonds to the $A$ transport-mediating site (18). We may now also conclude that the binding of the amino group very probably does not require a hydrogen bond, since the tetras alkylammonium group cannot form such a bond. These two conclusions cannot be extended to the leucine-preferring system, however, because in that case even a single $N$-methyl group eliminates or severely reduces affinity. We may be warned that a conclusion as to the character of the bonding of one of these systems may not apply to another, by the circumstance that the $\beta$ system scarcely differentiates between the carboxylate and the sulfonate groups. This system favors the latter anionic groups as presented by taurine, over the carboxylate group presented by $\beta$-alanine. In contrast, taurine or the sulfonic acid analogue of leucine has no observable inhibitory action on the transport of glycine or leucine. The low $pK_a$ of the sulfonic acid analogue of leucine deprives that result of clear significance; the contrast in the reactivities of taurine and $\beta$-alanine with the $A$ system (16) is, however, striking evidence of a fundamental difference.

**SUMMARY**

1. Introducing a single $N$-methyl group does not tend to decrease the reactivity of amino acids with the so-called alanine-preferring transport system of the Ehrlich cell, nor does it occasion perceptible transport by a different system.

2. An $N$-methyl group largely eliminates reactivity with the so-called leucine-preferring and lysine-accepting transport systems.

3. The differential effect of $N$-methylation has been applied first to eliminate selectively the characteristically dominant migration of phenylalanine by the latter agencies, so that the slower uptake by the alanine-preferring system was uncovered and could be identified. Conversely, $N$-methylamino acids have been used as inhibitors to eliminate the dominant migration of alanine by the alanine-preferring system, to uncover minor routes of migration. A portion of the residual migration has
characteristics attributed to the leucine-preferring system. In this way the evidence has been strengthened for the conclusion that the interaction among the ordinary neutral amino acids for transport systems is based largely on their mutual sharing of these routes, rather than a general presence of mutually inhibitory reactions abortive of transport.

4. The presence of two or three N-methyl groups eliminates reactivity neither with the alanine-preferring transport system, nor for the system serving for taurine and β-alanine. Most of the uptake of betaine by the Ehrlich cell (in contrast to uptake by segments of the hamster intestine) may well be accounted for by the alanine-preferring system. The reactivity of betaine strongly supports an earlier conclusion that the amino group reacts in its cationic form with the alanine-preferring transport system.

5. Betaine, like β-alanine, migrates, at most, only to a relatively minor extent by a slow, difficulty saturable route observed so far for all the neutral α-amino acids tested. The cumulative evidence for structural specificity for that route shows that it cannot represent simple diffusion.

REFERENCES
The Use of N-Methylation to Direct the Route of Mediated Transport of Amino Acids

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