# Discrimination of Single Transport Systems

## *The $Na^+$-sensitive transport of neutral amino acids in the Ehrlich cell*

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ABSTRACT Uptake of methionine, α-aminoisobutyric acid, and α-(methylamino)-isobutyric acid has been shown to occur by at least two transport systems, one sensitive and the other insensitive to the $Na^+$ concentration. For α-aminoisobutyric acid and its *N*-methyl derivative, the $Na^+$-insensitive uptake is not concentrative and its rate increases almost linearly with concentration within the range examined. In contrast, the $Na^+$-insensitive uptake of methionine is concentrative and subject to inhibition by such amino acids as phenylalanine, leucine, and valine, although not in a manner to indicate that the uptake is mediated by a single agency. This component is not produced by a residual operation of the $Na^+$-requiring transport system, handicapped by the absence of $Na^+$ or by its having combined with α-aminoisobutyric acid. The increase in the rate of methionine uptake is linear with concentration only above about 16 mM methionine. The $Na^+$-sensitive uptakes of methionine, α-aminoisobutyric, and α-(methylamino)-isobutyric acid appear to occur by the same population of transport-mediating sites. Both $K_m$ and $V_{max}$ of the $Na^+$-sensitive uptake of these three amino acids change with changes in the concentration of $Na^+$, an effect which is shown to have a theoretical basis. A similarity in the values of $V_{max}$ for ten amino acids entering principally by the $Na^+$-sensitive agency indicates that differences in their $K_m$ values probably measure differences in their affinities for that transport-mediating system.

In measuring the rate of uptake of an amino acid by a cell or tissue, one records the sum of the amounts migrating by several routes (2–6). By using a structural analog the uptake can often be partitioned into a component subject to inhibition and one not subject to competition. These two components are again not necessarily homogeneous. First of all the inhibited component may represent competition between the solute and the analog for two or more transport systems (2–6). The usual kinetic plots may well fail to give any evidence of heterogeneity unless the $K_m$ or $K_i$ values are separated by a whole order of magnitude (5). Instead they may yield a false $K_m$ or $K_i$ intermediate between two real ones. In some cases paired $K_m$ or $K_i$ values for a single amino acid are separated enough to permit their evaluation by curve-

fitting or other methods of successive approximation; nevertheless ancillary techniques for establishing homogeneity and for diminishing heterogeneity in analyzing modes of transport are badly needed. Some procedures have already been offered for the purpose in a recent report (4). Even if two analogs inhibit the transport of each other by what appears to be a homogeneous system, one needs to consider the possibility that their entry is by two discrete systems, each inhibited but not used by the other (4, 7).

Problems of heterogeneity occur also for the component of uptake of a solute not subject to inhibition by a given analog. That component is likely to include a part not observably subject to saturation or to any known competitive inhibitor (the so-called nonsaturable transport (8), which probably does not occur by simple diffusion, 9); but it may well also include migration by one or more additional transport systems that other analogs will inhibit. Still another possibility, which might greatly complicate analysis of transport, is represented by type Ib of Dixon and Webb (10), in which case the inhibitor combines with the transport system not at the same site as the primary substrate, but at a site near enough to decrease the affinity of the site for the primary substrate; in this scheme the ternary complex *EIS* is considered still to be able to transport amino acids at the same rate as the carrier substrate complex, *ES*. Such *partially competitive* inhibition of transport has been invoked in situations where the transport in question may well take place instead by two or more distinct agencies, only one of which is subject to ordinary competitive inhibition. A wrong decision for *partially competitive* inhibition causes a second or a third reactive site to be attributed incorrectly to a given transport-mediating system. An error in the other direction may lead to the proposal of nonexistent transport systems.

The present communication considers the case of interaction for uptake by the Ehrlich cell of three selected amino acids: methionine, an amino acid for which more than one mode of mediated transport has been observed (2, 6), $\alpha$-aminoisobutyric acid[1] and $\alpha$-(methylamino)-isobutyric acid,[1] the latter two showing, in contrast with methionine, an apparent simplicity of transport mediation. Specifically, we have selected the component of the transport of these amino acids that disappears when choline is used to replace $Na^+$ in the suspending medium, and have shown that it represents a single, apparently homogeneous transport agency, for which the three compete according to the simplest kinetic model. The residual uptake of these three amino acids therefore still includes all the differences included in their total uptake. For the purpose of the demonstration a method is described for defining the limit of the portion of uptake of a solute subject to competitive inhibition by an analog; kinetic criteria are also presented for defining the type

[1] The abbreviations used are: AIB for $\alpha$-aminoisobutyric acid; MeAIB for $\alpha$-(methylamino)-isobutyric acid.

of competition between analogs. In addition, the velocity of amino acid transport by the system has been shown to change with $Na^+$ concentration in the same way that it does with amino acid concentration, that is, according to the Michaelis-Menten equation in which the concentration of $Na^+$ appears as its first power.[2] Both the apparent $K_m$ and $V_{max}$ of the amino acids were found to be changed by varying the $[Na^+]$, a result shown to be predictable on a theoretical basis.

## EXPERIMENTAL METHODS AND MATERIALS

*Preparations of Cells* Cells collected from white Swiss mice 8 to 12 days after inoculation with the tumor were washed twice with 4 to 5 volumes of Krebs-Ringer bicarbonate buffer, pH 7.4, and separated in each case by centrifugation. After the supernatant solution had been removed completely, the cells were further washed twice with 5 volumes of isotonic choline chloride and again centrifuged to separate the cells. The cells were then resuspended in isosmotic choline chloride to obtain a known concentration, and aliquot portions transferred to several test tubes to yield after centrifuging about 0.15 g of packed cells in each tube. After removing the supernatant solution, the packed cells were kept in ice for not over 15 min before using them for the experiment.

*Incubation and Reisolation of Cells* After both the uptake medium and the test tubes containing packed cells had been brought to the desired temperature, 8 ml portions of the medium containing a radioactive test amino acid were added simultaneously to the several test tubes, one operator serving for each tube. The cells were gently resuspended by agitating with a spatula for 10 sec, and the suspension poured back into the flasks and held for 50 sec more in the water bath at 37°. Sharply at the end of this period, the cell suspensions were poured into weighed centrifuge tubes containing 4 ml of ice cold isotonic choline chloride solution, and centrifuged at top speed in an International Model PR-2 centrifuge for 2.5 min. The supernatant solution was removed from each tube, the last portion being withdrawn by touching it with a pointed filter paper strip, folded once along the length to stiffen it. After the fresh cells were weighed, 0.3 ml of 0.1 N acetic acid was added to the cell mass, the cells were broken up in an all-glass homogenizer, and then 1 ml of distilled water was introduced. Extraction and analysis for radioactivity were carried out by the method described previously (2). The trapped extracellular fluid was regularly determined and a correction made for its radioactivity. The relative volume trapped remained quite constant ($20\% \pm 2\%$) under the conditions used, swelling and shrinkage being minimal. The uptake rates, expressed in mmoles per kg cell water·min, can be converted to mmoles per kg dried cells·min by multiplying by 4. The 1 min interval of uptake used ordinarily permitted uptake to proceed not more than one-third of the way toward the steady state. Under these conditions the departure of the observations from initial rates will not lead to serious underestimates of $V_{max}$.

For these experiments a modified Krebs-Ringer bicarbonate medium was used,

[2] A preliminary report has been made of the relation of the constants for amino acid uptake to $[Na^+]$ (1).

containing $K^+$ = 26 (because choline bicarbonate was at first not available to us), $Na^+$ = 107, and choline$^+$ = 16 mEq per liter. Added amino acids replaced a portion of the choline chloride, so that [$Na^+$] was kept constant. Additional choline chloride served to replace NaCl in experiments in which concentrations of $Na^+$ less than 107 mEq per liter were used. The proportion of weight loss on drying the fresh cells was not changed measurably by 1 min incubation in the above solutions.

*Materials* Radioactive L-methionine (methyl-$^{14}$C) was purchased from Volk Radiochemical Company, AIB-1-$^{14}$C from the Isotope Specialities Company, and L-valine-1-$^{14}$C and L-leucine-1-$^{14}$C from Calbiochem. Both the MeAIB-1-$^{14}$C and the unlabeled MeAIB were prepared in this laboratory by the Strecker procedure for AIB (11), using acetone, methylamine hydrochloride, and K$^{14}$CN or K$^{12}$CN. Unlabeled methionine and AIB were purchased from the California Corporation for Biochemical Research. All optically active amino acids were studied in their L-forms.

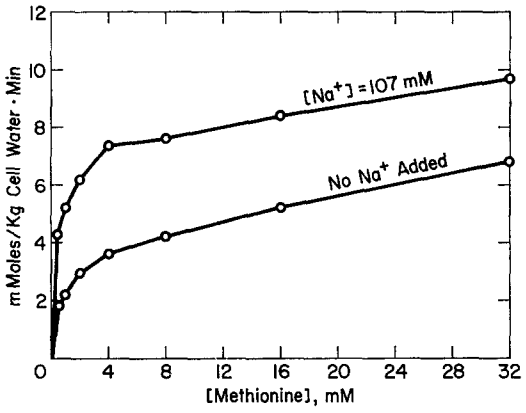


FIGURE 1. Rate of uptake of methionine in presence or absence of $Na^+$. Observations 1 min at 37°C, pH = 7.4 in all figures.

## RESULTS

*Relation between $Na^+$-Sensitive Components of Uptake of Methionine and of AIB*

Fig. 1 shows the relation between the rate of uptake of methionine and the methionine concentration in the modified KRB medium. Above 16 mM the uptake rate increased slowly and linearly (0.09 min$^{-1}$) with methionine concentration. When all $Na^+$ was eliminated from the medium by replacement, the initial rate was strongly diminished, although the uptake still appeared to be concentrative, the distribution ratio after 1 min being 2.25 (the calculated ratio, cellular level to extracellular level). In this case also the increase in the rate at methionine concentrations above 16 mM was small, with about the same linear slope as we saw in the presence of $Na^+$ (Fig. 1). A test of the inhibitory action of AIB on the uptake of methionine showed that only a part of the uptake was subject to inhibition (Fig. 2). For this and similar comparisons, we needed a method for obtaining the horizontal asymptotes of plots of $v$ vs. [$I$], as in Fig. 2. When the slope of that plot is governed by the Michaelis-Menten equation applied to the case of competing substrates, it will

have the form of a rectangular hyperbola, and as is shown in the theoretical part (part 1), a plot of the reciprocal of the diminution of velocity due to the presence of the inhibitor at various concentrations, $1/(V_o - V_i)$, against $1/[I]$ as illustrated in Fig. 3, will yield as the reciprocal of its intercept the magnitude of the inhibitable portion of uptake of the solute whose transport

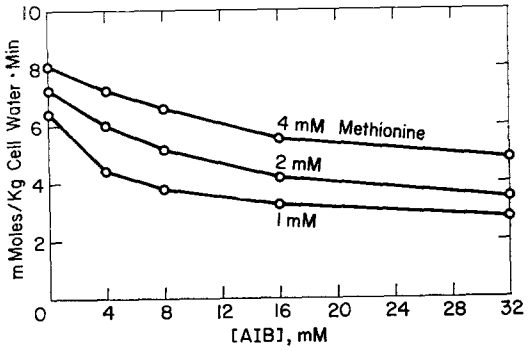


FIGURE 2. Inhibition by AIB of the uptake of methionine.

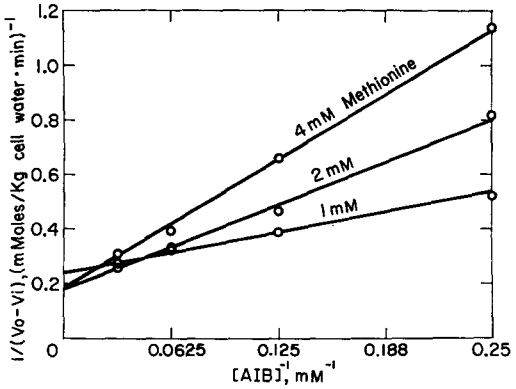


FIGURE 3. Plot to determine portion of the uptake of methionine subject to competitive inhibition by AIB. $[Na^+]$ = 107 mEq/liter; $V_o$ = rate of uptake of methionine without AIB; $V_i$ = rate of uptake of methionine at a given concentration of AIB. The intercept on the ordinate will be $\frac{1}{v_o}$, the reciprocal of the velocity that is subject to inhibition.

is under study. Using this method of estimating the AIB-sensitive component of methionine uptake, we find (Fig. 4) that the residual uptake of methionine obtained by the above method is very similar in magnitude to that of the $Na^+$-insensitive component, indicating that the component subject to inhibition by AIB is nearly equal to that sensitive to $Na^+$.

For the procedure of Fig. 3 to be valid the interaction represented in Fig. 2 must be a competitive one between AIB and methionine. Fig. 5 shows that the $Na^+$-sensitive uptake of methionine (obtained by the rate in the presence

of $Na^+$ less the rate in its absence, in experiments similar to those of Fig. 1), follows the Michaelis-Menten equation, yielding values of 0.3 mM and 4 mmoles/kg cell water·min for $K_m$ and $V_{max}$, respectively. Fig. 6 shows that the action of AIB on the AIB-sensitive portion of methionine uptake is competitive. In the theoretical part (part 2) it will be shown that a plot of the ratio,

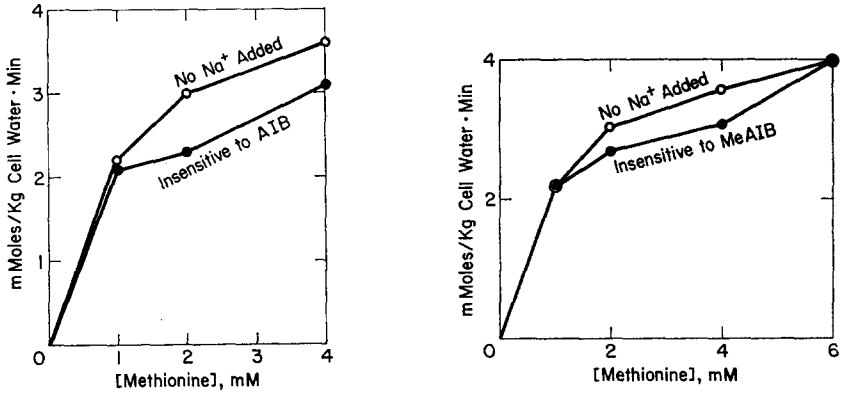


FIGURE 4. Uptake rate retained for methionine in absence of $Na^+$ and in presence of AIB (left) or MeAIB (right). Solid points show the residual uptake extrapolated to infinite concentration of AIB or MeAIB at a given methionine concentration.

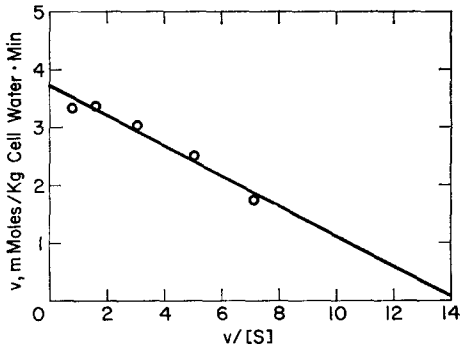


FIGURE 5. The $Na^+$-sensitive uptake of methionine. Unit for $[S]$ is mM. $K_m$ = 0.26 mM, $V_{max}$ = 3.7 mmoles/kg cell water·min.

slope/intercept for the line in Fig. 3, against $[S]$ will be linear only when the inhibition under description is competitive and of Dixon's type, Ia; i.e., when the complex $EI$ is totally inactive in producing transport of $S$. Fig. 7 shows that this description applies to the action of AIB on the transport of methionine.

When AIB is added to a suspending medium in which choline has replaced $Na^+$, it shows no significant inhibitory action on the uptake of methionine (Table I). The foregoing results show that the uptake of methionine is divided between two types of transport mediators, one sensitive and one insensitive to $Na^+$. This interpretation corresponds to our former prediction, that me-

thionine enters the Ehrlich cell partly by a system designated *A*, one with which AIB is principally reactive, and which is sensitive to the presence of $Na^+$; and partly by another agency, which we designated *L*, with which AIB appears to be unreactive within the range of concentrations studied, and which fails to show a distinct dependence on the presence of $Na^+$ (2). The present

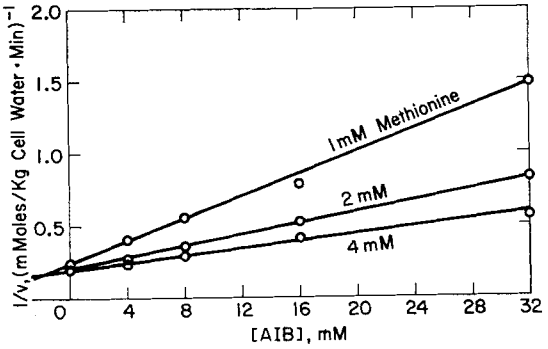


FIGURE 6. Inhibition by AIB of AIB-sensitive uptake of methionine. The AIB-sensitive uptake of methionine was obtained by subtracting the uptake rate for methionine retained at an infinite concentration of AIB (by the method of Fig. 3), from the uptake rate retained at a given concentration of AIB.

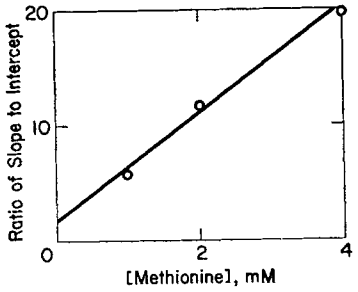


FIGURE 7. Relation between ratio of slope to intercept obtained by plotting $1/(V_o - V_i)$ against $1/[I]$, and concentrations of test amino acid, methionine. Inhibitor, AIB; $K_i$ of AIB = 2 mM. The ratio has mM as its unit.

investigation uses these sensitivities to associate quantitatively with each other, the $Na^+$-sensitive and the AIB-sensitive components of methionine uptake.

The foregoing interpretation is supported by parallel observations on AIB uptake. Fig. 8 shows the relation between the uptake rate and the concentration of this amino acid in the modified KRB medium. The lower curve shows that in the absence of added $Na^+$, the uptake of AIB, in contrast to that of methionine, rises almost linearly with AIB concentration throughout the range examined. The slope of the line describing this increase corresponds to

0.14 to 0.15 $min^{-1}$, our observations being for the first minute of uptake. This slope corresponds approximately to the rate of permeation observed during the same interval for AIB and many other neutral amino acids at high levels,

TABLE I

ABSENCE OF INHIBITORY ACTION FOR UPTAKE BETWEEN AIB (IN THE SECOND EXPERIMENT, MeAIB) AND METHIONINE WHEN NO $Na^+$ HAS BEEN INTRODUCED

The labeled amino acid in each case was at 1 mM. 1 min at 37°C, pH = 7.4.

| Inhibitor | Its concentration | Uptake of other amino acid |
|---|---|---|
| | *mM* | *mmoles/kg cell water·min* |
| AIB | 0 | 2.68 |
| | 4 | 2.63 |
| | 8 | 2.62 |
| MeAIB | 0 | 1.77 |
| | 8 | 1.68 |
| | 16 | 1.77 |
| | 32 | 1.67 |
| Methionine | 0 | 0.16 |
| | 0.5 | 0.17 |
| | 1 | 0.14 |
| | 2 | 0.19 |

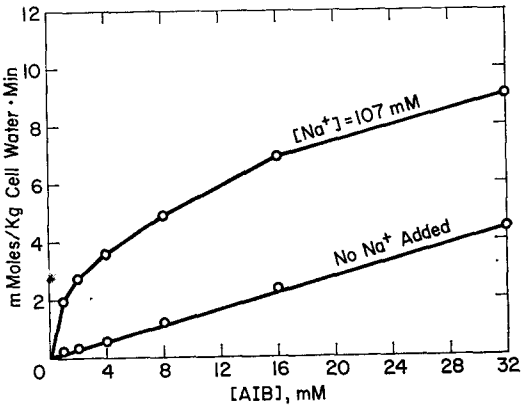


FIGURE 8. Uptake of AIB in the presence and in the absence of $Na^+$.

namely 0.1 $min^{-1}$, a slope which has been taken to measure their "nonsaturable" migration (2, 3). When this $Na^+$-insensitive component is deducted (Fig. 9), the initial rates of AIB uptake yield values for $K_m$ and $V_{max}$, respectively, of 2 mM and 5 mmoles/kg cell water·min, with good correspondence to the Michaelis-Menten equation.

In the presence of $Na^+$ at 107 mEq/liter, the inhibition of the saturable uptake of AIB by methionine is so nearly complete (Fig. 10) that the tech-

nique of plotting $1/(V_o - V_i)$ adds nothing to the precision. When $Na^+$ is replaced with choline$^+$, methionine shows a negligible inhibitory action on the residual uptake of AIB (Table I). For both methionine and AIB uptake, the possibility needed to be considered that the decreased uptake of the two test amino acids was due to the presence of choline rather than to the absence of $Na^+$. The results in Table II show that similar results were obtained whether sodium chloride was replaced with choline chloride or with sucrose. Hence

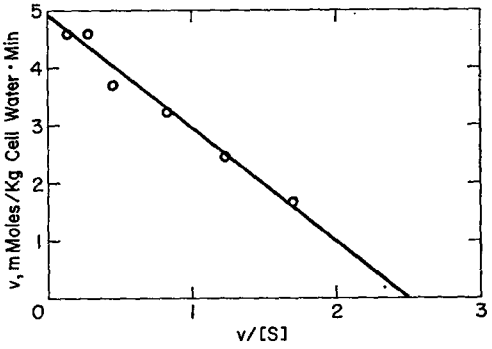


FIGURE 9. $Na^+$-sensitive uptake of AIB. Unit for $[S]$ is mM. $K_m$ = 2.0 mM, $V_{max}$ = 4.9 mmoles/kg cell water·min.

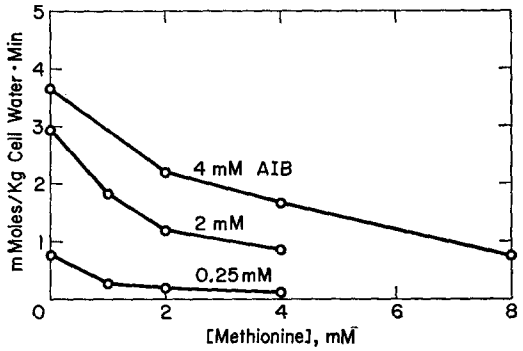


FIGURE 10. Inhibition by methionine of the uptake of AIB.

we conclude that the rates are diminished by the removal of $Na^+$ from the medium, and not by the addition of the substituting compounds.

In the study of the effect of methionine concentration on its inhibition of AIB uptake, the plot (not included here) of $1/(V_o - V_i)$ against $1/[I]$ shows a straight line at different concentrations of AIB. A replot, not included here, of the ratio of the slope to the intercept, both obtained in the above plot, against [AIB], shows that the inhibitory effect of methionine on the uptake of AIB corresponds to Dixon's type Ia, as is explained in the theoretical section. The $K_i$ describing this inhibitory action is 0.3 to 0.4 mM, in good agreement with the $K_m$ of 0.3 mM observed in this research for the $Na^+$-sensitive uptake of methionine.

As an additional test of the identity of the agencies serving for the $Na^+$-

sensitive uptake of AIB and methionine, we determined the ratio of the rates of uptake of AIB and methionine when they are present in a constant proportion but at various total concentrations. As will be shown in the theoretical part (part 3), if certain experimental conditions are met, this ratio should be a constant when the interaction is a competition for the same population of

TABLE II

INDEPENDENCE OF THE EFFECT OF $Na^+$ WITHDRAWAL ON THE NATURE OF THE SUBSTITUTE SOLUTE

AIB or methionine = 1 mM; time = 1 min

| [$Na^+$] | Solute substituting for NaCl | AIB uptake | | Methionine uptake | |
|---|---|---|---|---|---|
| | | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 |
| *mmoles/liter* | | *mmoles/kg cell water·min* | | *mmoles/kg cell water·min* | |
| 107 | | 2.65 | 3.43 | 4.74 | 5.98 |
| 0 | Choline chloride | 0.16 | 0.21 | 1.35 | 2.48 |
| 107 | | 3.06 | 3.37 | 4.33 | 5.31 |
| 0 | Sucrose | 0.21 | 0.29 | 1.44 | 2.79 |

TABLE III

TEST FOR CONSTANCY OF RATIO OF UPTAKE OF AIB AND METHIONINE AT A CONSTANT RATIO OF THEIR CONCENTRATIONS

Sodium-sensitive uptakes during 1 min at 37°C, pH 7.4, in mmoles/kg cell water·min. According to theory the ratio should be

$$\frac{V_{max} \text{ for Met}}{V_{max} \text{ for AIB}} \cdot \frac{K_m \text{ of AIB}}{K_m \text{ of Met}} \cdot \frac{[\text{Met}]}{[\text{AIB}]} = 1.0 \text{ to } 1.6$$

| Methionine | AIB | Sodium-sensitive uptake rate when the test amino acid is | | Ratio of uptake rates |
|---|---|---|---|---|
| | | Methionine | AIB | |
| *mM* | *mM* | | | |
| 0.25 | 1.0 | 0.58 | 0.51 | 1.1 |
| 0.5 | 2.0 | 0.99 | 0.64 | 1.5 |
| 1.0 | 4.0 | 1.04 | 0.77 | 1.4 |

sites. Table III shows that over a fourfold range of concentration a ratio is obtained which is approximately constant.

*The Relation between the Sodium-Sensitive Uptake Routes of Methionine and N-methylAIB* *N*-methylation was recently shown to serve to eliminate all or nearly all of the reactivity of $\alpha$-amino acids with the so-called *L* transport system (4). Therefore the interaction for transport between MeAIB and methionine was examined as a further test of the above interpretations and of the methods proposed here for characterizing competitive relations among solutes.

A series of results similar to those of Fig. 2 showed that when $Na^+$ is present, MeAIB inhibits only a part of the uptake of methionine. Again the residual uptake at various levels of methionine, on extrapolation to infinitely high levels of MeAIB, is almost the same as it is in the absence of $Na^+$ (Fig. 4, right). Furthermore MeAIB shows no significant action on the uptake of methionine in the $Na^+$-free medium (Table I). The relation between ratio of slope to intercept, both obtained by plotting $1/(V_o - V_i)$ against $1/[I]$, and the concentration of methionine (as in Fig. 7) showed that the inhibitory action of MeAIB on methionine uptake is also competitive and according to Dixon's type Ia. The $K_i$ for MeAIB in this action was observed to be 0.2 to 0.3 mM, in agreement with the $K_m$ for MeAIB uptake observed in this study, 0.2 to 0.3 mM (cf. reference 4).

Conversely, methionine inhibits almost completely the uptake of MeAIB in the presence of $Na^+$, as it did for AIB (Fig. 10). The inhibition was again proved to be a simple competitive one by tests corresponding to that of Fig. 6, the $K_i$ for methionine being 0.4 mM, in agreement with the $K_m$ of 0.3 mM observed for the $Na^+$-sensitive methionine uptake.

These results show that the *N*-methyl derivative of AIB, which is expected to have its transport, if anything, even more narrowly limited to the *A* system than AIB (4), gives results differing from those for AIB only in the quantitative values of the apparent constants, $K_m$ and $K_i$.

*Homogeneity of Phenylalanine Inhibition of the $Na^+$-Requiring Uptake of Methionine* The use of phenylalanine as an inhibitor already has revealed heterogeneity in two cases with the Ehrlich cell:

1. Of the agencies for lysine uptake that are reactive with neutral amino acids (16), it inhibits one and not the other (17).

2. A component of the $Na^+$-requiring uptake of alanine is much less readily inhibited by phenylalanine than is the component shared with AIB and MeAIB (Christensen and Liang, unpublished results). This additional system had already been detected in the results of Fig. 8 and Table II of a recent report (4).

Accordingly we tested whether a corresponding component is present in the $Na^+$-requiring uptake of methionine. This test showed that from 94 to 108% of that uptake was inhibited by phenylalanine in an apparently homogeneous manner; i.e., yielded a straight line when plotted as in Fig. 11.

*Relation between $Na^+$-Sensitive Uptake Process for AIB, MeAIB, and Methionine, and $Na^+$ Concentration* For a 1 mM methionine solution, the rate of uptake increases as the $Na^+$ level is raised until a near maximal value is attained at $[Na^+] = 115$ mEq/liter. As shown in Fig. 12 and in Fig. 3B of a preliminary note (1), plotting the rate $v$ for the $Na^+$-sensitive uptake, against $v/[Na^+]$ or $v/[S]$ yields straight lines for all three amino acids. The same is true for glycine. This behavior is in striking contrast to the finding of Vidaver for

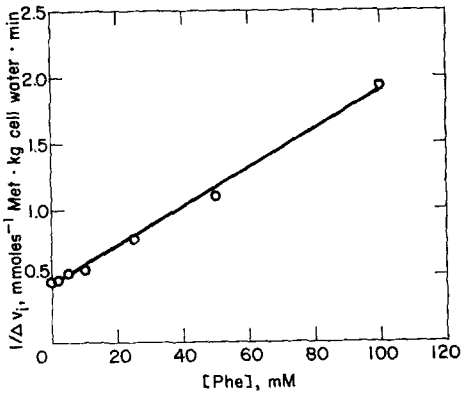


FIGURE 11. Phenylalanine inhibition of $Na^+$-requiring uptake of methionine. Uptake during 1 min from Krebs-Ringer bicarbonate medium, pH 7.4. The values for $\Delta v$, the gain in velocity on increasing $[Na^+]$ from 0 to 93 mEq/liter, were plotted according to the method of Fig. 3, which predicted that only 6% of the uninhibited rate would be retained at infinitely high phenylalanine levels. The rate subject to phenylalanine inhibition, $\Delta v_i$, was then plotted as shown. Heterogeneity as to the mode of methionine transport would be indicated by (*a*) departure of the plot from linearity, or (*b*) the presence of a saturable component not subject to phenylalanine inhibition. The latter was not detected.

glycine uptake by the pigeon red blood cell, showing that linearity is obtained when the second power, not the first power, of the $Na^+$ concentration is used in the Lineweaver-Burk plot (12), a result that we have confirmed for $Na^+$ concentrations above 15 mEq/liter (1), and found also to apply under similar conditions to the rabbit reticulocyte (13). The above results show that straight line relations apply equally for plots of $v$ against $v/[S]$ and $v$ against $v/[Na^+]$.

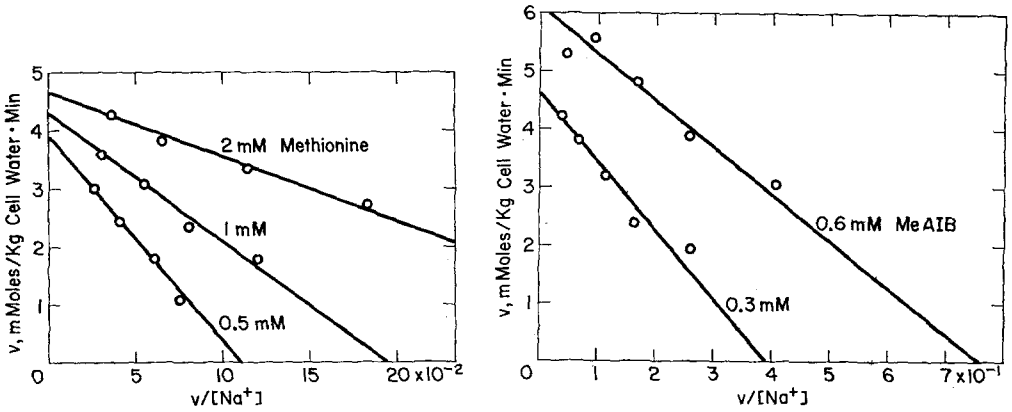


FIGURE 12. Relation between $Na^+$-sensitive uptake of test amino acids and the concentration of $Na^+$. Concentration range of $Na^+$ used, 15 to 115 mEq/liter. *Left,* L-methionine; *right,* MeAIB.

They also show that both parameters, $K_m$ and $V_{max}$, are modified by changing the $Na^+$ concentration. Extrapolation of the velocities for various methionine concentrations to infinite $[Na^+]$, and replotting the resultant velocities against $v/[S]$, gave a $V_{max}$ of 5.0 mM/kg cell water·min and a $K_m$ of 0.14 mM for methionine (data of Fig. 12 *left*), and a $V_{max}$ of 7.1 mM/kg cell water·min and a $K_m$ of 1.1 mM for AIB (data of Fig. 3A, reference 1).

From the experimental evidence that straight line relations apply equally between $v$ and $v/[S]$ and between $v$ and $v/[Na^+]$ one can derive the following equation which can be shown to arise uniquely from the separate dependence of the rate on $[S]$ and $[Na^+]$:

$$1/v = \frac{A_1}{[Na^+][S]} + \frac{A_2}{[Na^+]} + \frac{A_3}{[S]} + A_4$$

where $A_1$, $A_2$, $A_3$, and $A_4$ are constants. Rearranging the equation we get:

$$v = \frac{\frac{[Na^+]}{A_2 + A_4[Na^+]} \cdot [S]}{\frac{A_1 + A_3[Na^+]}{A_2 + A_4[Na^+]} + [S]}$$

In this equation the fraction in the numerator occupies the place of $V_{max}$ in the Michaelis-Menten equation; the fraction in the denominator, the place of $K_m$. Since the sodium ion concentration appears in both, together with constants, we conclude that both the $V_{max}$ and the $K_m$ will generally change with changes in the concentration of $Na^+$ in the uptake medium. The above equation does not depend on whether $\frac{A_1 + A_3[Na+]}{A_2 + A_4[Na+]}$ is equivalent to $\frac{k_{-1} + k_2}{k_1}$ or to $\frac{k_{-1}}{k_1}$; i.e., it is reached without making a choice between the equilibrium and the steady-state assumptions.

*Significance of $K_m$ for the $Na^+$-Sensitive System* Fig. 13 summarizes observations of $V_{max}$ for ten amino acids entering the Ehrlich cell mainly by the $Na^+$-sensitive system under study. The relative constancy of $V_{max}$, despite a more than 100-fold range of variation of $K_m$ (from a value of about 0.3 mM for MeAIB to one of about 40 mM for betaine) indicates that in the scheme,

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + S,$$

$k_2[E_t]$ is similarly constant. To restrict their entry to the $Na^+$-sensitive system, we redetermined the kinetic parameters for alanine and glycine using the difference method of Fig. 5. The $K_m$ values obtained were 0.4 and 4.1 mM; the $V_{max}$ values, 6.4 and 7.4 mmoles per kg cell water·min. If the rate measure-

ments approximate initial rates as we suppose, the relative constancy of $V_{max}$ shown in Fig. 13 means that $k_2$ tends to be nearly the same, whatever the chemical nature of $S$; the constancy of $k_2$ means in turn that the variation in $K_m$ arises largely from $k_{-1}$ and $k_1$ and therefore that the variation in $K_m$ actually measures (inversely, of course) the variation in the affinities of the several amino acids for the system. This relationship is a necessary consequence if the equilibrium assumption of Michaelis and Menten applies. It

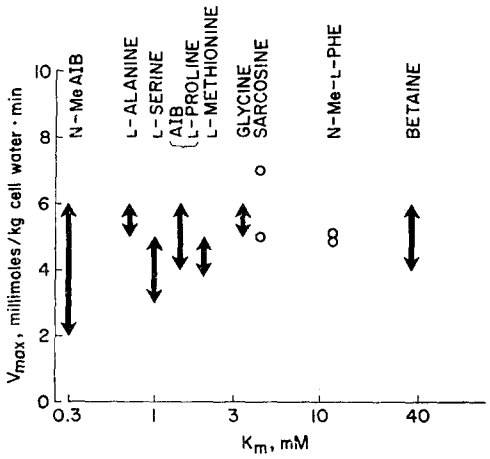


FIGURE 13. Relative constancy of $V_{max}$ for ten amino acids entering the Ehrlich cell mainly by a single transport system, despite wide variations in $K_m$. The abscissa scale is logarithmic to permit display of a wide range of $K_m$ values. In the case of methionine the $Na^+$-sensitive component only is included. Observations limited to the $Na^+$-sensitive component for alanine and glycine are recorded in the text. Sources, glycine, AIB, serine, and alanine, reference (2); sarcosine, betaine, and *N*-methylphenylalanine, reference (4); proline, unpublished work of this laboratory; others, the present paper.

implies that $k_1$ and $k_{-1}$ are large relative to $k_2$, although it does not require that they be very much larger than $k_2$. The observations of Fig. 13 make it inherently unlikely within the present system that the presence of one amino acid can accelerate the uptake of another by providing a faster pathway for the stage marked $k_1$ (14, 15), since that reaction is shown not the rate-limiting step. That conclusion must, of course, be restricted for the present time to the $Na^+$-sensitive system under study. An alternative explanation for the tendency of $V_{max}$ to be invariant would become available if the dissociation of $Na^+$ from the system should prove to be rate-limiting.

*Nature of $Na^+$-Insensitive, Mediated Transport of Methionine* The form of the lower line of Fig. 1 shows that much of the uptake of methionine retained in the absence of added $Na^+$ is saturable. In contrast to the considerable sensitivity of the total uptake of methionine to pH (upper curve of Fig. 5 in refer-

ence (2)), the uptake retained in the absence of $Na^+$ is nearly constant in the range, pH 5.6 to 7.0 (Table IV; (cf. reference 2)). Although almost totally insensitive to the action of AIB, MeAIB, or glycine, it is very easily inhibited by the presence of phenylalanine, valine, or leucine. Table V records estimates of the $K_i$ values applying to the interaction for uptake among methionine, valine, and leucine in the absence of added $Na^+$. Because they are

TABLE IV

ABSENCE OF pH DEPENDENCY FOR METHIONINE UPTAKE FROM $Na^+$-FREE MEDIUM

Uptake during 1 min from 1 mM solution in a Krebs-Ringer phosphate medium modified by replacement of NaCl by choline chloride. The phosphate was added as $KH_2PO_4$ and the pH adjusted with KOH. The proportion of water in the cellular mass was essentially the same at the pH values obtained.

| pH | Rate | |
|---|---|---|
| | Exp. 1 | Exp. 2 |
| | *mmoles/kg cell water·min* | |
| 5.6 | 3.03 | 2.99 |
| 6.3 | 3.03 | 3.08 |
| 7.0 | 2.86 | 2.87 |

TABLE V

INTERACTION OF METHIONINE AND VALINE OR LEUCINE FOR UPTAKE IN THE ABSENCE OF $Na^+$

Uptake from choline-containing modified Krebs-Ringer bicarbonate medium at 37°C, pH 7.4 during 1 min. Inhibitable portion determined as in Fig. 3. Dixon plot used to obtain $K_i$ values.

| Interaction | $K_i$ of methionine ($K_m$ = 0.13) | For the other amino acid | |
|---|---|---|---|
| | | $K_i$ | $K_m$ |
| | mM | mM | mM |
| Methionine and valine | 0.14 | 0.6 | 0.75 |
| Methionine and leucine | 0.15 | 0.1 | 0.15 |

based on 1 min observations of comparatively rapid processes, the rates observed were less than the initial rates, and the results of Table V must be regarded as only approximate. Within that limitation, they show a consistency between the values of $K_i$ and $K_m$ for each of the three amino acids suggesting that the uptake observed occurs by a single, homogeneous process. Results recorded in Table VI lead us, however, to reject that suggestion: When methionine and valine are present at identical concentrations, the ratio of the amounts of each taken up is strongly dependent on concentration. The result is not unexpected, because the uptake in question has already been shown to

include both a lysine-inhibitable and a lysine-insensitive component (16). Under the same conditions, heterogeneity was not clearly evident in the $Na^+$-insensitive interaction for uptake between methionine and leucine (Table VI); whether or not such evidence is obtained will, of course, depend upon the distribution of the several $K_m$ values involved. Presumably one can never properly assert that homogeneity has been established for a population of the transport-mediating structures, but only that no heterogeneity has yet been uncovered.

TABLE VI

TEST FOR HOMOGENEITY OF THE INTERACTION BETWEEN METHIONINE AND VALINE OR LEUCINE FOR UPTAKE IN THE ABSENCE OF $Na^+$

Same conditions as for Table IV.

| Interacting amino acids | Concentration of each amino acid | Ratio of uptake Methionine/other amino acid |
|---|---|---|
| | mM | |
| Methionine and valine | 0.25 | 3.2 |
| | 0.5 | 2.18 |
| | 1.0 | 1.65 |
| | 2.0 | 1.33 |
| Methionine and leucine | 0.2 | 0.99 |
| | 0.4 | 0.94 |
| | 0.8 | 0.92 |
| | 1.6 | 0.99 |

## DISCUSSION

These results show that methionine uptake can be divided into two components, one sensitive and one insensitive to $Na^+$. If the two components operated as the related portions of a single system, or were otherwise dependent on each other, so that a change in one would occasion a change in the other, then one would not expect the component of the total transport of methionine in the presence of $Na^+$ that can be inhibited by AIB or MeAIB to correspond as closely as it does (Fig. 4) to the component that can be eliminated by $Na^+$ removal, given as has been shown that the addition of AIB or MeAIB is without effect on methionine transport in the absence of added $Na^+$. The results further show that the transport of methionine retained in the presence of AIB or MeAIB is not produced by the residual operation of the same mediating system serving in their absence, handicapped by its having combined with AIB or MeAIB.

The equation showing the relation among three variables, the rate of the $Na^+$-sensitive uptake, the concentration of $Na^+$, and the concentration of test

amino acid

$$1/v = \frac{A_1}{[Na^+][S]} + \frac{A_2}{[Na^+]} + \frac{A_3}{[S]} + A_4$$

has the same form as that proposed by Dalziel to explain two-substrate enzyme kinetics (18).

In correspondence with such two-substrate kinetics, let us assume that the structure reacting with the amino acid and that reacting with $Na^+$ are on the same molecule and that the only form in which $S$ can enter transport is the ternary complex $ESNa^+$. We will consider the rather general case described below, in which the order of binding of $Na^+$ and $S$ to $E$ is not critical:

$$Na^+ + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ENa^+ + S \qquad \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}}$$

$$ESNa^+ \xrightarrow{k_5} \text{(transported } S\text{)}$$

$$S + E \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ES + Na^+ \qquad \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}}$$

There are four ways by which $Na^+$ and $S$ can bind to $E$, namely, (1) $E$—$Na^+$—$S$; (2) $E$—$S$—$Na^+$; (3) $E\begin{smallmatrix}\diagup Na^+\\ \diagdown S\end{smallmatrix}$; and (4) $Na^+$—$E$—$S$. If $S$ and $Na^+$ must bind to each other, in cases 1, 2, and 3, one would anticipate that the uptake of the test amino acid would be inhibited by increasing the concentration of either sodium or amino acid, while holding the other at a low, constant concentration, since in these cases either $Na^+$ or $S$ would compete with the complex, $Na^+$—$S$, for the subsequent binding to $E$. This effect was not observed. Accordingly, case 4, in which $E$ has 2 binding sites, one for the test amino acid, the other one for $Na^+$, seems to be the more probable one of those considered, although case 3 remains quite possible.

In order to make the discussion simple, let us assume that after the carrier–amino acid complex releases an amino acid, the rest of it returns very quickly to the orientation or locus at which $ENa^+$ and $ES$ are formed by reaction with the extracellular solutes. Treatment of the model under the steady-state assumption of Briggs and Haldane is more complex than that under the equilibrium assumption and does not give a linear relationship between $1/v$ and $1/[S]$, a situation already emphasized by Alberty (19). Under the equilibrium assumption, the dissociation constants for the several reactions are not independent of each other, but must be in special relation, $K_1 \cdot K_2 = K_3 \cdot K_4$, where $K_1 = \frac{k_{-1}}{k_1}$; $K_2 = \frac{k_{-2}}{k_2}$; $K_3 = \frac{k_{-3}}{k_3}$; and $K_4 = \frac{k_{-4}}{k_4}$. The initial

velocity is given by,

$$v = \frac{k_5 [E_t] [Na^+] [S]}{[Na^+] [S] + K_2 [Na^+] + K_4 [S] + K_1 K_2}$$

where $[E_t]$ is total carrier concentration. Rearranging this equation, one obtains

$$1/v = \frac{1}{k_5 [E_t]} \left\{ \frac{K_1 K_2}{[Na^+] [S]} + \frac{K_4}{[Na^+]} + \frac{K_2}{[S]} + 1 \right\}$$

This equation has the same form as that proposed by us. If this model is operative in the $Na^+$-sensitive uptake of AIB, we can introduce into this equation data corresponding to those obtained in Fig. 11 and in Fig. 3B of a preliminary report (1), to obtain the values for $K_1$, $K_2$, $K_3$, and $K_4$, of 45, 1.23, 6.0, and 9.2, respectively. These values suggest that the interaction between $E$ and $S$ is much stronger than that between $E$ and $Na^+$. If the affinity of $E$ for $S$ were not affected by binding $S$ to $Na^+$, $K_2$ should equal $K_3$. The experimental values for AIB just cited show that this is not the case. Therefore, provided, as assumed, that the ternary complex $Na^+ES$ is the only form in which $S$ can be transported by the system, and that its translocation or internal reorganization prior to breakdown is the rate-limiting step, we conclude that the two sites are probably located near enough to each other to permit interaction between them.

## THEORETICAL PART

### 1. *Analytical Method for Defining the Limit of the Portion of Uptake That Is Subject to Competitive Inhibition by a Given Analog*

Assumptions: (*a*) Uptake of $A$ occurs by 2 transport systems. (*b*) One system is inhibited by $B$ and its kinetics follows the Michaelis-Menten equation. (*c*) The other mode of uptake is not inhibited by $B$.

From the preceding conditions, we can reach the following equations ($K_i$ = dissociation constant for $EI$; see Fig. 14 for definition of other terms):

$$V_o = v_o + a; V_i = v_i + a$$

$$v_o = \frac{V_{max} [A]}{K_m + [A]}; v_i = \frac{V_{max} [A]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [A]}$$

$$V_o - V_i = v_o - v_i = V_{max} K_m [A] [I] \frac{1}{K_i (K_m + [A]) \left\{ K_m \left(1 + \frac{[I]}{K_i}\right) + [A] \right\}}$$

$$\frac{1}{V_o - V_i} = \frac{K_i(K_m + [A])^2}{V_{max}\, K_m \cdot [A]} \cdot \frac{1}{[I]} + \frac{K_m + [A]}{V_{max}\,[A]}$$

The equation shows that if the above conditions are satisfied, plotting $1/(V_o - V_i)$ against $1/[I]$ will result in a straight line, and the reciprocal of its intercept will correspond to the part of the uptake rate subject to inhibition.

2. *Types of Inhibition and Their Characteristics*

The assumptions involved are, (1) an equilibrium has been reached for each step, except for the last step, (2) the concentration of test amino acid and that of the inhibitor do not change appreciably during rate measurements, (3) the nature of the ternary complex *SEI* is independent of the mode by which it is formed, (4) the reverse velocity is zero, and (5) the last step, namely the movement or reorientation of the amino acid–carrier complex, is the rate-limiting step.

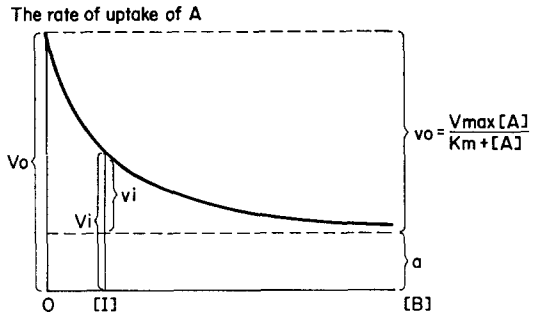


FIGURE 14. Method of defining limit to a component of uptake subject to given competitive inhibitor. $a$ = the residual uptake of $A$ extrapolated to infinite concentration of $B$; $v_o$ = rate of the uptake of $A$ sensitive to $B$; $v_i$ = rate of the uptake of $A$ sensitive to $B$ at a given concentration of $B$; $V_{max}$ and $K_m$ represent the maximal velocity of uptake and the Michaelis constant of $A$; $V_o$ = rate of the uptake of $A$ in the absence of $B$; $V_i$ = rate of the uptake of $A$ at a given concentration of $B$; and $[I]$ = concentration of $B$.

Cases of inhibition due to the four typical mechanisms proposed by Dixon and Webb (10) as type Ia, type Ib, type IIa, and type IIb are considered. In the first model the inhibitor-carrier complex does not transport the test amino acid; in the second model, the inhibitor does not bind at the same site as does the test amino acid, but at another site close enough to reduce the affinity of the site for test amino acid, the ternary complex still transporting the test amino acid at the same rate as does *ES*; in the third model, the inhibitor has equal affinity for both carrier and amino acid–carrier complex, but the ternary complex cannot transport the test amino acid; in the fourth case, the inhibitor has equal affinity for both carrier and amino acid–carrier complex; the ternary complex can still transport test amino acid but at a reduced rate.

For these four models, we can derive the following equations, in which the symbols, $K_s'$, $K_i$, $K_i'$, $k$ and $k'$, have the meanings given them by Dixon and Webb (10).

(case 1)
$$v_i = \frac{V_{max}\cdot[S]}{K_s\left(1+\frac{[I]}{K_i}\right)+[S]}\,;\; v_o = \frac{V_{max}\,[S]}{K_s+[S]}$$

$$v_o - v_i = V_o - V_i = \frac{V_{max}\cdot K_s\cdot[I]\cdot[S]}{(K_s+[S])\{K_i[S]+K_s(K_i+[I])\}}$$

$$\frac{1}{V_o - V_i} = \frac{(K_s+[S])^2K_i}{V_{max}\cdot K_s\cdot[S]}\cdot\frac{1}{[I]}+\frac{K_s+[S]}{V_{max}\cdot[S]}$$

(case 2)
$$v_i = \frac{V_{max}(K_i K_s' + K_s[I])[S]}{[S](K_1 K_s' + K_s[I] + K_s K_s'(K_i+[I]} \qquad (K_s \neq K_s')$$

$$v_o - v_i = V_o - V_i = \frac{V_{max}\,K_s(K_s' - K_s)[I][S]}{(K_s+[S])\{(K_i K_s' + K_s[I])[S] + K_s K_s'(K_i+[I])\}}$$

$$\frac{1}{V_o - V_i} = \frac{K_s'K_i(K_s+[S])^2}{V_{max}\,K_s(K_s' - K_s)[S]}\cdot\frac{1}{[I]}+\frac{(K_s+[S])(K_s'+[S])}{V_{max}\,[S](K_s' - K_s)}$$

(case 3)
$$v_i = \frac{V_{max}\,K_i[S]}{(K_s+[S])(K_i+[I])}$$

$$v_o - v_i = V_o - V_i = \frac{V_{max}\,[S][I]}{(K_s+[S])(K_i+[I])}$$

$$\frac{1}{V_o - V_i} = \frac{K_i(K_s+[S])}{V_{max}\,[S]}\cdot\frac{1}{[I]}+\frac{K_s+[S]}{V_{max}\,[S]}$$

(case 4)
$$v_i = \frac{V_{max}\,(K_i\,k + k'[I])[S]}{(K_s+[S])(K_i+[I])k} \qquad (k \neq k')$$

$$v_o - v_i = V_o - V_i = \frac{V_{max}\,[S][I](k-k')}{(K_s+[S])(K_i+[I])k}$$

$$\frac{1}{V_o - V_i} = \frac{(K_s+[S])K_i\,k}{V_{max}\,[S](k-k')}\cdot\frac{1}{[I]}+\frac{(K_s+[S])k}{V_{max}\,[S](k-k')}$$

The ratio of slope to intercept obtained by plotting $1/(V_o - V_i)$ against $1/[I]$ gives $K_i\left(1+\frac{[S]}{K_s}\right)$ for case (1), $\frac{K_s'K_i(K_s+[S])}{K_s(K_s'+[S])}$ for case (2), and $K_i$ for both cases (3) and (4), at a given concentration of $S$. These characteristics can be used to differentiate case (1) from the other cases. In case (1), the relation between the ratio, slope to intercept, and $[S]$ must be linear because in this case the slope equals $K_i/K_s$ and is independent of $[S]$.

### 3. *Kinetic Analysis of the Transport Phenomenon in Which Two Amino Acids Use the Same Carrier or Site*

Conditions: Transport of $A$ and $B$ meets the Michaelis-Menten equation, the transport site for these substances is identical, and each uses no other transport mechanism than the shared one. We can show the above condition as follows:—

$$A + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_2} A$$

$$B + E \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EB \xrightarrow{k_4} B$$

If we apply the steady-state approximation to this model, the initial velocity of $A$ or $B$ is given by the following equations,

$$V_a = k_2[E_t][A] \frac{K_2}{(K_2[A] + K_1[B] + K_1K_2)};$$

$$V_b = k_4[E_t][B] \frac{K_1}{(K_2[A] + K_1[B] + K_1K_2)}$$

$$\frac{V_a}{V_b} = \frac{V_{\max_a} \cdot K_2 \cdot [A]}{V_{\max_b} \cdot K_1 \cdot [B]}$$

If we keep the ratio $[A]/[B]$ constant, $V_a/V_b$ must be constant. (List of symbols: $[E_t]$, total concentration of site; $V_a$, the rate of uptake of $A$ in the presence of $B$; $V_b$, the rate of uptake of $B$ in the presence of $A$; $[A]$, concentration of $A$; $[B]$, concentration of $B$; $K_1 = \frac{k_{-1} + k_2}{k_1}$, $K_2 = \frac{k_{-3} + k_4}{k_3}$, $k_2[E_t] = V_{\max_a}$, and $k_4[E_t] = V_{\max_b}$.)

This work was supported in part by a grant (HD-01233) from the Institute of Child Health and Development, National Institutes of Health, United States Public Health Service.

*Received for publication 7 December 1965.*

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