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SODIUM DEPENDENCE OF MAXIMUM FLUX, J_M, AND K_m OF AMINO ACID TRANSPORT IN EHRLICH ASCITES CELLS

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SUMMARY

The Michaelis–Menten parameters, J_M and K_m of the initial 1-min fluxes of uptake of L-phenylalanine and of α-aminoisobutyric acid were determined for extracellular concentrations of Na^+ ranging from 0.5 to 110 mequiv/l for Ehrlich ascites tumor cells. The maximal initial flux, J_M, decreased with decrease in extracellular Na^+ for both α-aminoisobutyric acid and phenylalanine but the K_m for α-aminoisobutyric acid increased markedly as the Na^+ concentration fell whereas the K_m for phenylalanine decreased. Cycloleucine behaved like phenylalanine.

The data provide strong evidence that the Na^+-independent flux of phenylalanine is an exchange diffusion flux that can be varied by changing the intracellular level of amino acids such as phenylalanine. For phenylalanine, cycloleucine, and methionine this exchange diffusion flux appears to be additive with the Na^+-dependent initial flux. α-Aminoisobutyric acid also has an exchange diffusion that is Na^+-independent but it has a high K_m and is not additive with the Na^+-dependent flux.

INTRODUCTION

Transport of neutral amino acids in many cells is inhibited if Na^+ is omitted from the medium. The evidence that Na^+ actually enters with the amino acid in a cotransport has been reviewed recently by Schultz and Curran[1]. In many of the published studies on the effect of Na^+, the flux of amino acid is determined at some arbitrary concentration of amino acid, as a function of the extracellular concentration of Na^+. For a given, fixed concentration of Na^+ in the medium the initial flux of amino acid is well described by the relation given in Eqn 1.

$$J = \frac{J_M c_e}{K_m + c_e} + k(c_e - c_i) \tag{1}$$

In the above equation, k is the apparent permeability constant given by the linear component of the uptake process, J_M is the maximum saturable flux and K_m is the equivalent of the Michaelis–Menten constant for the saturable component of the uptake. Experimental examples are available of transport systems in which only

Abbreviations: BCH, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid; MPA, 4-amino-1-methylpiperidine-4-carboxylic acid.

K_m is affected by the Na^+ concentrations in the medium[2,3], only J_M is affected[4], and in which both J_M and K_m are Na^+ dependent[1,5]. Hence, a complete experimental analysis of the effect of Na^+ on the saturable component of an initial flux should include studies at a sufficient number of Na^+ and substrate concentrations to provide data on the Na^+ dependence of both J_M and K_m.

In a recent study Jacquez *et al.*[6] found that the J_M values for the initial fluxes of α-aminoisobutyric acid and phenylalanine in Ehrlich ascites cells were both markedly decreased in media in which choline replaced the sodium. However, the K_m for α-aminoisobutyric acid was increased whereas that for phenylalanine was decreased in the choline media. The present experiments were undertaken to determine the Na^+ dependence of J_M and K_m of the initial fluxes of α-aminoisobutyric acid and phenylalanine in this system in more detail with the expectation that this will help us to understand why the two differ and how this difference relates to the A and L systems as defined by Oxender and Christensen[7]. In the course of this work data on a few other amino acids were also obtained and are presented. The L system was originally defined[7] as a poorly concentrating system that serves well for exchange and does not require Na^+. The A system on the other hand was defined to be a Na^+-requiring transport system that was strongly concentrating. Although most amino acids appeared to show some affinity for each of these systems, phenylalanine was described as having a substantial L component whereas α-aminoisobutyric acid was considered to be taken up almost entirely by the A system. Since in the above-mentioned study[6] it was found that the J_M for both phenylalanine and α-aminoisobutyric acid were decreased by about 90% in choline media as compared to high Na^+ media it appeared plausible that both amino acids are strongly transportable by the A system and that the explanation for the apparent difference in their uptakes is related to the difference in the effects of Na^+ on their K_m values.

METHODS AND MATERIALS

The Ehrlich ascites cells used in these experiments were from a hypotetraploid line carried in this laboratory by weekly intraperitoneal injection into female Swiss albino mice. We carry two such lines which we call our Hi and Lo lines. These differ in that the Hi line grows faster, tends to be more hemorrhagic and kills the animals in 10–14 days whereas the animals carrying Lo line usually survive 21 days or even longer. When tested with glycine, methionine and tryptophan at 1 mM concentration the Hi line gave distribution ratios 1.2–1.5-fold higher than the Lo line for 2-min and for 1-h incubations. Previous studies reported from this laboratory have all been on the Hi line cells. However, because the Lo line is less hemorrhagic most of the studies reported in this paper have been on the Lo line. The general experimental methods used in this laboratory have been described in previous publications[6,8,9].

Two main types of experiments on the concentration dependence of 1-min initial fluxes are reported here. In one, only high Na^+ and Na^+-free media were used. The cells were collected into sodium Krebs–Ringer phosphate [100 ml 154 mM NaCl, 13.5 ml 100 mM K_2HPO_4, 1.5 ml 154 mM KH_2PO_4 and 0.25 ml of each of 110 mM $CaCl_2$ and 154 mM $MgSO_4$; pH 7.35]. After one wash the cells were resuspended in 150 ml sodium Krebs–Ringer phosphate and were incubated for ½ h at 37 °C. This incubation was used to load the cells with unlabeled

amino acid for the exchange experiments. The cells were then washed three times with cold choline Krebs–Ringer phosphate of the same composition as the sodium Krebs–Ringer phosphate except that choline chloride replaced NaCl mole for mole. At this stage the intracellular Na^+ level was generally in the range 5–10 mM. Thus up to the final incubation all cells were treated the same way and had the same intracellular Na^+ levels. This was an improvement in experimental design over the previous experiments[6] in which the cells incubated in the high Na^+ medium were washed with the high Na^+ medium and hence had higher intracellular Na^+ levels than the cells that were washed and incubated in the choline medium. 1 ml of cell suspension was placed in the sidearm of a 125-ml modified erlenmeyer flask and 5 ml of amino acid solution in choline Krebs–Ringer phosphate or in sodium Krebs–Ringer phosphate was placed in the center well. A set of flasks covering the range of amino acid concentrations 0.5–60 mM was set up in each experiment. The final medium concentration of K^+ was 25 mM and the final Na^+ concentration was 111 mM in the sodium Krebs–Ringer phosphate experiments and 0.5–1.5 mM in the choline Krebs–Ringer phosphate experiments. After a 2-min incubation in a shaker bath for temperature equilibration the sidearm was tipped in and the incubation continued for another minute. These final suspensions had packed cell volumes in the range 4.0–7.0%. The flasks were then chilled in ice water and taken to a cold room for further processing.

2 ml of the cell suspension was pipetted into 12 ml of ice cold medium and the cells packed by centrifugation. For counting, 0.5 ml of undiluted supernatant fluid and 2 ml of absolute ethanol were added to 13 ml of our scintillation counting fluid. This gave a clear one phase solution. The counting fluid is a mixture of 10 ml of a toluene solution of scintillators and 3 ml of absolute ethanol. The pellets were dissolved in 2 ml of NCS tissue solubilizer (Amersham-Searle) and added to 13 ml of counting fluid. For the amino acids used in this study we have found that for incubations of 1 min there is less than 1% difference between tracer label extractable from the pellets with 75% ethanol and that determined by dissolving the entire pellet in NCS tissue solubilizer. The extracellular spaces in the packed pellets were determined in separate but parallel flasks as the sucrose space or more often as the sorbitol space.

In the second type of experiment a more detailed examination of the Na^+ dependence of uptake of phenylalanine and α-aminoisobutyric acid was carried out. A Tris–phosphate buffered Krebs–Ringer phosphate was used so that these experiments could be run at lower concentrations of extracellular K^+. The Tris–phosphate Krebs–Ringer phosphate was made up by mixing 96 ml of a mixture of 154 mM NaCl and choline chloride, 8 ml 154 mM KCl, 0.5 ml 110 mM $CaCl_2$, 0.5 ml 154 mM $MgSO_4$ and 15 ml of isotonic Tris–phosphate buffer of pH 7.4. With this medium the extracellular K^+ was 10 mM and the Na^+ was set by controlling the ratio of NaCl and choline chloride. The Na^+ and K^+ concentrations were measured after the incubations.

The labeled amino acids used were all labeled with ^{14}C and were obtained from Amersham-Searle or New England Nuclear. The non-labeled amino acids were all of A grade from California Biochemicals. A Nuclear-Chicago Unilux II scintillation counter with external standard was used for all counting.

The fluxes and intracellular concentrations were calculated from the total

amount of amino acid found in the pellet, corrected for the material in the extracellular space of pellet. The basic data obtained for each flask were the extracellular concentration, the calculated intracellular concentration and the calculated 1-min initial flux. Fluxes were calculated in the units μmoles/g dry wt per min. The parameters J_M, K_m and k in Eqn 1 were then calculated with use of a non-linear least squares fitting program which was written in FORTRAN IV and was run on the IBM 360/67 at the University of Michigan Computing Center.

RESULTS

Fig. 1 shows concentration dependence curves of initial flux of phenylalanine at four different extracellular concentrations of Na^+ and Fig. 2 shows four such curves for α-aminoisobutyric acid. Both α-aminoisobutyric acid and phenylalanine obviously show a considerable dependence on Na^+. There is a difference between

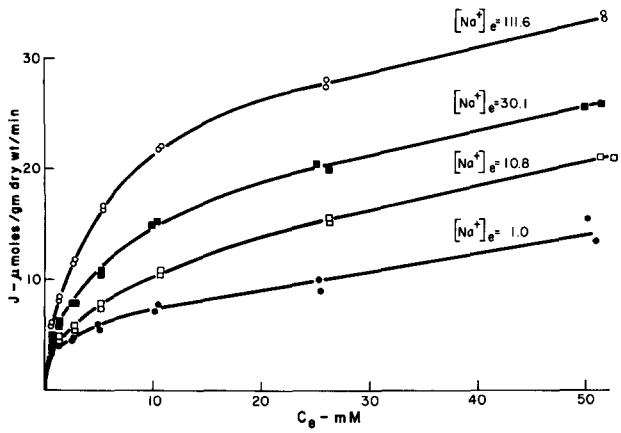

Fig. 1. Concentration dependence curves for flux of L-phenylalanine for different concentrations of Na^+ in the medium.

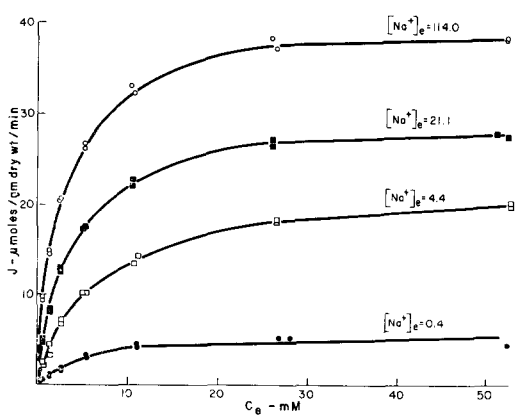

Fig. 2. Concentration dependence curves for the flux of α-aminoisobutyric acid for different concentrations of Na^+ in the medium.

the two which is reflected in the effect of Na^+ on the K_m values. For α-aminoisobutyric acid the initial slope of J *vs* c_e curve decreases considerably as $[Na^+]_e$ decreases whereas this effect is much less apparent for phenylalanine. Figs 3–6 show the Na^+ dependence of J_M and K_m. The data on the J_M and K_m, including the residual

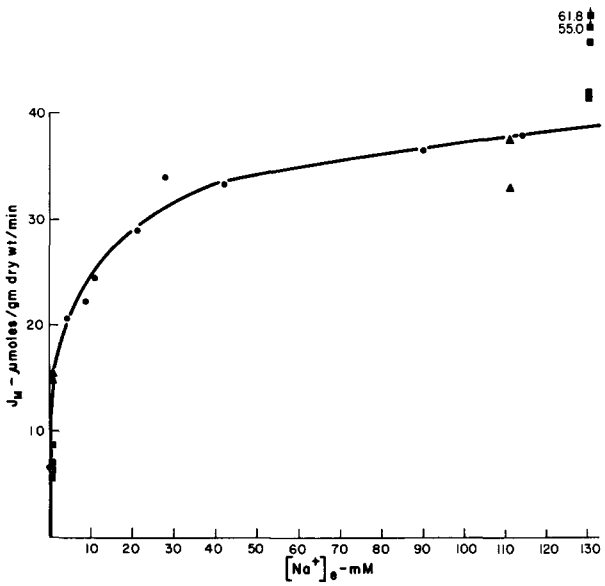

Fig. 3. Dependence of J_M on extracellular Na^+ for α-aminoisobutyric acid. ●—●, data from the Tris–phosphate experiments, $[K^+]_e = 10$ mM, pH 7.4; ▲—▲, data from potassium Krebs–Ringer phosphate reported in Table I, $[K^+]_e = 25$ mM, pH = 7.35; ■—■, data reported by Jacquez *et al.*[5], $[K^+]_e = 22$ mM, pH 7.0.

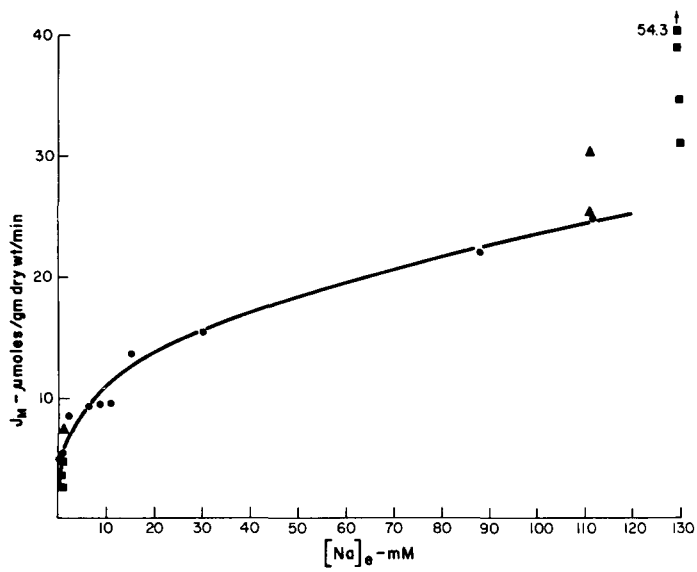

Fig. 4. Dependence of J_M on extracellular Na^+ for L-phenylalanine. The code for the data points is the same as that for Fig. 3.

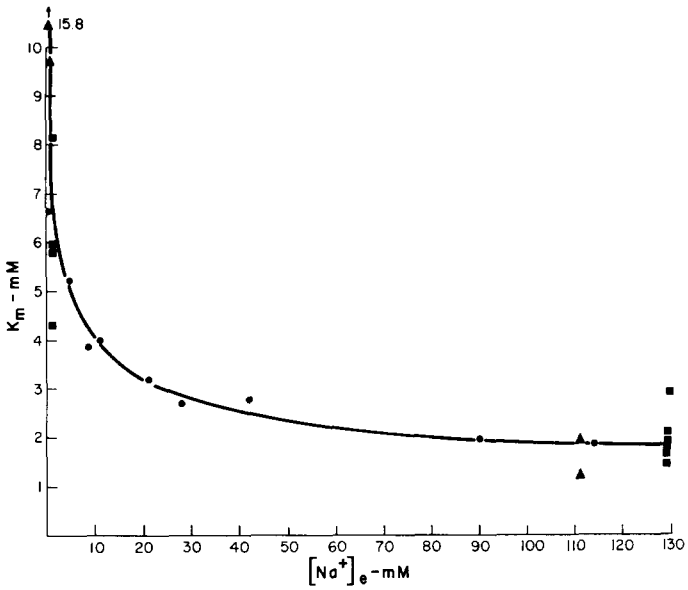

Fig. 5. Dependence of K_m on extracellular Na^+ for α-aminoisobutyric acid. The code for the data points is the same as that for Fig. 3.

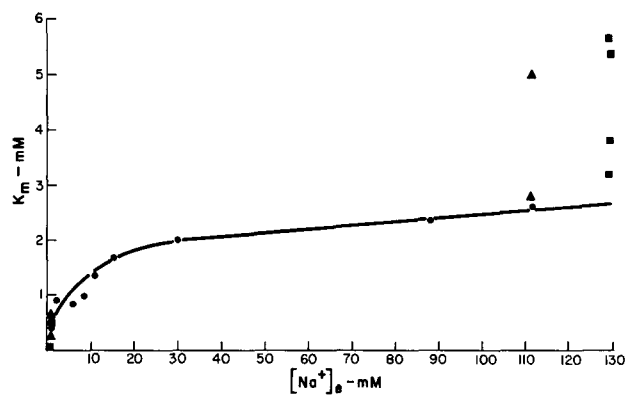

Fig. 6. Dependence of K_m on extracellular Na^+ for L-phenylalanine. The code for the data points is the same as that for Fig. 3.

sum of squares of deviations from the fit to Eqn 1, are given in Tables I and II for phenylalanine and α-aminoisobutyric acid, respectively. The curves are drawn by eye to fit the data from the Tris–phosphate experiments. Data from a previously published study on temperature dependence[6] and the data given in Table III are also included. The data from the latter two types of experiments are for different $[K^+]_e$ and slightly different pH values from those in the Tris–phosphate experiments and include results only at high Na^+ concentrations and low Na^+ concentrations. For both α-aminoisobutyric acid and phenylalanine the J_M increases with extracellular Na^+, the effect of Na^+ being larger on α-aminoisobutyric acid. The striking difference between the two is the effect on the K_m. For α-aminoisobutyric

acid the K_m decreases markedly as Na^+ increases but for phenylalanine the K_m increases as $[Na^+]_e$ increases.

The increase in the K_m of phenylalanine with increasing $[Na^+]_e$ might be a property of a single transport system, but it is also possible to interpret this in terms of the A and L systems. Thus one might argue that phenylalanine enters *via* these two systems and that the Na^+-independent L system has a very low K_m and J_M for phenylalanine. According to this argument the L system becomes saturated at low c_e, well below the K_m for the Na^+-dependent system so that the initial portions of all uptake curves should be close together as in Fig. 1. By this interpretation, the increase in K_m with increasing $[Na^+]_e$ reflects the increasing participation of the A system in the uptake. Hence, the uptake curve at 1 mM $[Na^+]_e$ should be almost entirely due to the Na^+-independent system and we should be able to correct for it. As is given in Table I, at $[Na^+]_e = 1$ mM, the parameters for the saturable component of uptake were $J_M = 5.5$ μmoles/g per min, $K_m = 0.42$ mM. The data were corrected by subtracting a component with a slightly smaller J_M, $J = 5.3\ c_e/(c_e + 0.42)$ and the k, J_M and K_m were then recalculated. The results for the original and for the corrected dara are given in Table I; in each case the residual sum of squares of deviations of the data around the fitted curve are also given. The change in the

TABLE I

PARAMETERS OF PHENYLALANINE UPTAKE BY EHRLICH ASCITES TUMOR CELLS

$[Na^+]_e$ (*mM*)	*Uncorrected*			*Residual*** *sum of squares* ($\times 10^5$)	*Corrected**			*Residual*** *sum of squares* ($\times 10^5$)
	k (*cm³/g per min*)	J_M (*μmoles/g per min*)	K_m (*mM*)		k (*cm³/g per min*)	J_M (*μmoles/g per min*)	K_m (*mM*)	
1.0	0.19	5.5	0.42	0.466				
2.0	0.12	8.6	0.92	0.246	0.10	4.3	4.6	0.190
6.2	0.11	9.3	0.85	1.498	0.08	5.5	4.6	0.304
8.4	0.10	9.5	0.99	0.767	0.06	6.8	6.8	0.474
10.8	0.25	9.6	1.37	0.521	0.12	14.9	25.9	0.074
15.3	0.18	13.7	1.70	1.11	0.12	12.2	7.1	0.527
30.1	0.24	15.5	2.05	1.09	0.16	15.4	8.3	0.356
88.1	0.28	22.2	2.37	2.20	0.21	21.1	5.8	1.200
111.6	0.23	25.1	2.63	0.86	0.17	23.3	5.5	0.286
	After 40% isotonic sodium Krebs–Ringer phosphate shock							
2.0	0.30	4.0	3.97	0.07				
85.0	0.43	39.4	13.3	1.97				
	After 50% isosmolar potassium Krebs–Ringer phosphate shock							
1.3	0.23	3.4	1.0	0.28				
85.0	0.27	32.0	4.6	3.40				

* The raw data are corrected by substracting $5.3\ c_e/(c_e + 0.42)$ from the fluxes.

** Residual sum of sqares of deviations of data points around best fitting curve of the form of Eqn 1.

TABLE II

PARAMETERS FOR α-AMINOISOBUTYRIC ACID UPTAKE BY EHRLICH ASCITES TUMOR CELLS

$[Na^+]_e$ (mM)	Uncorrected			Residual sum of squares ($\times 10^5$)	Corrected*			Residual sum of squares ($\times 10^5$)
	k (cm^3/g per min)	J_M (μmoles/g per min)	K_m (mM)		k (cm^3/g per min)	J_M (μmoles/g per min)	K_m (mM)	
0.4	—	6.6	6.6	0.033				
4.4	0.028	20.8	5.2	0.210	0.021	15.3	4.47	0.193
8.7	0.12	22.3	3.8	0.166	0.104	17.2	3.22	0.145
10.9	0.076	24.6	4.0	0.738	0.064	19.4	3.43	0.676
21.1	—	29.8	3.2	0.340	—	24.0	2.57	0.290
27.7	—	34.2	2.7	4.26	—	28.5	2.13	4.46
41.9	0.035	33.6	2.8	1.31	0.017	28.8	2.45	1.13
90.2	—	36.8	2.0	1.68	—	31.2	1.52	1.97
114.0	—	40.1	2.2	1.27	—	34.5	1.75	1.07

* The initial fluxes were corrected by substracting the value $0.62\ c_e/(10+c_e)$ from them.

residual sum of squares is not enough to clearly prefer one over the other. However, the uptake curves remaining after the correction show a new pattern; J_M still increases with $[Na^+]_e$ but except for one unusually high value, K_m is now practically constant over the range of Na^+ values. If this correction is applicable to phenylalanine it should also be applicable to the α-aminoisobutyric acid data so the curve for uptake of α-aminoisobutyric acid at $[Na^+]_e = 0.4$ mequiv/l should be primarily uptake due to Na^+-independent mechanisms. For α-aminoisobutyric acid the flux $0.62\ c_e/(10+c_e)$ was subtracted from the raw data. Although the K_m at $[Na^+]_e = 0.4$ was 6.6, the curve of K_m *vs* $[Na^+]_e$ (Fig. 5), rises sharply as $[Na^+]_e$ approaches zero so the value, $K_m = 10$, was chosen. The original and corrected values are given in Table II. The general pattern remains the same in the corrected data, J_M increases with $[Na^+]_e$ and K_m decreases with $[Na^+]_e$.

There are appreciable levels of free amino acids in Ehrlich ascites cells[10] and there is reason to believe that the Na^+-independent uptake may be due to an exchange diffusion with the free amino acids normally present in these cells because exchange diffusion has been shown to be independent of Na^+ for a number of amino acids[6,11–13]. If this is so it should be possible to decrease the Na^+-independent uptake by lowering intracellular free amino acid levels. To do this the cells were treated with 40% isotonic sodium Krebs–Ringer phosphate at room temperature or were incubated with 50% isosmolar potassium Krebs–Ringer phosphate at 37 °C and then with isosmolar potassium Krebs–Ringer phosphate prior to the experiment. Eddy has found that the latter treatment removes over 80% of the endogenous amino acids of LS mouse ascites tumor cells (personal communication). The experiments on concentration dependence of uptake of phenylalanine were then repeated in sodium Krebs–Ringer phosphate and in choline Krebs–Ringer phosphate. The results, Fig. 7, show that the initial slopes of the J *vs* c_e curves are now clearly different for the low and high $[Na^+]_e$ as for α-aminoisobutyric acid in Fig. 2. The hypotonic sodium Krebs–Ringer

phosphate shock procedure gives an increase in the linear component of uptake but with both shock procedures there is a decrease in J_M at the low $[Na^+]_e$ indicating that there is a decrease in the Na^+-independent uptake mechanism. Surprisingly the J_M at high $[Na^+]_e$ is increased over that in cells not treated with hypotonic shock. Possibly this is also directly related to the decrease in intracellular amino acids given by the osmotic shock treatment and that the intracellular amino acids decrease the J_M of the Na^+-dependent system. However, further investigation of this effect was deferred. The summary of the parameters found is given in Table I.

If the Na^+-independent uptake of phenylalanine is actually an exchange diffusion with some intracellular free amino acids, not only should it be possible to decrease it by decreasing intracellular amino acids but it should also be possible to increase it by preloading the cells with phenylalanine and the results on "untreated" cells should fit in as a transition between the results on cells treated with hypotonic shock and those preloaded with increasing levels of phenylalanine. By the definition of the L system this uptake of phenylalanine should be inhibited slightly if at all by an amino acid such as α-aminoisobutyric acid that is for the most part an A type amino acid so it was important to compare the Na^+-independent uptake and that not inhibitable by α-aminoisobutyric acid. For such experiments three 1-min initial fluxes were generally obtained on the same preparation of cells, each in duplicate and all at an extracellular concentration of phenylalanine of 1 mM. These were, the flux in sodium Krebs–Ringer phosphate, the flux in choline Krebs–Ringer phosphate

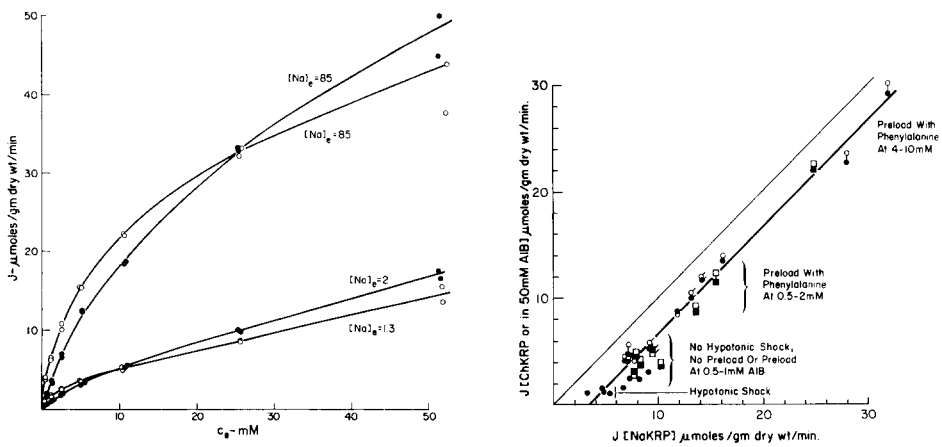

Fig. 7. Concentration dependence curves for initial flux of L-phenylalanine for cells subjected to hypotonic shock. ●—●, hypotonic shock with 40% sodium Krebs–Ringer phosphate at room temperatures; ○—○, hypotonic shock by incubation at 37 °C in 50% isosmolar potassium Krebs–Ringer phosphate.

Fig. 8. Initial flux of phenylalanine from 1 mM phenylalanine for cells in choline Krebs–Ringer phosphate or in 50 mM α-aminoisobutyric acid in sodium Krebs–Ringer phosphate (NaKRP) plotted against the initial flux for the same preparation of cells in sodium Krebs–Ringer phosphate. Hi line: □ in choline Krebs–Ringer phosphate, ■ in 50 mM α-aminoisobutyric acid (AIB); Lo line: ○ in choline Krebs–Ringer phosphate (ChKRP), ● in 50 mM α-aminoisobutyric acid (AIB). Fluxes in choline Krebs–Ringer phosphate and 50 mM α-aminoisobutyric acid for the same preparation of cells are linked by a vertical line. A slant line on a symbol, *e.g.* ◌́, means the medium was a Krebs–Ringer bicarbonate.

in which $[Na^+]_e$ was usually less than 2 mM, and the flux in sodium Krebs–Ringer phosphate in the presence of 50 mM α-aminoisobutyric acid. The concentration of 50 mM α-aminoisobutyric acid was chosen because it was found that over 90% of the inhibition of the phenylalanine flux obtainable with α-aminoisobutyric acid was obtained at that concentration and above 50 mM α-aminoisobutyric acid the inhibition increased very slowly with increase in α-aminoisobutyric acid concentration. The flux of phenylalanine inhibited by 50 mM α-aminoisobutyric acid was close in value to the Na^+-dependent flux, *viz.*, the difference between the phenylalanine flux in sodium Krebs–Ringer phosphate and in choline Krebs–Ringer phosphate, although it was usually a little larger. Inui and Christensen[5] reported the same sort of results for methionine. Fig. 8 presents the results and includes data on the Hi line and Lo line as well as from a few experiments in which a bicarbonate buffered Krebs–Ringer was used. In Fig. 8 the fluxes in choline Krebs–Ringer phosphate and in 50 mM α-aminoisobutyric acid in sodium Krebs–Ringer phosphate are plotted on the ordinate against the flux in sodium Krebs–Ringer phosphate for the same preparation of cells. As is shown in the figure, the Na^+-dependent and α-aminoisobutyric acid inhibitable component is approximately constant at a level of 3.5 μmoles/g dry wt per min for an extracellular concentration of 1 mM phenylalanine. The remainder, the Na^+-independent flux is variable and can be decreased by subjecting the cells to hypotonic shock or increased by loading the cells with phenylalanine. Thus for cells at the lower left end of Fig. 8 most of the flux is Na^+-dependent and α-aminoisobutyric acid inhibitable whereas for cells at the upper right of Fig. 8 only a small fraction of the flux is Na^+-dependent and α-aminoisobutyric acid inhibitable, but for both the Na^+-dependent flux is approximately the same in absolute magnitude. Thus these data provide strong evidence that for phenylalanine the Na^+-independent uptake is an exchange diffusion and that its contribution to initial flux measurements depends on the level of readily exchanging free amino acids in the cells and this may change from one preparation to another depending on the condition and treatment of the cells. As has been suggested by one of the referees, the final proof of this would be to show that there is a direct correlation between the Na^+-independent uptake of phenylalanine and a concomitant loss of endogenous amino acids. This remains to be done.

It would appear from Fig. 8 that the Na^+-dependent component of phenylalanine uptake is practically the same at all levels of the exchange component, suggesting that they are independent and additive. In a previous paper from this laboratory[6] the J_M for exchange and active transport were tested for additivity for a number of amino acids and it was found that they were not simply additive. However, the cells that were incubated in choline Krebs–Ringer phosphate or sodium Krebs–Ringer phosphate were each washed in their own media prior to the measurement and so differed in their levels of intracellular Na^+. These experiments were repeated but after the preliminary incubation that was used to load the cells all were washed with choline Krebs–Ringer phosphate and all started with intracellular Na^+ levels of 5–10 mM. The results are given in Table III. As shown in the previous paper[6] the test of additivity is given by Eqn 2, in which $J_M(X,Na)$ is the maximal flux in an exchange experiment (X) in high Na^+ medium (Na), $J_M(T,Ch)$ is the maximal flux in a transport experiment in choline medium.

$$J_M(X,Na) = J_M(T,Na) - J_M(T,Ch) + J_M(X,Ch) \tag{2}$$

TABLE III

PARAMETERS OF AMINO ACID UPTAKE FOR 1-MIN INITIAL FLUXES FOR EHRLICH ASCITES TUMOR CELLS

Amino acid	*Type of experiment* **	J_M (*μmoles/g dry wt per min*)		K_m (*mM*)		*k* (*cm³/g per min*)	
		Na^+	*Choline*	*Na^+*	*Choline*	*Na^+*	*Choline*
L-Alanine	T	39.3	23.0	0.96	17.1	0.22	0.13
		36.2	21.3	1.08	10.4	0.28	0.18
	X	54.0	51.4	1.44	10.7	0.33	0.20
		72.2	52.7	1.66	16.9	0.12	0.22
α-Aminoisobutyric acid	T	37.9	14.9	1.95	9.7	0.0*	0.0*
		33.2	15.4	1.25	15.8	0.11	0.0*
	X	26.6	23.0	5.6	34.6	0.25	0.35
		30.5	36.2	4.0	91.5	0.30	0.26
L-Methionine	T	34.4	14.8	0.87	2.05	0.08	0.19
		28.7	12.7	0.71	1.71	0.16	0.20
	X	131.3	106.4	1.77	1.88	0.22	0.38
		128.0	104.2	1.57	1.62	0.12	0.47
L-Phenylalanine	T	30.7	5.0	5.0	0.24	0.12	0.15
		25.5	7.5	2.8	0.66	0.25	0.11
	X	64.9	33.3	1.13	0.75	0.21	0.11
		45.1	32.4	1.21	0.85	0.40	0.52
Cycloleucine	T	36.3	11.3	1.09	0.84	0.04	0.14
		31.4	8.7	1.46	0.58	0.09	0.06
	X	123.1	104.9	2.45	2.90	0.20	0.29
		135.4	101.6	3.14	2.66	0.35	0.36

* These uptake curves had a flat plateau at high concentrations and the best fit in the least squares sense was obtained for $k=0$.

** The results of two independent experiments are given for initial 1-min uptakes by cells not loaded (T) and by cells that had first been loaded (X) by incubation with the same, unlabeled amino acid at 60 mM.

By this test the exchange and transport maximal fluxes are close to being additive for methionine, phenylalanine, and cycloleucine. This conclusion is reinforced for phenylalanine by the results of the studies shown in Fig. 8. The data on the J_M of alanine show too much fluctuation in J_M(X,Na) to allow a definite conclusion; further tests will have to be run. For α-aminoisobutyric acid the J_M for exchange and transport are not additive and in fact J_M(X,Na) is less than J_M(T,Na) suggesting that loading the cells with unlabeled α-aminoisobutyric acid actually inhibits uptake. These findings cannot be attributed to a carryover of unlabeled α-aminoisobutyric acid from the initial loading and to leak from the cells in the preliminary incubation so diluting the specific activity of the α-aminoisobutyric acid used in the subsequent measurement of initial flux as to decrease the measured J_M. In other experiments on α-aminoisobutyric acid this contamination, measured at the point where the initial flux measurement starts, amounts to an extracellular concentration of 0.07–0.5

mM for loading concentrations of 1–50 mM. Such concentrations might affect the measured K_m but could not appreciably affect the estimates of J_M because these are determined by the initial fluxes at extracellular concentrations of α-aminoisobutyric acid above 20 mM.

DISCUSSION

This study was undertaken to clarify what appeared to be uncertainties about the definition of the L system. Oxender and Christensen[7] originally classified phenylalanine as one of the amino acids that had a substantial L component to its uptake. The fraction of the uptake of phenylalanine that is Na^+ independent has been found to depend on the concentration of phenylalanine[14,15] and in this laboratory, prior to the present study, it has been found to vary somewhat for different preparations of cells, apparently depending on the extent of washing and pretreatment of the cells. The results of the present study provide evidence for the view that for phenylalanine the L system represents an exchange diffusion, that phenylalanine has a low K_m for this mode of uptake and that its relative contribution to measured initial fluxes depends on the level of freely exchanging amino acids in the cells. The active transport of phenylalanine is Na^+-dependent but its K_m is much higher than the K_m for exchange diffusion so the fraction of uptake that is Na^+ independent increases markedly as the extracellular concentration of phenylalanine is decreased. Cycloleucine is similar to phenylalanine; most of its J_M for uptake is Na^+-dependent but it also shows a lower K_m for the Na^+-independent uptake than for the Na^+-dependent uptake. Recently, in a search for amino acids that show only an L system type of uptake, Christensen *et al.*[15] have prepared 2-aminobicyclo-[2,2,1]-heptane-2-carboxylic acid (BCH) and have reported that very little of BCH uptake is *via* the A or the ASC systems. However, McClellan and Schafer (personal communication) have found that the J_M for BCH uptake increases from about 4.2 μmoles/g dry wt per min at $[Na^+]_e = 2.2$ mequiv/l to about 10.5 μmoles/g dry wt per min at medium concentrations of Na^+ above 100 mequiv/l. The K_m on the other hand was Na^+ independent with an average value of 0.16 mM.

The results presented here taken in conjunction with the data of Belkhode and Scholefield[12] and of Potashner and Johnstone[13] on methionine can be interpreted as evidence that, for the neutral amino acids, the L system is simply an exchange diffusion and that it is particularly prominent in Ehrlich ascites cells because of their high levels of free amino acids. Since only a few amino acids of the set of possible neutral amino acids have been tested in this regard, it should be considered a hypothesis that is unproven but worth testing. The recent report of Christensen[16] that 4-amino-1-methylpiperidine-4-carboxylic acid (MPA) is concentrated to levels that exceed the total endogenous pool of amino acids in ascites cells in a medium to which no Na^+ had been added and that this uptake is inhibitable by metabolic inhibitors appears to contradict this. However, MPA is not a simple neutral amino acid; it has three forms in solution, an α-zwitterion, δ-zwitterion, and a cationic form. Presumably only the α-zwitterionic form enters *via* the L system and we must consider the possibility of contributions to uptake due to other effects so it is probably not as clear a test of the hypothesis as one might wish. We can say that for phenylalanine and methionine the evidence favors the view that the L mode of uptake is an exchange

diffusion. A plausible but unproven extrapolation of the data in Table III is that all neutral amino acids undergo exchange diffusion *via* this system. If this holds then the difference between amino acids such as α-aminoisobutyric acid and alanine on the one hand and phenylalanine and cycloleucine on the other is that α-aminoisobutyric acid and alanine have very high K_m values whereas phenylalanine and cycloleucine have very low K_m values for exchange diffusion. This would explain why α-aminoisobutyric acid uptake appears to be almost entirely Na^+-dependent and why loading cells with α-aminoisobutyric acid did not appear to increase the exchange uptake of phenylalanine (see Fig. 8). Whatever the nature of the L system, the results presented here suggest some simple tests to use in the search for L-specific amino acids. These are obtained by measuring the J_m and K_m in a high Na^+ medium and in a choline medium in which $[Na^+] \leqslant 1$ mequiv/l. The J_M in high Na^+ medium, $J_M(Na)$, measures a sum of the capacities for Na^+-dependent and Na^+-independent uptakes whereas the J_M in choline, $J_M(Ch)$, measures the capacity of Na^+-independent uptake processes. If the two are independent and additive as for phenylalanine, then $[J_M(Na)/J_M(Ch) - 1]$ is the ratio of maximal capacities for Na^+-dependent uptake mechanisms to maximal capacities for Na^+-independent mechanisms for that amino acid. The ratio of the K_m on the other hand would provide a measure of the kinetic separability of the two modes of uptake.

From the data on phenylalanine the fluxes of the Na^+-independent exchange uptake and of the Na^+-dependent active transport are additive suggesting that the two uptakes are mediated by independent systems. However, α-aminoisobutyric acid also shows a Na^+-independent exchange diffusion and this is not simply additive with the Na^+-dependent active transport. This problem remains unresolved and requires further investigation. However, a few features of this exchange should be pointed out. The uptake of phenylalanine at low Na^+ concentrations is, if not entirely, almost entirely an exchange uptake. If one thinks of the L carrier as having one binding site and that the carrier is a two-state carrier that shifts back and forth between two positional states, one on each side of the membrane, then the results imply that the rate of transition between the two states for carrier–substrate complex must be greater than that of the free carrier. Alternatively, one might invoke a carrier model that has two sites, one available to substrate outside the other to substrate inside, the transition being an exchange of the two sites both of which must be loaded. There is now evidence that the facilitated diffusion system for glucose in human erythrocytes has features that cannot be explained by the traditional two-state carrier model[17]. Perhaps we are faced by a similar problem in the exchange uptake of amino acids.

There is another discrepancy in the data on Na^+ and phenylalanine cotransport that requires further investigation. In work done in this laboratory Schafer[18] and Schafer and Jacquez[19] found that the increment in Na^+ uptake accompanying uptake of α-aminoisobutyric acid gave a ratio of approx. 1 for $\Delta Na^+/$α-aminoisobutyric acid whereas there was no increase in Na^+ uptake accompanying uptake of phenylalanine. In fact there was a small decrease in Na^+ uptake during uptake of phenylalanine and the higher the concentration of phenylalanine the larger the concomitant decrease in Na^+ uptake. Since the decrease in Na^+ uptake was small this appeared to fit in with the idea that α-aminoisobutyric acid uptake was mediated almost entirely by a Na^+-dependent system and phenylalanine uptake was mediated

by a Na^+-independent mechanism. But the present studies show clearly that at high concentrations phenylalanine uptake is mostly Na^+-dependent and that its J_M increases with Na^+ but that the K_m for the Na^+-dependent uptake is either constant or increases some as extracellular Na^+ increases. How can these two observations be reconciled. One possible explanation is that the uptake in Schafer's studies was mostly by the Na^+-independent exchange. However, the effect was found at extracellular phenylalanine concentrations of 10 and 25 mM, well above the K_m for the Na^+-dependent uptake of phenylalanine so that there should have been an appreciable Na^+-dependent component to the uptake. Is it possible then to have a transport system for which J_M increases with extracellular Na^+ but with which there is no obvious increase in Na^+ influx? One possibility was suggested by the data on Na^{22} influx in Ehrlich ascites cells. These have a high Na^+ influx and efflux. Schafer[18] found that the increment in Na^+ influx accompanying uptake of α-aminoisobutyric acid was in all but one case less than one-half the control level of Na^+ influx. This suggests the following explanation. Let us suppose that the carrier complexes with Na^+ and with Na^+ and substrate can both cross the membrane and that in the absence of extracellular amino acid a considerable portion of the one-way Na^+ influx is actually by way of the Na^+–carrier complex. Then if the complex Na^+–carrier–substrate crosses at the same rate as Na^+–carrier there will be no increased influx of Na^+ accompanying uptake of substrate even though the transport J_M increases with Na^+. This explanation introduces the possibility that if Na^+–carrier–substrate complex crosses more slowly than Na^+–carrier or if the complex carrier–substrate is formed in appreciable amounts so as to sequester the carrier in this form that there might be a decrease in Na^+ influx accompanying a Na^+-dependent uptake. It is possible to show that if the above conditions hold that this is indeed a consequence of the models of ion cotransport that have been published by Heinz *et al.*[20] and by Jacquez[21]. However, this explanation is at the moment a conjecture and is not supported by any direct experimental evidence. The assumption that Na^+–carrier readily crosses the membrane in effect assumes a large leak around the Na^+–K^+ pump and would have to be demonstrated experimentally to make this explanation plausible.

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