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Na^+/Li^+ Selectivity in Transport System *A*: Effects of Substrate Structure

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Summary. The effect of amino acid structure on the selectivity between Na and Li as co-substrates for transport System *A* in the Ehrlich cell has been explored to localize relative binding positions. By various tests the relative effectiveness of the two cations varies over fivefold. Changes in structure of the amino acid that lower its response to Na tend to decrease its selectivity for Na over Li, but with many exceptions. The higher the Li level required to half-maximize amino acid entry, the slower tends to be the entry attainable for both Li and amino acid. Our attention fell on strong departures from these trends. An atypically fast uptake is produced by Li in the presence of a second amino group $pK_2' < 8.5$, in exceptional association with the known fast uptake in Na. The hydroxyl group of serine yields exceptionally strong uptake, whereas hydroxyl groups in restrained orientation (as in threonine and hydroxyprolines) sharply limit co-substrate interaction. Despite the absence of a sidechain, glycine shows unexceptional relative co-substrate responses. A sidechain in the α_2 position, as in D-alanine, lowers tolerance for both ions, an aberration largely corrected by the insertion of a second (α_1) methyl group, and surprisingly, even by an N-methyl group. For L-alanine, an N-methyl group has in contrast unfavorable effects on co-substrate interaction. These factors point to disturbance by the α_2 methyl group of the position taken by the amino acid at the site, largely rectifiable by balancing effects of a second methyl group. They also point to a position of the alkali ion quite close to the α-carbon and far from the position taken in System *ASC*. Addition of an ethylene bridge between the α-methyl groups of α(methylamino)-isobutyric acid leads to the strongest discrimination seen against Li^+ relative to Na^+, suggesting through crowding of the area that the alkali ion adjoins the three methyl groups of this analog.

Only one of the Na^+-dependent transport systems has so far revealed the spatial position taken by the sodium ion with respect to the substrate molecule at the receptor site. That system, *ASC*, differs from System *A* in its intolerance for an N-methyl group on the substrate, for branching of the sidechain, and for Li^+ as a substitute for Na^+ as co-substrate. It functions in all the nucleated or reticulated red blood cells studied so far, as well as in the Ehrlich cell [2, 9, 11, 15, 18, 19, 20], in both erythroblastic and myeloblastic leukemia cells [Wise, *personal communica-*

tion], in the placenta [12], and no doubt in many other cells. In that system, Na^+ appears to associate itself with an O or N atom lying ideally on carbon 4 of the sidechain of L-amino acid substrates and oriented *trans* to the carboxyl group, as determined through study of the several isomeric hydroxyprolines [2, 15, 18, 19]. Beyond this feature, apolar sidechains, e.g. the methyl group of alanine, also play a role in enhancing the co-substrate role of Na^+ in System *ASC*. The present investigation seeks to determine whether Na^+ takes an identifiable position similarly near the amino acid molecule in System *A*, using the Ehrlich ascites tumor cell.

The signal for co-substrate proximity used in the present study is the strength of the preference of Na^+ over Li^+ as co-substrate. We assign to System *A* the Na^+- or Li^+-dependent uptake of neutral amino acids known not to be transported by System *ASC*, and also the uptake of Na^+ or Li^+ dependent on the presence of these amino acids. Alkali ion-independent uptake of amino acids measured in simultaneous tests was in all cases deducted. For amino acids known to migrate by both systems, we assign to System *A* the component of their uptake blocked by excess 2-(methylamino)-isobutyric acid (MeAIB).[1] This agent does not, of course, block alkali ion uptake by System *A*, but instead produces a high rate characteristic of MeAIB. Hence, we had access to no basis for dividing between the two systems the Na^+ uptake dependent on the presence of such amino acids. This limitation does not apply to Li^+ influx, since System *ASC* makes no observable contribution here. The wider range of observations could be made by selecting System *A* substrates whose molecules are too short (glycine), too long (e.g. methionine, norleucine), too branched, or of the D configuration [11, 19] and thus unsuitable for System *ASC*.

Materials and Methods

Experiments of three scopes were used:

1. Study of effects of Na^+ and Li^+ concentration on amino acid influx assignable to System *A*. These experiments yield $K_m^{aa/Na}$ and $K_m^{aa/Li}$[2], the concentrations of the alkali-

1 *Abbreviations used:* AIB = 2-aminoisobutyric acid; MeAIB, its N-methyl derivative; cycloleucine = 1-aminocyclopentane carboxylic acid.

2 To deal with the complexity inherent in describing the kinetic measurements used here, we adopt the provisional convention that the first superscript symbol following V_{max} or K_m represents the substrate whose movement is measured, and the second the substrate whose concentration is varied. Thus $K_m^{aa/Li}$ represents the concentration of Li permitting a half-maximal augmentation of amino acid uptake; whereas the expression $K_m^{aa/aa}$ would represent a normal determination of K_m for the transport of an amino acid under some specified condition.

ions producing half-maximal amino acid influx, and for $V_{max}^{aa/Na}$ and $V_{max}^{aa/Li}$, the alkali ion-dependent amino acid influxes obtained at saturating levels of each cation. These tests were usually made at 0.5 mM external amino acid, 1.0 mM for DL amino acids, and in selected cases rather higher levels (as for 2,4-diaminobutyric acid) to cause System *A* uptake to predominate. The selected concentrations were nevertheless low enough so that moderate levels of the MeAIB served to block uptake. This experiment can validly be applied to known System *A* substrates in the Ehrlich cell, even to those that also show uptake by System *ASC*. The possible presence of another Na^+-dependent component of uptake in other tissues might limit extension of the approach.

2. Determination by flame photometry of the net uptake of Li^+ (at 100 mM in the suspending medium) stimulated by test amino acids at saturating levels, usually 25 mM, but 75 mM for amino acids showing very high K_m values for uptake. This experiment can also be applied for apparently the full range of System *A* substrates.

3. Determination of the amino acid-dependent uptake of Na^+ and Li^+, each at 100 mM in parallel tests at various amino acid levels, accompanied by measures of the alkali ion dependent amino acid entry. In this way we obtained for comparison the flux augmentation ratios, $\Delta v_{Li}/\Delta v_{aa}$ and $\Delta v_{Na}/\Delta v_{aa}$. Because the latter fraction will include any uptake of Na^+ occurring by System *ASC*, we limited this full experiment to amino acids showing little or no uptake by that system.

The suspending solutions used were modifications of Krebs-Ringer's bicarbonate medium at pH 7.4 under an atmosphere of 5% CO_2 and 95% O_2, in which Na^+ was often replaced by Li^+, and in which choline was used to replace a part or all of the Na^+ or Li^+ to obtain desired concentrations of the alkali metal at an unaltered osmotic pressure. The preparation of such solutions is discussed elsewhere [4]. The preparation of the Ehrlich ascites-tumor cell and the measure of amino acid uptake followed earlier procedures [11, 14]. Uptake of amino acids was usually observed during 1 min, but for more slowly migrating amino acids, intervals of up to 5 min were used. Amino acids tritiated by the Wilzbach or catalytic techniques were purified to good chromatographic radiopurity before use. Beyond that, it proved necessary to monitor for any atypical, rapidly fixed radioactive contaminant in such preparations. When radioactively labeled amino acids were not available, we used the automated "amino acid analyzer", selecting the type of column and the eluting buffer that allowed accurate separation and analysis of the amino acid in question during a relatively brief elution. Na^+ uptake was measured using liquid scintillation counting of $^{22}Na^+$, whereas Li^+ uptake was determined by an Instrumentation Laboratory flame photometer. The uptake of Li^+ observed accordingly represents a net uptake of that ion, presumably offset to an undetermined extent by simultaneous Na^+ exodus; the uptake of $^{22}Na^+$ was likewise to an undetermined extent offset by $^{23}Na^+$ exodus, since efflux is also stimulated. The results shown are supported by numerous agreeing determinations over a range of concentrations. Determinations of kinetic parameters that figure significantly in our argument were duplicated, and the mean result is shown.

Results

Effect of Alkali-Ion Concentration on the Rate of Amino Acid Uptake

The raw results of the experiments of type 1 allow us to compare amino acids as to selectivity for Li^+, relative to Na^+, on two bases:

1. By the ratio $K_m^{aa/Na}/K_m^{aa/Li}$, which shows the fractional degree to which Li^+ is accepted as a substitute for Na^+ in half-saturating uptake.

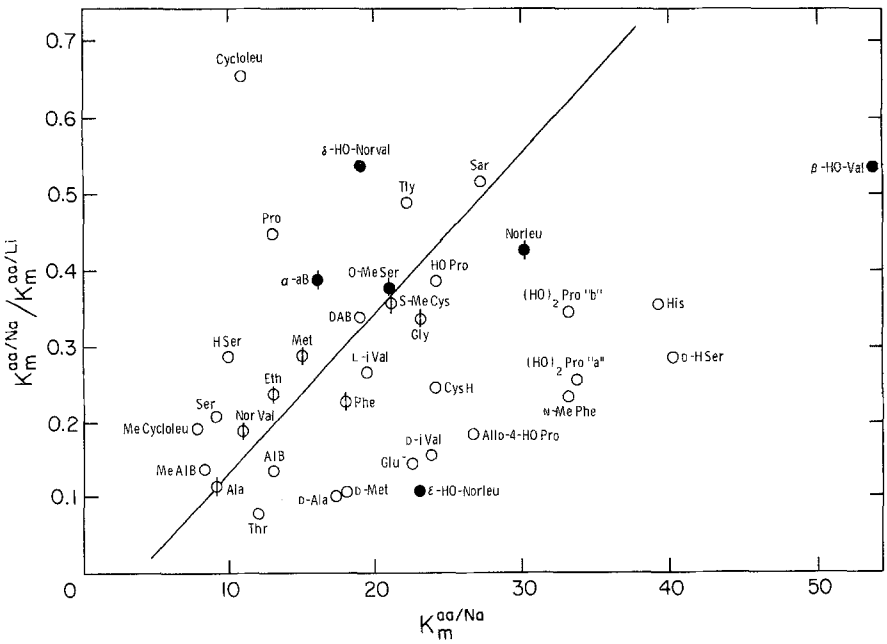

Fig. 1*A*

Fig. 1. (*A*) Relation between the ratio of the concentration of Na^+ and Li^+ half-maximizing uptake of a given amino acid, and the concentration of Na^+ half-miximizing uptake of the same amino acid. Amino acids at 0.5 mM, except 1 mM for DL amino acids, which are indicated by solid circles. Less common abbreviations: α-Bu, α-amino-*n*-butyric acid; cycloleu, 1-aminocyclopentanecarboxylic acid; DAB, 2,4-diaminobutyric acid; Eth, ethionine; HSer, homoserine; HO-Norleu, ε-hydoxynorleucine; HO-Norval, δ-hydroxynorvaline; HOPro, hydroxyproline, *cis* or *trans* as indicated; $(HO)_2$ pro, 3,4-dihydroxyproline, two isomers, *a* and *b* in both of which the hydroxyl groups are *cis* to each other; iVal, isovaline or α-ethylalanine; MeAla, MeAIB, Mecycloleu, MePhe, all N-methyl derivatives; MeCys, S-methylcysteine; MeSer, O-methylserine; TLy, thialysine or S(-2-aminoethyl)-cysteine. In this and other Figures a vertical line through the point marks results for primary amino acids (and not D isomers) without strongly polar groups on their sidechains as follows: glycine, alanine, α-aminobutyric acid, norvaline, norleucine, O-methylserine, S-methylcysteine, methionine, ethionine, phenylalanine. The lines on this and the other Figures were drawn arbitrarily to allow us to examine for a relation, and have no theoretical basis. (*See* Materials and Methods for other details.) (*B*) Ratio of the velocities of amino acid uptake attainable on raising the Li^+ and Na^+ concentrations, respectively, plotted against the velocities attainable for the same amino acid on raising $[Na^+]$. Same experiments as in Fig. 1*A*. The inset identifies the more aberrant amino acid falling near the origin

Fig. 1*A* shows on the ordinate scale that the relative tolerance for Li^+ varies from less than 0.1 to more than 0.5. Fig. 1*A* also suggested to us that as the value of $K_m^{aa/Na}$ increases, the relative tolerance for Li^+, $K_m^{aa/Na}/K_m^{aa/Li}$, tends also to increase. Note here the points bearing cross-bars, which apply to primary amino acids, not D isomers, and not bearing strongly polar groups on their sidechains. As we proceeded to look

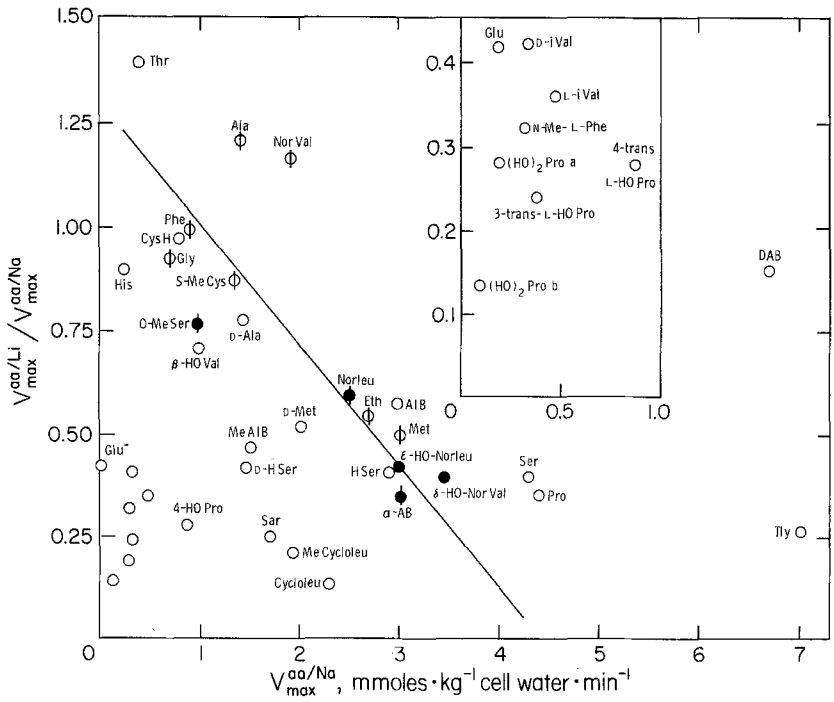

Fig. 1*B*

for aberrant System *A* substrates, this relation, as suggested by the line on Fig. 1*A*, was seen to be of somewhat restricted application.

2. By the ratio of the rates of amino acid uptake attained at saturating levels of the two cations, written $V_{max}^{aa/Li}/V_{max}^{aa/Na}$ so as again to measure tolerance for Li substitution. At one extreme for some amino acids Li^+ allows less than one-fourth as rapid uptake as Na^+; at the other extreme for other amino acids Li^+ allows about 1.4 times as fast uptake as Na^+ (ordinate scale, Fig. 1*B*). The plot examines the proposition parallel to that of Fig. 1*A* that as V_{max} rises with respect to $[Na^+]$, the tolerance for Li^+ by this test tends to fall. Here again we accrued strong exceptions to the suggested relation as we included diamino acids and amino acids of structure especially unsuited for rapid transport by System *A*.

If the relations proposed by the lines in Fig. 1 have even the restricted validity we suggest, then a linear relation might also hold for the plot of Fig. 2, where we consider the ratio, V_{max}/K_m, of the parameters studied in Fig. 1. This quotient will be recognized as a fundamental measure of transport rate. In the present case this quotient is the first-order

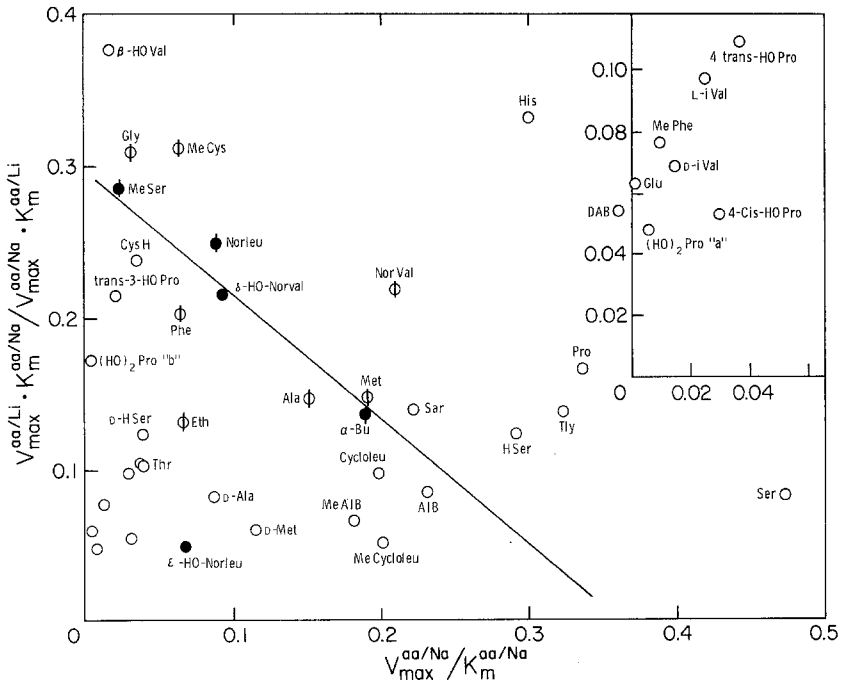

Fig. 2. Replot of the experiments of Fig. 1, calculating the quantity V_{max}/K_m for each amino acid, and plotting the value of this quantity in Li^+ solutions relative to its value in Na^+ on the ordinate, against its value in Na^+ solutions on the abscissa. Since the equation $v = V_{max}[B^+]/(K_m + [B^+])$ reduces to $v = (V_{max}/K_m)[B^+]$ at low values of $[B^+]$, the quantitity V_{max}/K_m is the first-order rate coefficient for transport as a function of $[B^+]$ at low values. The inset identifies the more aberrant amino acids falling near the origin

coefficient describing the transport rate of the amino acid as a function of the concentration of the selected cation, when that concentration is well below saturating levels. The ordinate scale shows that differences in amino acid structure cause the relative tolerance for Li^+ to vary about 10-fold by this test. The line drawn with a slope of minus one corresponds to a tendency among about 15 amino acids for those which move slowest in Na^+ to be least slowed by Li^+ substitution. Roughly supporting this relation are five homologous, straight-chain amino acids plus sarcosine, AIB and phenylalanine; also three analogously apolar amino acids containing ethereal O or S in their sidechains; also the homologous sulfhydryl analog of one of them. Other quite different slopes examined for validity proved to represent heterogeneous structures.

Our hope was to extract such generalizations and then to look along

the sloping lines, first of all for the structural features that enhance selectivity for Na^+. Secondly, we hoped to look for the structural features that a) allow Na^+ to be highly effective without the usual cost in the relative effectiveness of Li^+, also for features that b) allow neither Li^+ nor Na^+ to be effective. For the latter case we accumulated many examples (lower left, Fig. 1 *B* and Fig. 2).

Association of effectiveness of and specificity to Na^+ (Fig. 2) were quite high when the sidechain was a methyl, hydroxymethyl, ethyl or hydroxyethyl group. The second α-methyl group of AIB enhanced the effect of the first for this association. When no sidechain was present (glycine) or when the sidechain had three atoms or more, these two properties decreased in association. An earlier study from this laboratory of the stereospecificity of transport [16] indicated that longer sidechains (e.g. that of methionine) decreased the degree of stereospecificity, perhaps because additional elements in the sidechain pull the β-carbon out of the statistical position it takes in site-bound alanine. The results of Fig. 2 are consistent with a binding position for the alkali ion near the β-carbon of the site-bound L-amino acid, but have little force in revealing that position.

Cysteine and serine provide a more emphatic contrast. Serine underwent the fastest uptake of any amino acid at nonsaturating levels of Na^+, a rate not decreased to any typical degree by Li^+ substitution (Fig. 2). Cysteine by contrast showed one of the lowest rate coefficients with respect to Na^+ concentration. No other position of the hydroxyl group has the effect shown by its position in serine. Furthermore, the presence of the 3-hydroxyl group in threonine, in β-hydroxylvaline and in 3-hydroxyproline led to poor response to Na^+. This difference from serine may arise from the relative restraint placed on the positions that the hydroxyl group can take, in the three former amino acids.

The ordinary and the *allo* forms of 4-hydroxyproline earlier showed enormous differences for cooperativity with Na^+ uptake in System *ASC*, to help fix the relative positions taken by the two co-substrates in that system. For System *A*, the insertion of the 4-hydroxyl group lowers the maximal rates attainable by either Na^+ or Li^+ (Fig. 1 *B*). These results show that the alkali-ion takes entirely different relative positions in the two Na^+-dependent systems. In two isomeric 3,4-dihydroxyprolines [13], the two hydroxyl groups being in one case *cis* and in the other case *trans* to the carboxyl group, hydroxylation was also unfavorable to response to the alkali-ions.

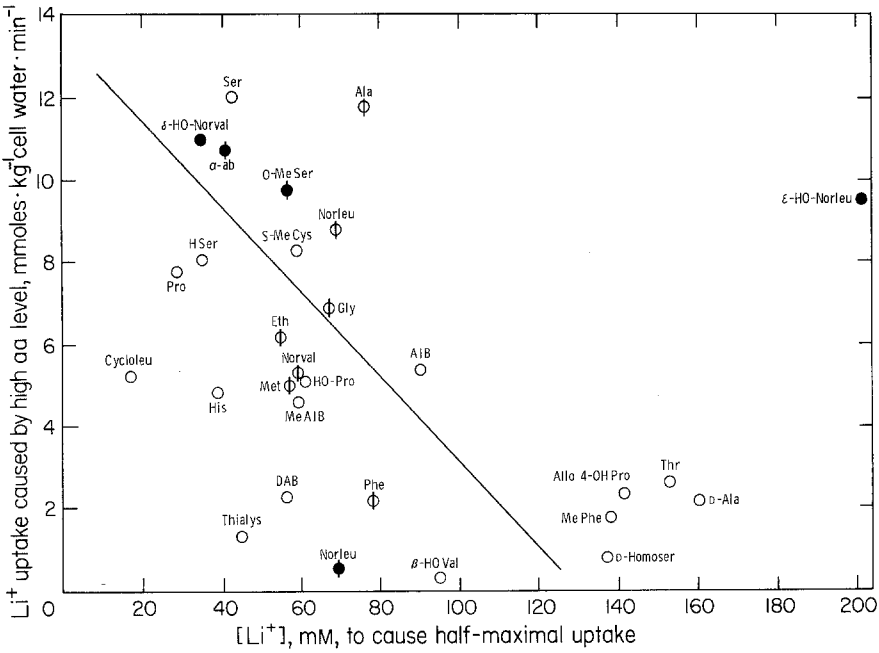

Fig. 3. Relation between the influx of Li^+ (at 100 mM) caused by a given amino acid at high levels (25 to 75 mM), and the Li^+ concentration needed to produce half-maximal uptake of that amino acid. *See* Materials and Methods for details

Li^+ Uptake Occasioned by Various Amino Acids

In the second type of experiment we compare the amino acids as to their effectiveness in stimulating the uptake of Li^+. In Fig. 3 the ordinate scale shows about a 50-fold range in the uptake of Li (at 100 mM) stimulated by various amino acids, each at a level nearly saturating its transport. The Figure plots the Li^+ uptake produced by each amino acid against the concentration of Li^+ required to half-maximize influx of that amino acid at 0.5 mM (1 mM for DL isomers), as obtained in the results of Fig. 1*A*. Although a tendency to a negative relation may be noted, one may note that for the 10 less polar amino acids the Li^+ required does not vary greatly. The stimulation by serine of the highest Li^+ uptake should be noted, with threonine again in strong contrast (Fig. 3).

Table 1 shows the effects of substituting 100 mM Li for 100 mM Na^+ on the K_m and V_{max} values shown by several amino acids in modifying their own uptake. The advantage of Na^+ appeared in about half the cases to arise from more favorable values for K_m and in about half

Table 1. Kinetic comparison of 100 mM Li^+ and 100 mM Na^+ in determining effectiveness of amino acids in driving their own transport[a]

Amino acid taken up	Uptake measured in 100 mM Li^+			Uptake measured in 100 mM Na^+			f
	V_{max}	K_m	V_{max}/K_m	V_{max}	K_m	V_{max}/K_m	
L-Methionine	7.35	1.83	4.0	6.91	1.26	5.5	0.73
L-Ethionine	7.12	1.28	5.6	8.9	0.50	18.0	0.31
L-Norleucine	11.8	6.38	1.85	17.7	3.96	4.4	0.21
S-Me-L-cysteine	3.74	1.92	1.96	4.1	0.91	5.2	0.38
O-Me-DL-Serine	14.4	4.1	3.5				
L-Histidine	1.48	3.51	0.42	4.36	2.64	1.65	0.16
L-2,4-Diamino-butyric acid	4.3	14	0.30	14.4	25	0.68	0.45
L-Thialysine	8.9	40	0.22	16.8	15.9	1.06	0.21
Glycine	10.5	5.75	1.83	17.5	3.18	5.52	0.33
Sarcosine	7.1	9.4	0.76	7.6	4.05	1.89	0.40
D-Alanine	4.9	40.2	0.12	10.9	16.5	0.66	0.18
AIB	6.9	2.91	2.4	13.4	1.11	12.1	0.20
MeAIB	3.3	1.35	2.4	4.3	0.29	14.7	0.16
Cycloleucine	6.2	3.7	1.67	15.4	1.95	7.9	0.21
N-Me-Cycloleucine	3.1	5.8	0.54	5.7	1.20	4.7	0.11

[a] The amino acids studied here are not to a perceptible degree substrates of System *ASC*, so the alkali ion dependent uptake is attributed to System *A*. Uptake of the amino acid into the Ehrlich cell measured during a time yielding approximately an initial rate, 1 to 5 min at 37 °C, pH 7.4 from Krebs-Ringer's bicarbonate medium containing the selected alkali metal ion at 100 mM, and choline chloride to compensate for the variation introduced in amino acid concentration. The kinetic parameters for the amino acids have the usual meaning, i.e. $V_{max}^{aa/aa}$ and $K_m^{aa/aa}$. The ratio in the last column is between the quantities in the third and sixth numerical quantities, and shows a sixfold range in the effectiveness of Li^+ relative to Na^+ by this test.

the cases from more favorable values of V_{max}. Table 2 compares the stoichiometry of the interdependent fluxes of the alkali ion and the amino acid, for the same group of amino acids. In agreement with preliminary results of Dr. Lynton R. Smith in this laboratory [2], these results show that somewhat less than one lithium ion is usually taken up for each amino acid molecule; furthermore the ratios show no tendency to approximate unity. For Na^+, less than half an equivalent of Na^+ usually accompanied each amino acid molecule. A structure-dependent, nonintegral stoichiometry of co-transport has already been shown for System *ASC* in the pigeon red blood cell [15], although for that system well over one Na^+ accompanied each amino acid, except in the case of proline. These discrepancies from integral stoichiometry ap-

Table 2. Stoichiometry ratios in the inward co-transport of several amino acids with Li^+, compared with the corresponding ratios for Na^+[a]

Amino acid taken up	ΔvLi/Δvaa (Li)	ΔvNa/Δvaa (Na)	Amt. of Li relative to amt. of Na entering with one amino acid molecule
L-Methionine	0.46	0.68	0.68
L-Ethionine	0.92	0.50	1.8
L-Norleucine	0.78	0.32	2.4
S-Me-L-Cysteine	3.1	1.50	2.1
L-Histidine	3.2	1.35	2.3
L-2,4-Diaminobutyric acid	0.79	0.53	1.5
L-Thialysine	1.35	0.61	2.2
Glycine	0.74	0.30	2.5
Sarcosine	0.65	0.59	1.1
D-Alanine	0.23	0.26	0.9
AIB	0.67	0.51	1.3
MeAIB	1.25	0.46	2.7
Cycloleucine	0.89	0.28	3.2
N-Me-Cycloleucine	0.88	0.17	5

[a] Except for an omission, these are the same amino acids and the same experiments listed in Table 1. Li^+ uptake measured by flame photometry of the cell extract; $^{22}Na^+$ uptake measured by liquid scintillation counting. The usual corrections have been made for interstitial suspending fluid, for the amino acid-independent alkali ion entry, and for the alkali ion-independent amino acid entry. Hence these are augmentation ratios, e.g. ΔvLi = amino acid-dependent uptake of lithium. Δvaa(Li) = lithium-dependent amino acid uptake.

peared to arise from a variable and independent release of the two co-transported species from the carrier [5, 15], which probably occurs also for System *A*.

These results show that even though Li^+ appears the less favorable co-substrate by the tests of Figs. 1 and 2, strikingly more Li^+ than Na^+ is usually co-transported with the amino acid (Table 2). The amino acids for which this sentence applies are taken up from 2.4 to 6 times as fast in Na^+ as in Li^+. Fig. 4 seeks to correlate the relative amounts of Li^+ and Na^+ taken up for each accompanying amino acid molecule, from Table 2, with the relative rate coefficients in Li^+ and in Na^+, from Fig. 2. Even though the amino acids selected for test in Tables 1 and 2 are a heterogeneous lot, we note in Fig. 4, if we except the three amino acids with two alkyl groups attached to the α-carbon (one by ring formation), a tendency for the relative rate constant with respect to B^+ at nonsaturating levels to rise in correlation with the proportion

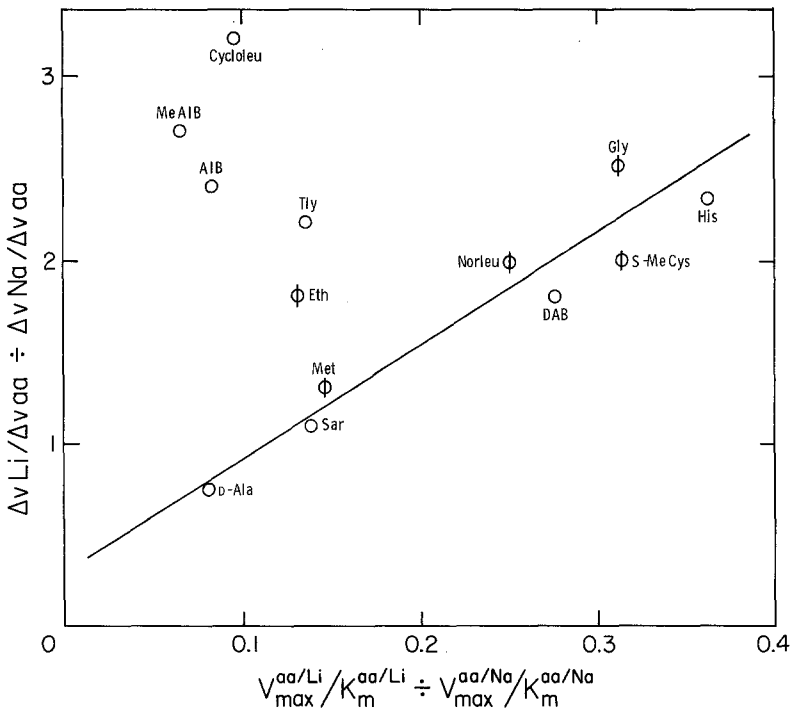

Fig. 4. Flux-augmentation ratio for Li^+ and for a given amino acid, relative to the flux-augmentation ratio for Na^+ and the same amino acid (from Table 2), plotted against the relative rate coefficient measuring the alkali-ion stimulation of the uptake of the alkali ion concentration (from Fig. 2). The line has been drawn to represent a rough relation for the amino acids in which the alpha carbon is not tertiary

in which the two alkali ions move inward in association with a single amino acid. The relative low rate for the three exceptional amino acids in Li^+, to be discussed later, could arise from the necessity that larger amounts of Li^+ need to co-migrate in relation to the amount of Na^+.

Structural Features that Allow Neither Na^+ nor Li^+ to be Highly Effective as Co-Substrates. The D *Configuration*

The low first-order rate constant for the transport of D-amino acids allowed by Na^+ was not accompanied by an elevated tolerance for Li^+ (Fig. 2). On the contrary D-alanine showed a much lower relative tolerance for Li^+ than did L-alanine by the criterion of Fig. 2, and the two isomers of homoserine show indistinguishable relative tolerances for Li^+ despite the large differences in their response to Na^+. A line, not

shown, approximately described by the four D-amino acids at the lower right of Fig. 1*A* does not correspond to that of any other group of amino acids. In association, the rates of Li^+ uptake allowed by high concentrations of the D isomers were lower than for any other amino acids studied (ordinate of Fig. 3). The augmentation of Li^+ entry due to the presence of D-alanine was also exceptionally low at low amino acid levels, whether stated in relation to the Li^+-augmentation of the amino acid uptake, or to the corresponding augmentation ratio for Na^+ uptake (Table 2).

Glycine does not share these aberrations of the D-amino acids. Its behavior by the tests of Figs. 2 and 3 falls with that of amino acids with sidechains in the ordinary α_1 position. During its uptake glycine occasions 2.5 times as rapid uptake of Li^+ per molecule of entering amino acid at it does of Na^+ in Na^+-containing medium (Table 2).

These results mean that the presence of a sidechain in the α_2 position, as in D-alanine, rather than its absence in the α_1 position, is the feature unfavorable to the binding of both alkali ions, and to the relative action of Li^+ in particular. In AIB and its N-methyl derivative, an α-methyl in the α_1 position, superimposed on the one in the α_2 position, largely led to the correction of the aberration shown by D-alanine, whether by the criterion of slowness of uptake in Na^+, or of the relative effectiveness of the two alkali ions according to Fig. 2, or of the relative stoichiometries of co-transport of the two ions (Table 2). Some peculiarities persist, however: Uptake attainable by these two α,α-dimethyl derivatives of glycine is only 0.20 and 0.17 as fast in 100 mM Li^+ as in 100 mM Na^+, handicaps larger than for any L isomer studied in Table 1. Recall also the preculiarity they share in Fig. 4 with the analogous cycloleucine. We conclude that the α_2-methyl group, although modifying co-transport, does not *per se* greatly restrict or deform the subsite for the alkali ion by obtruding into it. Instead the α_2-methyl group appears to cause D-alanine to assume a somewhat faulty position at the site, a position not taken to any great extent by glycine, and one that fails to complete well the subsite otherwise available for the alkali ion. The introduction of an α_1-methyl group appears to correct, in a statistical sense, the position taken by the D molecule. This righting effect on the amino acid from a presumably somewhat "tumbled" position at the site may arise either from steric factors or from apolar forces on the α_1-methyl group. In contrast to the α_1-methyl group, an α_1-ethyl group in isovaline fails to correct the difficulty seen with D-alanine (Fig. 2).

Effects of N-methylation. The N-methylation of glycine accelerates

Table 3. Contrasting effects of N-methylation of L and D isomers on interaction between substrate and co-substrate[a]

	Δv for Li^+ due to amino acid (mEq kg^{-1}) (cell water·min^{-1})	Δv for Na^+ due to amino acid (mEq kg^{-1}) (cell water·min^{-1})	Δv for amino acid due to alkali ion (mmoles/kg^{-1}) (cell water·min^{-1})	
			Li^+	Na^+
L-alanine	11.7	5.9	[b]	[b]
N-Me-L-alanine	6.7	2.9	0.76	2.2
D-alanine	0.0	1.3	0.12	0.63
N-Me-D-alanine	4.8	3.3	0.31	1.1

[a] The central columns compare in simultaneous 1-min tests Li^+ and Na^+ uptake due to the presence of the amino acid at 25 mM. The right-hand columns show the uptake of the amino acid at 1 mM due to the presence of Li^+ or Na^+ at 100 mM measured in a separate experiment using a 60-min heating period to develop the color with ninhydrin of effluent from the amino acid analyzer.

[b] Not determined. Parallel uptake of Na^+ with L-alanine by System *ASC* would strongly distort this comparison.

its uptake as a function of $[Na^+]$ with an unexceptional gain in specificity to that ion (Fig. 2). The methylation handicaps the apparent binding of Na^+ (Fig. 1*A*) but accelerates the rate-limiting event of transport (Fig. 1*B*). This behavior is consistent with the idea that most of the bound glycine molecules, if not propped by an N-methyl group, take positions at the site favorable to cation binding but not to a subsequent translocative event.

An N-methyl group introduced into L-alanine is unfavorable to the migration of Na^+ and more particularly to that of Li^+. In contrast, N-methylation of D-alanine greatly increases the quantities of Na^+, Li^+ and the amino acid taken up under standard conditions (Table 3). The N-methyl group has minimized the difference in the abilities of the two isomers to occupy the site effectively, particularly when Li^+ is the co-substrate. This change would not be expected if the ideal position of the alkali ion were perfectly symmetrical with respect to the asymmetric carbon atom. When two α-methyl groups are present as in AIB, N-methylation has only a small effect on the relative responses to Na^+ and Li^+, although both K_m and V_{max} for the amino acid with respect to its own concentration are sharply decreased (Figs.1, 2; Table 4). Only for cycloleucine, N-methylation causes a major exclusion of Li^+, the K_m for this ion being increased 2.5-fold, or 3.7-fold with respect to Na^+

Table 4. Difference in effect of N-methylation on the selectivity of 3 amino acids between Na^+ and Li^+. Uptake of the amino acid, 0.5 mM, observed during 1 min at various concentrations of the alkali ion at pH 7.4 and 37 °C

	Alkali-ion concentration half-maximizing amino acid uptake, mM				Coefficient for change in Na^+ preference on methylation[a]
	not methylated		methylated		
	Na^+ *a*	Li^+ *b*	Na^+ *c*	Li^+ *d*	
Glycine	22.5	67	28.5	52	0.61
2-Aminoisobutyric acid	13.1	90	8.4	59	0.98
1-Aminocyclopentane carboxylic acid	11.7	16.5	7.8	41	3.7

[a] $d/c \div b/a$.

(Table 4). According to the test of V_{max}/K_m in Table 1, an N-methyl group intensifies the relative disadvantage of Li^+ not at all for glycine, only slightly for AIB, but by 1.9 times in the case of cycloleucine.

Structural Effects that Allow Both Na^+ and Li^+ to be Highly Stimulating

Diamino acids. Some diamino acids with $pK'_2 \leqq 8.5$ show paradoxical intensity of transport by two neutral systems [5, 6]. 2,4-Diaminobutyric acid and thialysine show extreme positions for their rate responses to Na^+ (Figs. 1 *B*, 2). Their uptake as a function of $[Li^+]$ is very fast in relation to their fast uptake in Na^+ (Fig. 2). At 75 mM, these two amino acids stimulate quite ordinary rates of uptake of both alkali ions. An unusually high specificity of System *A* to the L isomer of diaminobutyric acid, compared with the isosteric homoserine (Fig. 3 in Ref. 7) shows that the distal amino group must take a definite position in space for uptake by this system to be fast. The similar transport behavior of 2,3-diaminopropionic acid points to a position for the responding structure near carbons 3 and 4 of the site-bound amino acid molecule. We see no reason for suspecting the bound alkali ion of participating in the recognition of the omega amino group.

Concluding Discussion

The foregoing results show a widespread sensitivity of kinetic aspects of the selectivity between Na^+ and Li^+ as the co-substrates for System *A*

to various details of the structure of the amino acid substrate. These results argue but do not prove that the alkali ion is bound for System *A* transport in close juxtaposition to the amino acid molecule, as shown for System *ASC* of the pigeon red blood cell [18, 19] (*see* Fig. 5). A very different position must apply, however, for System *A*. The detailed kinetic relations support at some points but violate at others the several intuitions one might set up, which we will not list here.

Some of the strongest effects on co-substrate interaction have been produced by structural changes close to the α-carbon. Recall the sensitivity to the unrestrained hydroxyl group of serine. These changes often concern apolar structures. Recall the potent effects of inserting the first methyl group on the α-carbon, or in repositioning it from the α_1 to the α_2 locus. Recall also the reversal of most of the effects of the α_2-methyl group on adding a second α-methyl group to AIB. Recall also the effects of introducing a single N-methyl group, which is favorable to co-substrate reactivity only when no sidechain is present or when the sidechain is present in the "wrong" α_2 position; otherwise its effect is unfavorable. These results urge our attention to the possible importance of the attitude taken by the amino acid molecule at the site, and they support a position for the alkali ion close enough to allow it to respond to the presumed changes in attitude, even when the molecule may thrust nothing but two hydrogen atoms, or one hydrogen atom and a methyl group, into the space on the side of the molecule opposite to the α-amino and α-carboxyl groups.

As one examines space-filling atomic models, three approximate positions for the alkali ion with respect to the amino acid molecule may be considered, using the AIB molecule for reference. Each of these is accessible to bonding with the oxygen atom lying on the L-serine sidechain.

1) A position more or less directly beyond the α-carbon, i.e. on the side of the molecule opposite to the α-amino and α-carboxyl groups. This position would have to lie beyond the two methyl groups of AIB, since there is insufficient space between them.

2) A position adjoining and intruding into the shallow crypt described by the amino group and the two methyl groups of AIB.

3) A position correspondingly near the shallow crypt formed by the same two methyl groups and the carboxylate group of AIB. We have seen no support for this position in our results.

The first position is not decisively excluded by the strong System *A* transport of cycloleucine, because the two-carbon bridge which it possesses attached to the two methyl groups of AIB might be inclined toward

either the amino or the carboxyl group, and therefore not obstruct the space directly beyond the β-carbon atoms. The unusual properties shown in the co-substrate interaction of cycloleucine indicates that this added structure falls in a sensitive position: Although it resembles AIB in the test of Fig. 4, it shows a uniquely low selectivity for Na^+ with respect to Li^+ for apparent binding of the alkali metal (Fig. 1 *A*) and a uniquely high selectivity for Na^+ as estimated by the apparent relative activity of the two cations for catalyzing amino acid transport (Fig. 1 *B*). Note that these two opposed effects are produced here without introducing any functional group into the amino acid molecule. It would be helpful for further probing the region around the β-carbon atoms to be able to restrain the orientation taken by the two-carbon bridge, e.g. by further bonding it to the amino or N-methyl group. Sidechain groups introduced onto the α- or β-carbons of cycloleucine (or its cyclohexane analog) have so far largely eliminated reactivity with System *A*, showing how restricted space is in this area. A single methylene group in the form of a one-carbon bridge (i.e. in the norbornane amino acid, [8]) brings the reactivity with System *A* below detectible levels in our hands and for our line of the Ehrlich cell. Perhaps its unreactivity with System *A* arises from the exclusion of Na^+ from its subsite, rather than from an overcrowding limited to the amino acid molecule *per se*.

α,α-Diethylglycine shows immeasurably slow Na^+-dependent uptake by the Ehrlich cell and no measurable inhibition of uptake by System *A* [3, 10]. We now find that Li^+ also fails to augment uptake of this amino acid. Clearly its two terminal methyl groups take positions unfavorable to binding of the combination of the two co-substrates. The transport consequences of drawing together the distal carbon atoms of this analog to form the cyclopentane ring [17], as discussed previously [10], are remarkable.

A position in region No. 1 corresponds well to the behavior described for serine, but it leaves unexplained the potent effects of the introduction of an N-methyl group into the smaller amino acid molecules or of an α-methyl group into D-alanine.

Position No. 2 for the alkali ion is the one supported by this research, most specifically by the high sensitivity of the transport and of the co-substrate interaction of amino acids to the introduction of an N-methyl group. Some of these effects appear to include a stabilization of the attitude of the amino acid molecule with respect to the site (e.g. of glycine or D-alanine) and to the space occupied by the metal ion – in a way that does not necessarily imply contact between the methyl

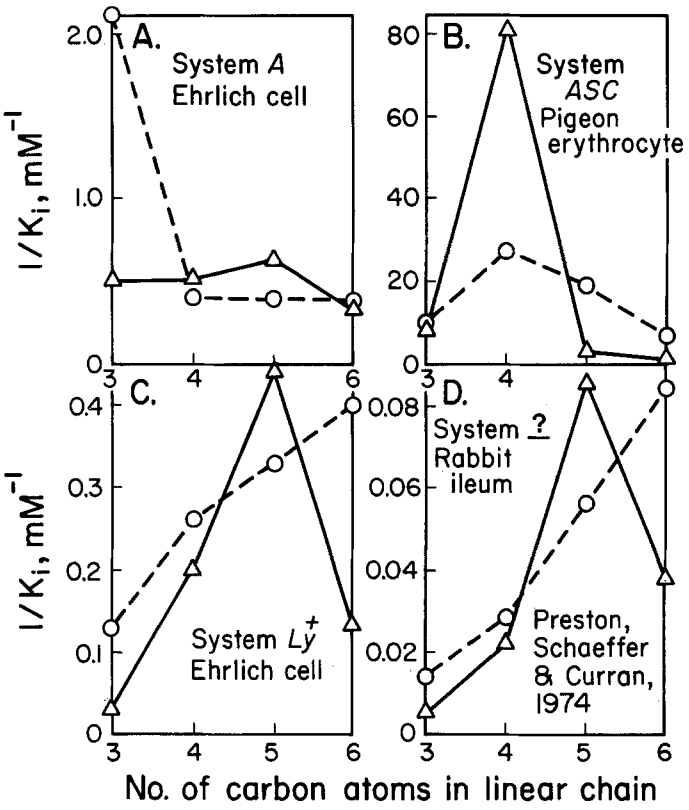

Figure introduced in proof.

Fig. 5. *Comparison of transport systems as to sensitive position for the omega hydroxyl group on a linear amino acid side chain.* Dashed lines and circles show the apparent affinity of the 3-, 4-, 5- and 6-carbon amino acids, alanine, α-amino-*n*-butyric acid, norvaline, and norleucine; solid lines and triangles show the corresponding values for the omega-hydroxy derivatives of these 4 amino acids, in Part *A* on the basis of inhibition of uptake of 2-(methylamino)-isobutyric acid, i.e., by System *A*, as determined in the present research. Part *B* compares affinities of the same 8 amino acids by their inhibition of uptake of L-serine by the pigeon erythrocyte, i.e., by System *ASC* [19]. Part *C* compares affinities through the Na^+-dependent inhibition of uptake of L-homoarginine by the Ehrlich cell, i.e., by System *Ly*$^+$ (E.L. Thomas, T.-C. Shao, H.N. Christensen (1971). *J. Biol. Chem.* **246**:1677). Part *D* compares affinities through inhibition of uptake of 2 mM L-methionine by sections of the mucosal surface of rabbit ileum (replotted from R.L. Preston, J.F. Schaeffer, and P.F. Curran (1974) *J. Gen. Physiol.* **64**:443). δ-Hydroxynorvaline and ε-hydroxynorleucine were used in their DL forms and the inhibitory action attributed to the L-form. Uptake during 1 min from modified Krebs-Ringer bicarbonate media containing fixed Na^+ levels in the range 118 to 140 mM. In contrast to the other systems, System *A* shows a large influence by the ω-hydroxy group only when it is on carbon 3, and then in the negative direction. For System *ASC* the sensitive position is C4, for *Ly*$^+$ and the intestinal system, *C5*. We believe we have shown for Systems *ASC* and *Ly*$^+$ (and now for System *A*) that these positional specificities are determined in each case by the position taken by the alkali ion with respect to the membrane-accepted amino acid. The intestinal system studied in Curran's Laboratory resembles in this regard Systems *Ly*$^+$ and *ASC*, but not System *A*

group and the alkali ion. But in the transition from AIB to cycloleucine, the effect of N-methylation appears much more informative as to the position taken by the inorganic ion. N-Methylation of AIB leads as for glycine and D-alanine to enhanced co-substrate reactivity with Li^+. But when the ethylene bridge is added, in a way of speaking, across the two α-methyl groups of AIB to produce cycloleucine, the K_m of the N-methyl derivative for Li^+ increases 3.7 times with respect to that for Na^+. The N-methyl group has apparently changed its contribution from one of helping make a place suitable for Na^+ or Li^+ to one of obstructing the entry of the larger lithium ion, whether judged on a relative or absolute basis. It matters little which way we picture the ethylene bridge as inclined; whether toward the amino or toward the carboxyl group, it can decrease the space available between the N-methyl group and two methylene groups of the sidechain structure. One inclination would place the two β-methylene groups, the other the two γ-methylene groups, into region No. 2.

Although largely without support, position No. 3 has not lent itself to probes of the same sort. We know as yet no means for crowding the area of the carboxyl group in a way corresponding to the effect of a methyl group on the amino group.

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