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TEMPERATURE DEPENDENCE OF AMINO ACID TRANSPORT IN EHRLICH ASCITES CELLS: WITH RESULTS WHICH BEAR ON THE A–L DISTINCTION

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SUMMARY

We have examined the temperature dependence of maximal flux, J_M, the half-saturation value, K_m, and of the linear component, k, of the initial transport flux for L-methionine, L-tryptophan, glycine, L-alanine, α-aminoisobutyric acid and L-phenylalanine in Ehrlich ascites cells. For L-alanine, α-aminoisobutyric acid and L-phenylalanine we examined the initial flux in transport and in exchange for cells in Na^+-containing and in Na^+-free media.

The maximal flux in transport falls with decrease in temperature, the activation energies falling in the range 11–20 kcal/mole.

The K_m decreased with decrease in temperature for L-phenylalanine and L-tryptophan with apparent activation energies of 20 and 15 kcal/mole respectively. For L-methionine, glycine and L-alanine the K_m showed no dependence on temperature whereas for α-aminoisobutyric acid it increased as the temperature fell. For all amino acids, the K_m appeared to be more variable a parameter of the initial flux than was the maximal flux.

The results for the linear component of the uptake, k, also were highly variable, but k did decrease with a fall in temperature with activation energies falling in the range 5.8–17.7 kcal/mole.

INTRODUCTION

Many biological processes show a marked dependence on temperature. Much of the early work on the effects of temperature on biological processes is summarized in the book by JOHNSON *et al.*[1]. Recently, STEIN[2] has reviewed the data on temperature coefficients and activation energies for Na^+ and K^+ movement in a number of cell types as well as the activation energies for the permeabilities to water and a number of organic solutes. The present study examines the temperature dependence of amino acid transport in Ehrlich ascites tumor cells. An excellent review of amino acid transport in tumor cells, including some effects of change in temperature, is the article by JOHNSTONE AND SCHOLEFIELD[3].

A marked temperature dependence has been one of the characteristics repeatedly reported for amino acid transport[4–17]. Table I summarizes the Q_{10}'s or activation

energies reported for various aspects of amino acid transport. In most cases Q_{10}'s were reported and we have calculated the activation energy from the simple Arrhenius theory. For the most part the data reported in Table I were collected as minor parts of larger studies and the Q_{10}'s were calculated from data obtained at two or occasionally three temperatures. Detailed studies of temperature dependence of maximum flux, J_M, and of K_m for amino acid transport have not been reported. For transport of glucose into the human red cell such studies are available. SEN AND WIDDAS[18] carried out a study of the temperature variation of K_m and J_M for glucose and found an 'energy of activation' of 10 kcal/mole for K_m. They reported that J_M was not a linear function of the reciprocal of the absolute temperature. However a replot of their data gives a straight line for temperatures below 30° from which we obtain an activation energy of 22.5 kcal/mole.

The concentration dependence of the initial flux of uptake of neutral amino acids in Ehrlich ascites cells is fairly well described by a relation of the form of Eqn. 1.

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

J_M is the maximum saturable flux, K_m is a parameter similar to the Michaelis–Menten constant of enzyme kinetics, c_e and c_i are mean extracellular and intracellular concentrations. The term $k(c_e - c_i)$ is called the linear component or non-saturable component; it probably does not represent solely a crossing of the membrane by simple diffusion but may also contain a mediated component[7,19]. At the least, J_M, K_m and k are descriptive parameters; the significance of J_M and K_m in terms of mechanism is certainly not as clear as is their significance in the description of enzyme reactions.

OXENDER AND CHRISTENSEN[10] have presented evidence for the existence of two distinct mediating systems for the transport of neutral amino acids in Ehrlich ascites cells. By their description, one of these, the A system, has higher affinity for the short side chain amino acids and requires Na^+, the other, the L system does not require Na^+ and prefers the large side chain and aromatic side chain amino acids. INUI AND CHRISTENSEN[20] presented stronger evidence for this distinction and SCHAFER AND JACQUEZ[21] and JACQUEZ[22] have shown that the assumption that there are two systems, one Na^+ dependent, the other not, appears to explain the findings on competitive stimulation[23,24], that is, the stimulation of initial flux of uptake of one amino acid by another when both are initially present extracellularly. JOHNSTONE AND SCHOLEFIELD[25] have shown that although approximately one-half of the transport flux of methionine depends on the presence of extracellular Na^+, the exchange flux does not. The present work was undertaken to examine the temperature dependence of J_M, K_m and k for glycine, L-alanine, α-aminoisobutyric acid, L-methionine, L-phenylalanine and L-tryptophan. For some of these amino acids the temperature dependence of J_M, K_m and k was examined for both transport and exchange diffusion, for Na^+-containing media and for Na^+-free media in which NaCl was replaced by choline chloride.

METHODS

Experimental

The methods of collecting and preparing the suspensions of ascites tumor cells and the general experimental methods used in this laboratory have been described

in previous publications[24,26]. The basic experiment carried out was one in which 1-min fluxes were measured at seven different concentrations of an amino acid, each concentration being run in duplicate. Two-armed reaction vessels were used for the experiments; the cell suspension was pipetted into one side, the solution of amino acid into the other. The experiments reported here were done over a period of 3 years and during that time the methods used were changed a number of times. Most of the experiments with Na^+-containing media were run with use of Krebs–Ringer phosphate[22] solution made up with sodium phosphates or potassium phosphates; the latter solution had a higher K^+ concentration and a somewhat lower Na^+ concentration than the former but the results with the two did not differ appreciably so these media will be referred to as Krebs–Ringer phosphate in this paper. Na^+-free media were made up with choline chloride replacing NaCl and with potassium phosphate buffers; these media will be referred to as Na^+-free Krebs–Ringer phosphate. The pH's of all media, all measured at room temperature, were 6.95–7.05.

The following gives, in outline form, the methods used with each of the amino acids studied. Because Na^+ is involved in transport *via* the A system it is possible that different methods of preparing the cells could affect the initial net fluxes by way of an effect on intracellular Na^+ levels. For this reason we give the cellular Na^+ levels, Na_i^+, for the different methods of preparing the cells. These were determined recently after all experiments in this study had been completed.

L-*Methionine and* L-*tryptophan*. Osmotic shock with 50% Krebs–Ringer phosphate. Wash with Krebs–Ringer phosphate. At this stage $Na_i^+ = 56$ mequiv per kg cell water. Incubate 10 min at temperature of experiment, mix and incubate 1 min.

Glycine. Osmotic shock with 50% Krebs–Ringer phosphate. Wash with Krebs–Ringer phosphate. Incubate 10 min at 37° and chill. Na_i^+ was 44 mequiv per kg cell water. Incubate 5 min at temperature of experiment, tip in glycine and incubate 1 min.

L-*Alanine*. No osmotic shock. Wash cells with Krebs–Ringer phosphate or Na^+-free Krebs–Ringer phosphate. Intracellular Na^+ was 33 mequiv per kg for Krebs–Ringer phosphate-washed cells and 7 mequiv per kg for cells washed with Na^+-free Krebs–Ringer phosphate. For exchange studies load cells by incubating for 30 min with 100 mM alanine, chill, and wash with Krebs–Ringer phosphate or Na^+-free Krebs–Ringer phosphate. Incubate 2 min at chosen temperature, tip in alanine and incubate for 1 min.

α-*Aminoisobutyric acid and* L-*phenylalanine*. No osmotic shock; wash cells with Krebs–Ringer phosphate. For transport studies resuspend cells to 140 ml with Krebs–Ringer phosphate and incubate 30 min at 37°. For exchange studies use this incubation to load cells with α-aminoisobutyric acid (100 mM) or phenylalanine (60 mM). Chill and wash cells with Krebs–Ringer phosphate or Na^+-free Krebs–Ringer phosphate. At this stage intracellular Na^+ concentration was 51 mequiv per kg for cells washed with Krebs–Ringer phosphate and 10 mequiv per kg for cells washed with Na^+-free Krebs–Ringer phosphate. Incubate 2 min at chosen temperature, tip in amino acid and incubate for 1 min.

In all experiments the incubations were terminated by chilling and samples were taken. Pellets were obtained for analysis by pipetting a measured volume of suspension into a large volume (7–13 ×) of cold medium and centrifuging. This was done to dilute the material present in the extracellular space of the pellet. The methods used to prepare extracts of pellets and supernatant fluid have been described[26]. Cor-

rections for extracellular water and amino acid in the extracellular space of the pellet were calculated with the use of sucrose spaces which were read from a standard curve. Sucrose spaces were measured in a series of independent experiments and a standard curve was prepared relating the extracellular space of pellets to the pellet volumes.

The labeled amino acids used were all labeled with ^{14}C and were obtained from Nuclear-Chicago or New England Nuclear. The non-labeled amino acids were all A grade amino acids from California Biochemicals. For the experiments on L-methionine, L-tryptophan, glycine and L-alanine a Nuclear-Chicago Model No. 725 scintillation counter was used to obtain counts/min on the extracts and the channels-ratio method was used to obtain efficiencies of counting. For the experiments on α-aminoisobutyric acid and L-phenylalanine a Nuclear-Chicago Unilux II scintillation counter with external standard was used.

Calculations

The fluxes and intracellular concentrations were calculated with use of the corrections for extracellular water and amino acid in the pellets. Thus the basic data obtained in each experiment consisted of extracellular concentration, calculated intracellular concentration and the calculated 1-min initial net flux. We assume the data can be described by Eqn. 1. Three main systems of units are in use for reporting data on fluxes. These are: mmoles per cm^2 of surface area per min, mmoles per g dry wt. per min, and mmoles per g intracellular water per min. Corresponding to these units, the units of k in Eqn. 1 are cm/min, cm^3/g per min and min^{-1} respectively. K_m has the same units as concentration and is reported in mmoles per kg water here. The flux J was calculated for each of these systems of units. We use the notation J_M (1), J_M (2), J_M (3) and K_m (1), K_m (2), K_m (3) and k_1, k_2, k_3 for the results in the three systems of units, in the order given above. To obtain J_M (1), the cell counts on the suspensions and the pellet volumes were used, with the assumption that the cells are uniform in size and perfect spheres. The relative error in the cell counts is higher than that in dry weight or cell water and the calculated surface area probably underestimates the true cell surface area because of folding in the cell membrane. Nonetheless the general pattern of the results was the same in the different units. For this reason the results are reported primarily for one of these systems of units, J_M (3), K_m (3) and k_3. Although the conversion factors show some fluctuation from experiment to experiment the following are average factors, with standard deviations, found for converting the fluxes from one set of units to another: J_M (1) $= (2.14 \pm 0.15) \cdot 10^{-4}$ J_M (3); J_M (2) $= (3.91 \pm 0.48)$ J_M (3).

The parameters J_M, K_m and k were calculated with use of a non-linear least squares fitting program which was written in Fortran IV and was run on the IMB 360/67 system at the University of Michigan Computing Center. The energies of activation reported here are obtained by plotting, for example, J_M against $1/T$ where T is the absolute temperature and if the data appear reasonably linear, fitting a straight line by eye. The energy of activation is calculated from the simple Arrhenius theory, *i.e.*, $J_M = Ae^{-E_a/RT}$, where E_a is the energy of activation. There was sufficient variability in the data that we thought it did not warrant the application of least squares fitting or of the refinements of the modern theory of rate processes[1]. As will be seen from the graphs the fluctuations in our data are such that the estimated energies of activation could well be off by 1–2 kcal/mole, an amount which is larger than the cor-

rection term, RT, which is subtracted from the energy of activation to obtain enthalpy of activation, so we report only energies of activation.

There is no problem in applying the term energy of activation to k and J_M, both describe rate processes and have the units of a rate. The same cannot be said for K_m. In models of active transport[2,27–29] K_m is a ratio which contains rate constants for crossing the membrane and dissociation constants or rate constants for binding of substrate to carrier; but its units are those of a concentration. For this reason we use the term apparent energy of activation when referring to K_m. However it is possible that one of the rate constants dominates the temperature dependence of K_m. Furthermore J_M is also given by a relatively complex expression in various rate constants for crossing the membrane, so that its temperature dependence may not be easy to interpret.

RESULTS

Figs. 1–4 show examples of the data obtained in the basic experiments. Fig. 1 shows the concentration dependence of the 1-min flux of α-aminoisobutyric acid at 37° for two transport experiments, for the upper curve Krebs–Ringer phosphate was the medium, for the lower curve Na^+-free Krebs–Ringer phosphate was the medium. Fig. 2 shows the results of the corresponding exchange experiments. Figs. 3 and 4 give results of the corresponding experiments with L-phenylalanine. We would like to emphasize that we have measured the 1-min initial fluxes of uptake in cells which have not been loaded (transport experiments) and in cells which have been loaded to high levels with the same but unlabeled amino acid whose uptake was being measured (exchange experiments). Operationally the measured fluxes represent 1-min net fluxes for the labeled amino acid which was introduced extracellularly at the start of the incubation. These are the fluxes usually measured and reported in the literature on amino acid uptake. One of the referees of the first draft of this paper expressed concern that the measured fluxes might measure different things in the transport experiments as compared to the exchange experiments. In particular he pointed out

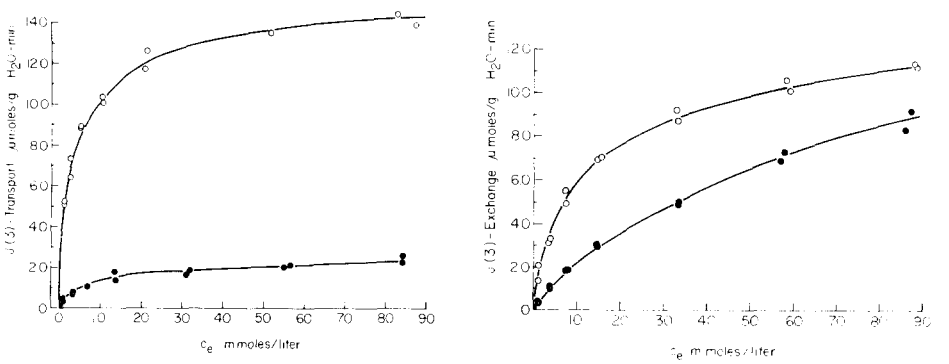

Fig. 1. Concentration dependence of 1-min initial flux of uptake of α-aminoisobutyric acid in transport experiments. Suspending media: ○—○, Krebs–Ringer phosphate; ●—●, Na^+-free Krebs–Ringer phosphate.

Fig. 2. Concentration dependence of 1-min initial flux of uptake of α-aminoisobutyric acid in homoexchange. Suspending media: ○—○, Krebs–Ringer phosphate; ●—●, Na^+-free Krebs–Ringer phosphate.

that at high extracellular concentrations of amino acid the 1-min initial flux in the exchange experiments might be a fairly good measure of a one-way influx because the entering amino acid is well diluted in the large amount of intracellular amino acid with which the cells were loaded. In the transport experiments, on the other hand,

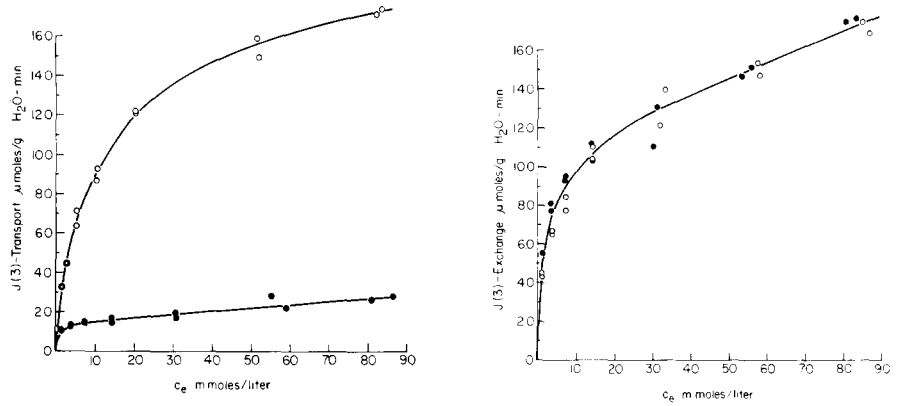

Fig. 3. Concentration dependence of 1-min initial flux of uptake of L-phenylalanine in transport experiments. Suspending media: O—O, Krebs–Ringer phosphate; ●—●, Na^+-free Krebs–Ringer phosphate.

Fig. 4. Concentration dependence of 1-min initial flux of uptake of L-phenylalanine in experiments in homoexchange. Suspending media: O—O, Krebs–Ringer phosphate; ●—●, Na^+-free Krebs–Ringer phosphate.

the entering amino acid would be diluted only by the small amounts of endogenous amino acids in the cells and if the efflux rate is significant this 1-min initial flux in transport would underestimate the one-way influx. However we can estimate the significance of this effect. Efflux rates, though not exchange rates, for the amino acids for Ehrlich ascites cells are quite small[10,30]. Furthermore if this effect were significant we would expect the measured initial transport flux to be lower than the measured initial flux in exchange experiments. But as can be seen from Table II, $J_M(3)$ in Krebs–Ringer phosphate did not differ significantly between transport and exchange experiments for α-aminoisobutyric acid and phenylalanine. For alanine $J_M(3)$ was indeed lower in the transport experiments. However, we would put less weight on the alanine experiments as far as comparisons between the transport and exchange experiments are concerned because the cells used for the transport experiments did not receive the 0.5-h incubation comparable to that used for loading the cells with alanine for the exchange experiments (see METHODS) and thus we cannot say the cells used in the transport and exchange experiments were handled the same way in all respects.

Occasionally one to three points were lost as the result of accidents during the experiment and in a few experiments the top concentration was omitted. Therefore, the number of data points obtained in such an experiment varied from 10 to 14. Each curve was fitted with Eqn. 1, giving rise to the three parameter values $J_M(3)$, $K_m(3)$, k_3. Table II summarizes the results of all experiments carried out at 37°. For comparison the results of the same experiments are given in Table III in terms of $J_M(2)$, $K_m(2)$ and k_2. The general pattern is much the same although there are relative

TABLE I

SUMMARY OF REPORTED DATA ON TEMPERATURE DEPENDENCE OF AMINO ACID TRANSPORT

Cell type or tissue	*Amino acid*	*Process*	*Temp. range*	Q_{10}	*Activation energy (kcal/mole)*	*Ref.*
Ehrlich ascites	L-α-γ-Diaminobutyric	Distribution ratio,				
	L-tryptophan	incubation ⩾ 1 h	27–37°	1.8	10.9	4
Ehrlich	Glycine	1 min initial flux	24–32.5°	1.6	8.6	5
		maximal flux	28–37°	1.3	5.3	
Ehrlich	L-Methionine	Efflux	20–37°	2.0	12.8	7
Ehrlich	Glycine	1 min initial flux	27–37°	3.5	23.2	
	L-Alanine			3.2	21.5	
	α-Aminoisobutyric acid			3.5	23.2	10
	L-Methionine			3.0	20.3	
	L-Leucine			2.5	17.0	
	L-Valine			2.7	18.4	
S-37	L-Tyrosine	Uptake 10 min	17–37°	1.9	11.9	8
Lymph cells guinea pig	α-Aminoisobutyric acid	Initial uptake 5–10 min	17–37°	2.2	13.5	9
Human erythrocytes	L-Leucine	Initial flux 2 min	27–37°	1.7	9.8	11
Rat diaphragm	α-Aminoisobutyric acid	Influx	27–37°	1.9–2.6	11.9–17.7	12
Rabbit ileum	L-Alanine	Initial flux 1 min	23–37°	4.5	27.7	13
Bone cells-rat	α-Aminoisobutyric acid	Distribution ratio 1 h	21–37°	2.2	13.5	15
Neurospora crassa	L-Phenylalanine	Uptake 1 h	0–30°	1.6	8.6	16
	L-Tryptophan	Initial rate of uptake	20–30°	2.0	12.8	17

TABLE II

PARAMETERS OF AMINO ACID UPTAKE FOR EHRLICH ASCITES TUMOR CELLS AT 37°

The number in parentheses is the number of experiments. Values are in mean ± S.E.

Amino acid	*Type of experiment*	$J_M(3)$ (*μmoles per g water/min*)		$K_m(3)$ (*mmoles per kg water*)		k_3 (min^{-1})	
		Na⁺	*Choline*	*Na⁺*	*Choline*	*Na⁺*	*Choline*
L-Tryptophan	Transport	2.3 ± 0.3 (4)	—	1.1 ± 0.3 (3)	—	0.09 ± 0.003 (4)	—
L-Methionine	Transport	7.7 ± 0.4 (3)	—	1.9 ± 0.3 (3)	—	0.08 ± 0.01 (3)	—
Glycine	Transport	9.1 ± 0.8 (5)	—	5.3 ± 0.3 (5)	—	0.11 ± 0.02 (5)	—
L-Alanine	Transport	14.1 ± 1.2 (3)	4.0 ± 0.7 (4)	1.9 ± 0.6 (3)	10.8 ± 2.1 (4)	0.04 ± 0.02 (3)	0.04 ± 0.01 (4)
	Exchange	19.1 ± 1.6 (7)	13.3 ± 1.2 (3)	2.9 ± 0.4 (7)	16.0 ± 2.6 (2)	0.06 ± 0.01 (7)	0.06 ± 0.01 (3)
α-Aminoisobutyric acid	Transport	12.6 ± 0.9 (6)	2.2 ± 0.2 (4)	1.9 ± 0.2 (6)	6.1 ± 0.8 (4)	0.02 ± 0.004 (6)	0.01 ± 0.01 (3)
	Exchange	10.5 ± 0.3 (3)	6.5 ± 0.2 (3)	6.0 ± 0.3 (3)	18.8 ± 2.6 (4)	0.04 ± 0.02 (3)	0.05 ± 0.01 (4)
L-Phenylalanine	Transport	10.4 ± 1.3 (4)	1.1 ± 0.02 (3)	4.5 ± 0.5 (4)	0.25 ± 0.1 (2)	0.05 ± 0.01 (4)	0.01 ± 0.002 (3)
	Exchange	12.3 ± 0.8 (3)	11.1 ± 0.7 (3)	1.9 ± 0.2 (3)	1.4 ± 0.3 (3)	0.07 ± 0.01 (3)	0.08 ± 0.01 (3)

TABLE III

PARAMETERS OF AMINO ACID UPTAKE FOR EHRLICH ASCITES TUMOR CELLS AT 37°

The number in parentheses is the number of experiments. Values are in mean ± S.E.

Amino acid	*Type of experiment*	$J_M(2)$ (*μmoles per g dry wt./min*)		$K_m(2)$ (*mmoles per kg water*)		k_2 (*cm³ per g/min*)	
		Na⁺	*Choline*	*Na⁺*	*Choline*	*Na⁺*	*Choline*
L-Tryptophan	Transport	6.5 ± 0.8 (4)	—	1.1 ± 0.2 (3)	—	0.29 ± 0.02 (4)	—
L-Methionine	Transport	21.6 ± 0.4 (3)	—	2.1 ± 0.4 (3)	—	0.19 ± 0.03 (3)	—
Glycine	Transport	29.4 ± 2.6 (5)	—	6.0 ± 0.4 (5)	—	0.34 ± 0.05 (5)	—
L-Alanine	Transport	55.8 ± 4.7 (3)	13.1 ± 2.3 (4)	2.1 ± 0.6 (3)	10.4 ± 2.0 (4)	0.15 ± 0.04 (3)	0.14 ± 0.03 (4)
	Exchange	88.5 ± 6.8 (7)	54.5 ± 5.1 (3)	3.1 ± 0.4 (7)	16.7 ± 2.4 (2)	0.26 ± 0.04 (7)	0.25 ± 0.05 (3)
α-Aminoisobutyric acid	Transport	49.7 ± 3.1 (6)	6.9 ± 0.7 (4)	2.0 ± 0.2 (6)	6.0 ± 0.8 (4)	0.05 ± 0.02 (6)	0.03 ± 0.02 (3)
	Exchange	46.2 ± 1.4 (3)	23.3 ± 3.0 (3)	5.9 ± 0.3 (3)	18.9 ± 2.5 (4)	0.16 ± 0.08 (3)	0.19 ± 0.04 (4)
L-Phenylalanine	Transport	40.1 ± 5.0 (4)	3.7 ± 0.7 (3)	4.5 ± 0.6 (4)	0.26 ± 0.2 (2)	0.16 ± 0.04 (4)	0.04 ± 0.005 (3)
	Exchange	53.3 ± 3.6 (3)	39.6 ± 1.7 (3)	1.9 ± 0.1 (3)	1.3 ± 0.3 (3)	0.24 ± 0.02 (3)	0.08 ± 0.01 (3)

changes in the ratios, intracellular water/dry weight, for the different conditions. For example the conversion factors c, $J_M(2) = cJ_M(3)$, obtained from the experiments on alanine, α-aminoisobutyric acid and phenylalanine were 4.45 ± 0.23 (S.D.) for exchange in Krebs–Ringer phosphate, 3.83 ± 0.36 for exchange in Na^+-free Krebs–Ringer phosphate, 3.81 ± 0.10 for transport in Krebs–Ringer phosphate and 3.31 ± 0.35 for transport in Na^+-free Krebs–Ringer phosphate. Note how close $K_m(3)$ and $K_m(2)$ are for the same experiments. A number of points stand out. First if we look only at the transport experiments we note that k_3 is somewhat larger for the amino acids glycine, L-methionine and L-tryptophan. Because osmotic shock was used only with these three it is possible that the difference is related to this treatment. For the three amino acids for which experiments were run with Na^+-free Krebs–Ringer phosphate there was a marked drop in $J_M(3)$ in the transport experiments when choline was substituted for Na^+. For alanine and α-aminoisobutyric acid, $K_m(3)$ increases, for phenylalanine it decreases on going to Na^+-free Krebs–Ringer phosphate. The maximal fluxes of transport and of exchange in Krebs–Ringer phosphate clearly do not differ significantly for α-aminoisobutyric acid or for phenylalanine and the difference found with alanine is not impressive in terms of the standard errors of $J_M(3)$ for alanine. Again we would caution the reader that the experiments on transport and exchange may not be as comparable for alanine as for α-aminoisobutyric acid and phenylalanine. For phenylalanine the exchange experiments in Krebs–Ringer phosphate and Na^+-free Krebs–Ringer phosphate are indistinguishable. The same cannot be said for α-aminoisobutyric acid and L-alanine; although the drop in $J_M(3)$ in going from Krebs–Ringer phosphate to Na^+-free Krebs–Ringer phosphate is much less in the exchange than in the transport experiments it is accompanied by an approximately 5-fold increase in $K_m(3)$ for alanine and a 3-fold increase for α-

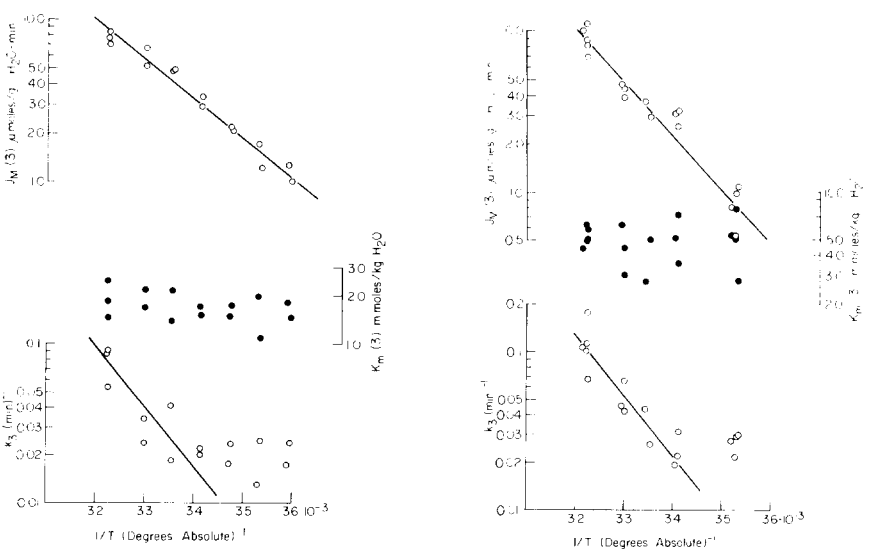

Fig. 5. Temperature dependence of $J_M(3)$, $K_m(3)$ and k_3 for L-methionine from experiments on 1-min initial fluxes in transport. Data on $K_m(3)$ are given by ●.

Fig. 6. Temperature dependence of $J_M(3)$, $K_m(3)$ and k_3 for glycine from experiments on 1-min initial fluxes in transport. Data on $K_m(3)$ are given by ●.

aminoisobutyric acid. Interestingly the increase in $K_m(3)$ in going from Krebs–Ringer phosphate to Na^+-free Krebs–Ringer phosphate in the transport experiments is also 5-fold and 3-fold for alanine and α-aminoisobutyric acid respectively.

Figs. 5–7 summarize all of the temperature studies on methionine, glycine and tryptophan respectively. $J_M(3)$ falls markedly with temperature for all three amino acids; the energies of activation for methionine, glycine and tryptophan being 11.1, 15.5 and 13.0 kcal/mole. Interestingly, the $K_m(3)$ for glycine and methionine show no temperature dependence whereas $K_m(3)$ for tryptophan decreases with a fall in temperature, the apparent energy of activation being 14.7 kcal/mole. The behavior of k_3 is of interest. For glycine, methionine and tryptophan k_3 decreases as the temperature falls to about 20° but there seems to be no further drop below that temperature. It seemed possible that this behavior might be explained by a phase change in the lipids of the membrane if one assumed that the linear component of the uptake was made up of at least two parallel components, a mediated component which passed through the lipids of the membrane and a simple diffusion through possibly only a few aqueous channels. Then the lipid component might be expected to show a considerable temperature dependence whereas diffusion through aqueous channels would not and might represent the level remaining below 20°. Such an explanation is particularly appealing because studies of NMR spectra of lecithins show a transition near 25° which has been interpreted as a phase change between liquid and crystalline forms[31]. This behavior of k_3 was not seen with alanine, α-aminoisobutyric acid and phenylalanine. Osmotic shock was not used in the experiments with the latter three amino acids and the k_3's were uniformly higher in the experiments in which osmotic shock was used. It may be that osmotic shock damages the cell membrane so as to leave some aqueous channels through the lipid membrane.

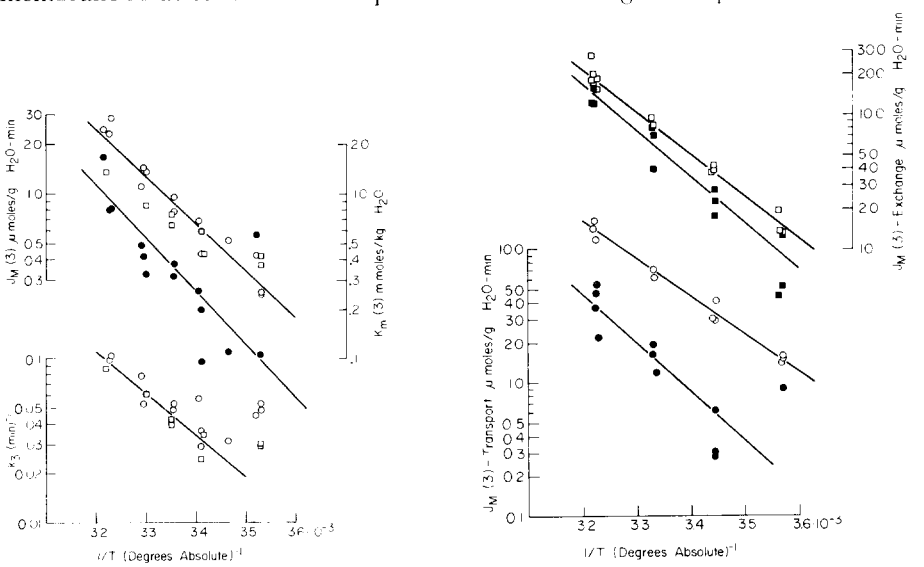

Fig. 7. Temperature dependence of $J_M(3)$, $K_m(3)$ and k_3 for L-tryptophan from experiments on 1-min initial fluxes in transport. Data on $K_m(3)$ are given by ●.

Fig. 8. Temperature dependence of $J_M(3)$ for L-alanine in transport in Krebs–Ringer phosphate (○—○) and in Na^+-free Krebs–Ringer phosphate (●—●), and in homoexchange in Krebs–Ringer phosphate (□—□) and in Na^+-free Krebs–Ringer phosphate (■—■).

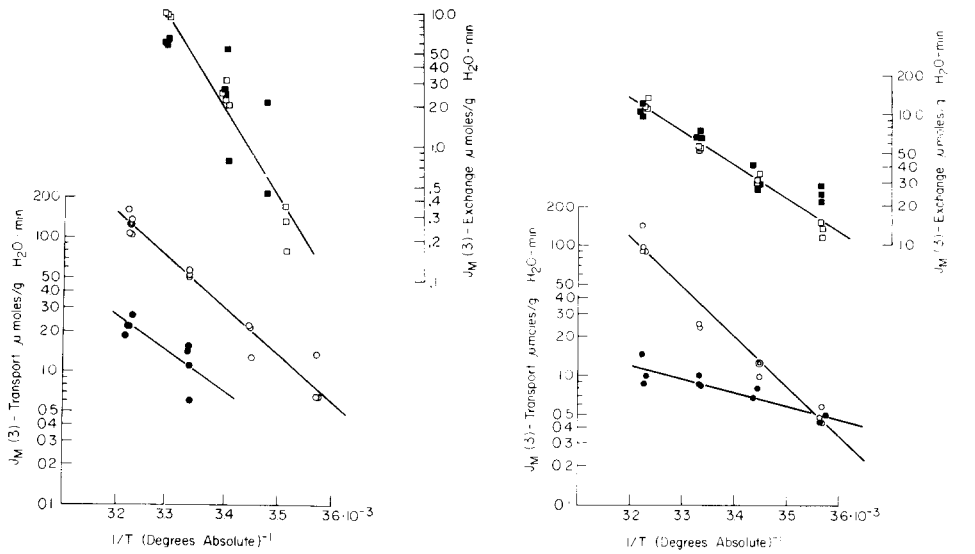

Fig. 9. Temperature dependence of $J_M(3)$ for α-aminoisobutyric acid in transport in Krebs–Ringer phosphate (○—○) and in Na^+-free Krebs–Ringer phosphate (●—●), and in homoexchange in Krebs–Ringer phosphate (□—□) and in Na^+-free Krebs–Ringer phosphate (■—■).

Fig. 10. Temperature dependence of $J_M(3)$ for L-phenylalanine in transport in Krebs–Ringer phosphate (○—○) and in Na^+-free Krebs–Ringer phosphate (●—●), and in homoexchange in Krebs–Ringer phosphate (□—□) and in Na^+-free Krebs–Ringer phosphate (■—■).

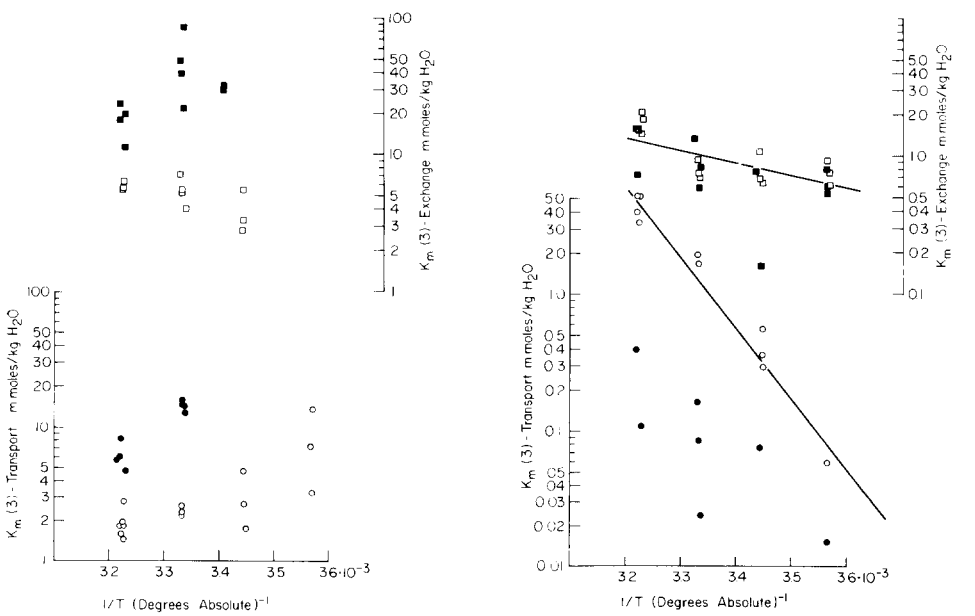

Fig. 11. Temperature dependence of $K_m(3)$ for α-aminoisobutyric acid in transport in Krebs–Ringer phosphate (○) and in Na^+-free Krebs–Ringer phosphate (●), and in homoexchange in Krebs–Ringer phosphate (□) and in Na^+-free Krebs–Ringer phosphate (■).

Fig. 12. Temperature dependence of $K_m(3)$ for L-phenylalanine in transport in Krebs–Ringer phosphate (○—○) and in Na^+-free Krebs–Ringer phosphate (●—●), and in homoexchange in Krebs–Ringer phosphate (□—□) and in Na^+-free Krebs–Ringer phosphate (■—■).

Figs. 8–10 show the temperature dependence of $J_M(3)$ in transport and in exchange, each for Krebs–Ringer phosphate and Na^+-free Krebs–Ringer phosphate, for alanine, α-aminoisobutyric acid and phenylalanine respectively. Note that $J_M(3)$ in exchange is slightly lower in Na^+-free Krebs–Ringer phosphate than in Krebs–Ringer phosphate for alanine, but that this appears not to be true for α-aminoisobutyric acid and clearly is not true for phenylalanine. The $J_M(3)$ obtained in the exchange experiments with α-aminoisobutyric acid showed much more variability than did that of phenylalanine. An interesting difference between phenylalanine and α-aminoisobutyric acid or alanine is that although the $J_M(3)$ in transport is much lower in Na^+-free Krebs–Ringer phosphate than Krebs–Ringer phosphate for all three, $J_M(3)$ in Na^+-free Krebs–Ringer phosphate has an unusually low energy of activation for phenylalanine. Furthermore phenylalanine is similar to tryptophan in that its $K_m(3)$ in transport experiments shows a marked temperature dependence whereas the $K_m(3)$'s for alanine and α-aminoisobutyric acid show little or no temperature dependence. In fact the $K_m(3)$ for α-aminoisobutyric acid increased with a drop in temperature but the results were extremely variable. Figs. 11 and 12 show the behavior of $K_m(3)$ for α-aminoisobutyric acid and phenylalanine.

Table IV summarizes the apparent energies of activation calculated for $J_M(3)$, $K_m(3)$ and k_3 for all of the amino acids studied.

TABLE IV

ENERGIES OF ACTIVATION

Numbers in parentheses are common estimates for Krebs–Ringer phosphate and Na^+-free Krebs–Ringer phosphate. Values are given in kcal/mole.

Amino acid	*Type of experiment*	$J_M(3)$		$K_m(3)$		k_3	
		Na^+	*Choline*	*Na^+*	*Choline*	*Na^+*	*Choline*
L-Methionine	Transport	11.1	—	0	—	17.3*	—
L-Tryptophan	Transport	13.0	—	14.7	—	11.8*	—
Glycine	Transport	15.5	—	0	—	17.7*	—
L-Alanine	Transport	13.0	16.9	0	0	(5.6)	
	Exchange	14.3	15.9	0	3.9	(5.8)	
α-Aminoisobutyric acid	Transport	16.3	13.2	**	**	(10.8)	
	Exchange	(20.4)		3.8	**	(9.9)	
L-Phenylalanine	Transport	17.5	4.8	20.3	15.8	15.9	12.3
	Exchange	(11.8)		(5.8)		(13.8)	

* For range 20–37° C.
** $K_m(3)$ increases as temperature falls.

DISCUSSION

On reviewing our data we were struck by the fact that for a given set of conditions the values of K_m generally showed more fluctuation than did those of J_M. For instance, compare the ratios, S.E./mean for $J_M(3)$ and $K_m(3)$ in Table II and compare the fluctuation in $K_m(3)$ with that in $J_M(3)$ in the figures. One plausible explanation for this finding is that perhaps Eqn. 1 is only an approximation to the true flux equation, an approximation which gives more precise estimates of J_M than of K_m. Many reasonable models[27,28] for the active transport process predict the general form for the net flux equation given by Eqn. 2.

$$J = \frac{J_M c_e - A c_i}{K_m + c_e + B c_i + D c_e c_i} + k(c_e - c_i) \qquad (2)$$

Constants A, B and D as well as J_M and K_m are lumped constants which contain various rate constants and dissociation constants in their defining expressions. Eqn. 2 was derived[27,28] before the role of Na^+ in transport was established but would still hold for the same models, including a Na^+ effect, for a fixed concentration of Na^+. In general one would expect Ac_i to be small in comparison to $J_M c_e$ and $Bc_i + Dc_e c_i$ to be small in comparison to $K_m + c_e$ so long as c_i is far below its steady-state value. This condition is certainly satisfied for initial fluxes but it should be pointed out that for most of the amino acids $c_i > c_e$ after 1 min of incubation for low values of c_e but $c_i < c_e$ for the high values of c_e. For the sake of this argument assume that Eqn. 2 is the true relation describing the initial fluxes measured in our experiments. What effects would we expect to see if we tried to fit the data with Eqn. 1? The points in the data (see Figs. 1–4) which contribute most to the estimation of J_M are those at high values of c_e; for these points $c_i < c_e$ and the terms Ac_i, Bc_i and $Dc_e c_i$ contribute less relative to the other terms than when $c_i > c_e$. Thus the estimates of J_M might well show little effect due to the terms neglected in Eqn. 1. However, the terms which contribute most to the estimation of K_m are those at the lower c_e. For these, $c_i > c_e$, and furthermore for given experimental conditions and c_e, c_i varies by as much as a factor of 1.5 at the lower c_e, from experiment to experiment. Since the denominator in the first term of Eqn. 2 is $K_m + c_e + Bc_i + Dc_e c_i$ the terms Bc_i and $Dc_e c_i$ may contribute significantly to the increased variability of the estimated K_m.

The activation energies for J_M for transport and exchange fall in the band of values 11–20 kcal/mole, except for one, the transport of L-phenylalanine in choline media. For all but alanine the energies of activation for k_3 fall in the same range. The temperature dependence of K_m shows more variation from one amino acid to another. For transport of L-tryptophan and L-phenylalanine $K_m(3)$ decreases with drop in temperature with apparent activation energies of 14.7–20.3 kcal/mole, but $K_m(3)$ shows no change with temperature for methionine, glycine and alanine and for α-aminoisobutyric acid transport it increases as temperature falls. Considering the compound nature of J_M and K_m it is difficult to interpret the values of activation energies obtained. Nonetheless it is worthwhile to compare the values of activation energies we have obtained with those reported for the transport or permeability of other compounds. STEIN[2] reviews the activation energies for water reported for a number of cell types and these fall in a wide range, 4.1–20.3 kcal/mole. TIEN AND TING[32] have found an activation energy of 6.8 kcal/mole for the osmotic permeability coefficient for water movement across bilayers of oxidized cholesterol. For ascites tumor cells and human erythrocytes the reported energies of activation[2] for Na^+ and K^+ influx and efflux fall in the range 12.4–25 kcal/mole, except for one value of 3.3 kcal/mole reported for Na^+ influx in ascites tumor cells. The activation energies for the permeabilities of many polar organic compounds fall in the range 10–25 kcal/mole and STEIN[2] argues that for polar molecules the major energy barrier is due to the number of hydrogen bonds which must be broken in moving from the aqueous phase into the lipid layer of the cell membrane. This argument is still cogent for polar molecules which are transported; it seems reasonable to think that the transition of an amino acid from the aqueous phase to a form bound with carrier in the lipid phase involves the breaking of hydrogen bonds. STEIN[2] would assign five hydrogen

bonds to a neutral amino acid in aqueous solution and if we take 11–20 kcal/mole as the activation energy for $J_M(3)$, we obtain 2.2–4 kcal/mole per hydrogen bond, not unreasonable values for the enthalpy of activation per hydrogen bond.

The data in Table II provide an explanation for the finding that the initial flux of uptake is greater in homoexchange than in transport for some amino acids and is less for others[26,29,30]. For both α-aminoisobutyric acid and phenylalanine, J_M in Krebs–Ringer phosphate does not differ significantly in transport and exchange. However, for α-aminoisobutyric acid K_m goes from 1.9 mmoles per kg water in transport to 6.0 in exchange; hence at all concentrations, but particularly for low extracellular concentrations we expect the initial uptake in exchange experiments to be lower in transport experiments. For phenylalanine on the other hand K_m is 4.5 in transport and 1.9 in exchange; hence for this amino acid the initial uptake in exchange experiments should be greater than in transport. If both J_M and K_m differ between exchange and transport the change in both must be taken into account in evaluating relative uptakes in transport and exchange. In the experiments on alanine J_M was larger in exchange. However, we would put less weight on the transport–exchange comparison with alanine because we believe our experimental methods were not quite comparable in the transport and exchange experiments with alanine (see METHODS). On the basis of the results obtained with α-aminoisobutyric acid and L-phenylalanine it seems possible that J_M might be the same in transport and exchange (if the cells are loaded to high levels as in these experiments) provided the cells are treated the same way, except for the presence of the loading amino acid in the preliminary incubation of the cells for the exchange experiments. In any case it is clear that the comparisons between transport and exchange are best made in terms of the parameters J_M and K_m rather than comparisons at one or two extracellular concentrations of the amino acid under study.

Implications for the A–L distinction

Although not directly related to the problem of the temperature dependence of amino acid transport, our results on the uptake of phenylalanine differ considerably from those reported by CHRISTENSEN and co-workers[10,34] and this difference raises some questions about the rather widely accepted distinction made by OXENDER AND CHRISTENSEN[10] between two systems for transport of neutral amino acids, the A and L systems. One of the major distinctions between these two is that the A system requires Na^+ and is primarily an active transport system whereas the L system does not require Na^+ and is described as primarily a system for exchange[10,33]. The most striking evidence for the existence of two distinct systems was the demonstration by INUI AND CHRISTENSEN[20] that the Na^+-dependent portion of the initial flux of uptake of methionine was approximately equal to the portion of the initial flux inhibitable by α-aminoisobutyric acid. α-Aminoisobutyric acid is an example of an amino acid which enters almost entirely by the A system whereas phenylalanine is supposed to enter primarily *via* the L system. CHRISTENSEN *et al.*[34] report that at an extracellular concentration of about 1 mM only 20 % of the initial flux of phenylalanine is mediated by the A system. However, in our experiments we find that at an extracellular concentration of 1 mM 60–65 % of the initial flux of phenylalanine requires Na^+ and in terms of the maximal flux, $J_M(3)$, 89.4 % of $J_M(3)$ is Na^+ dependent. Indeed we find that only 82.5 % of $J_M(3)$ is Na^+ dependent for α-aminoisobutyric acid. It is possible that

differences between the lines of Ehrlich ascites or differences in technique account for some of the differences between the two laboratories. We use a phosphate-buffered Krebs–Ringer solution whereas CHRISTENSEN and co-workers generally use one buffered with bicarbonate and in this study we have used a 0.5-h preliminary incubation which CHRISTENSEN and co-workers do not use. We are looking into the effect of these factors now. But if the difference between our results is due to a difference in lines of Ehrlich ascites, why is phenylalanine primarily an L-mediated amino acid in one line and an A-mediated amino acid in another? On the other hand if the difference is simply due to a difference in methods one would doubt that the need for Na^+ represents a fundamental part of the distinction between A and L systems.

However, it appears to us that the discrepancy goes even deeper and may bear on the question of the very existence of two distinct systems. When OXENDER AND CHRISTENSEN[10] made the distinction between the A and L systems they pointed out that the L system served well for exchange of amino acids but that the A system was primarily an uphill transport system and served poorly for exchange. Since then JOHNSTONE AND SCHOLEFIELD[25] have shown that methionine homoexchange is Na^+ independent but that approximately one-half of the transport flux of methionine is Na^+ dependent. More recently BELKHODE AND SCHOLEFIELD[35] have shown that heteroexchange between L-methionine and 1-aminocyclopentane carboxylic acid is independent of Na^+ concentration. In our experiments homoexchange of phenylalanine does not require Na^+. Furthermore OXENDER[35] has reported that there are appreciable levels of free amino acids in Ehrlich ascites cells and that these levels can be changed by the methods of preparing the cells. Hence, is the distinction between A and L systems only a distinction between transport and exchange? If so it seems appropriate to ask whether there are really two distinct systems or whether they are merely two aspects of but one system. In theory, the data on J_M can answer this question. To demonstrate this we first introduce a new notation for the experimentally measured quantities. We make the following definitions. $J_M(T,Na)$, the maximal flux in transport experiments with Krebs–Ringer phosphate; $J_M(T,Ch)$, the maximal flux in transport experiments with Na^+-free Krebs–Ringer phosphate; $J_M(X,Na)$, the maximal flux in exchange experiments with Krebs–Ringer phosphate; $J_M(X,Ch)$, the maximal flux in exchange experiments with Na^+-free Krebs–Ringer phosphate.

Now let us consider what the predicted value, $J_M(X,Na)_{pred.}$, would be for the following two hypotheses, (i) there are two distinct systems, one for transport, one for exchange, and (ii) there is only one system which can take part in transport or exchange.

Hypothesis 1. There are two independent systems, one an uphill transport system which requires Na^+, the other a purely exchanging system which does not require Na^+. Then the fluxes mediated by the two systems should be independent and additive. Let $J_M(T)$ be the maximal flux mediated by the transport system and $J_M(X)$ be the maximal flux mediated by the exchange system. Clearly, in terms of experimentally measured quantities, $J_M(T)$ and $J_M(X)$ are given by Eqns. 3.

$$J_M(T) = J_M(T,Na) - J_M(T,Ch)$$
$$J_M(X) = J_M(X,Ch) \qquad (3)$$

Hence, if this hypothesis is true, we would predict that $J_M(X, Na)$ is given by the sum of $J_M(T)$ and $J_M(X)$ as in Eqn. 4.

$$J_M(X,Na)_{pred.} = J_M(T,Na) - J_M(T,Ch) + J_M(X,Ch) \quad (4)$$

From our data in Table II we obtain values of $J_M(X,Na)_{pred.}$ of 20.4 and 16.9 for phenylalanine and α-aminoisobutyric acid respectively whereas the values found were 12.3 and 10.5.

Hypothesis 2. There is only one system which can take part in transport or exchange. Now let $J_M(T)$ and $J_M(X)$ be the maximal transport and exchange fluxes respectively which this single system can give. We do not have an exact estimate of $J_M(T)$. $J_M(T,Ch)$ is not zero and on this hypothesis it measures an exchange flux. But $J_M(T,Ch)$ is small so we can assume that $J_M(T,Na)$ is a fairly good measure of $J_M(T)$, although there may be a small exchange flux included in this measurement. Furthermore $J_M(X,Ch)$ is a good measure of the maximal exchange flux for this system for the particular amino acid under consideration. Hence for the exchange experiment in the presence of Na^+ there is in effect a competition between transport and exchange for the carrier. Let p, $0<p<1$, be the fraction of the system used for transport and $(1-p)$ be the fraction used for exchange in the exchange experiment in Krebs–Ringer phosphate. Then $J_M(X,Na)_{pred.}$ is given by Eqn. 5.

$$J_M(X,Na)_{pred.} = pJ_M(T,Na) + (1-p)J_M(X,Ch) \quad (5)$$

In other words, this hypothesis leads to the prediction that the maximal flux in homo-exchange experiments in Krebs–Ringer phosphate must lie between $J_M(T,Na)$ and $J_M(X,Ch)$. Our data on phenylalanine and α-aminoisobutyric acid adequately satisfy this criterion. The data on alanine satisfy neither hypothesis but fall between the two but as we have pointed out we have some reservations about the comparability of the transport and exchange experiments with alanine.

Obviously our evidence relates to our line of Ehrlich ascites. We cannot say that the A–L distinction is not valid for the line of CHRISTENSEN and co-workers. Furthermore they have presented evidence which is rather persuasive and we believe that the strongest such evidence is the approximate equality of the Na^+-dependent and the α-aminoisobutyric acid-inhibitable flux of methionine[20]. But the distinction does not appear to hold for our line of Ehrlich ascites. Our findings therefore do raise some doubts about the A–L distinction in general. We think that measurement of $J_M(T,Na)$, $J_M(X,Na)$, $J_M(T,Ch)$ and $J_M(X,Ch)$ on the line of CHRISTENSEN and co-workers, with use of their techniques would help to resolve these doubts. We are now planning such experiments on another line of Ehrlich ascites which we carry and which has transport characteristics rather close to those CHRISTENSEN and co-workers have reported for their line.

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