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CARRIER–AMINO ACID STOICHIOMETRY IN AMINO ACID TRANSPORT IN EHRLICH ASCITES CELLS

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SUMMARY

1. The initial flux of L-tryptophan in "exchange diffusion" and in "competition" experiments with other amino acids is significantly increased by certain of the amino acids.

2. For the straight-chain amino acids with aliphatic side chains, there is a progressive, almost constant increase in the stimulatory effect obtained in these experiments for each additional CH_2 group in the side chain.

3. In the competition experiments, branching the chain, decreases the stimulation of initial flux.

4. A model of the transport system in which the ratio of amino acid to carrier is 1:1 can predict the stimulation in the exchange-diffusion experiments. An amino acid to carrier ratio of 2:1 must be assumed to also predict the stimulation observed in competition experiments.

INTRODUCTION

HEINZ AND WALSH[1] reported that the initial influx of an amino acid may be increased in Ehrlich ascites cells if the cells are first loaded with another amino acid. This heteroexchange diffusion has been well documented by reports from a number of laboratories[2–6]. A similar phenomenon in sugar transport in erythrocytes has been called counter flow or counter-transport by WILBRANDT AND ROSENBERG[7,8]. These experimental findings have provided crucial support for carrier hypotheses of active transport. The increase in flux owing to counter flow cannot be explained by models of active transport which involve a set of fixed binding sites; mobile binding sites must be invoked[7–9].

Many of the amino acids compete for active transport. However we have found that for some pairs of amino acids the initial flux of one amino acid is greater when the other amino acid is present in roughly equimolar amounts[6]. This raises the question,

Abbreviation: KRB, Krebs–Ringer–bicarbonate.

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"What stoichiometry between amino acid and carrier is necessary to explain these findings?" We have shown that the findings on heteroexchange diffusion can be explained by a carrier model in which the carrier–amino acid binding ratio is 1:1 (see ref. 9). It was not possible to solve the differential equations describing this model to check whether it could also predict the stimulation of initial flux of one amino acid by another amino acid. As will be shown later, this is unlikely. Shortly after the appearance of this paper[9], J. WONG suggested in a personal communication that it may be necessary to postulate a ternary complex to explain the stimulation of uptake of one amino acid by another.

In order to resolve these questions it was necessary to obtain more extensive data on the concentration dependence of the stimulatory effects seen in the competition experiments, and on the effect of amino acid structure on the effects seen in exchange diffusion and competition experiments, and to examine the possible relations between these effects. This is the raison d'être for the present work.

METHODS AND MATERIALS

The Ehrlich ascites cells used in these experiments were from a hypotetraploid line carried in this laboratory by weekly intraperitoneal inoculation of 0.1-ml ascites into male Swiss albino mice.

Collection and preparation of ascites cells

Mice with 6-day-old ascites tumors were killed by cervical dislocation; the abdomens were opened and the ascites removed with a pipette. Ascitic fluids which were very hemorrhagic were not used. A total of 20–50 ml of ascitic fluid was collected into 100 ml KRB containing 0.2 mg heparin. The resulting suspension was diluted with an equal volume of distilled water. The cells were concentrated by centrifugation, resuspended in KRB, centrifuged once more and resuspended in KRB. This treatment does not impair the ability of the Ehrlich ascites cells to concentrate amino acids. Subsequent procedures other than the incubations were carried out in a cold room maintained at 2–4°.

Exchange diffusion experiments

Solutions, 40 mM in an amino acid, were prepared. The acidic and basic amino acids were neutralized to pH 7.0 with NaOH or HCl and the solutions were made up with KRB and distilled water in the proportions calculated to correct for the osmolarity of the amino acid and any added HCl or NaOH. Equal parts of such a solution and a suspension of the cells in KRB were mixed and incubated with gentle shaking for 30 min in a water bath at 37°. For a control, a mixture of equal parts of KRB and the suspension of cells in KRB was incubated at the same time. The high concentrations were used to obtain high intracellular concentrations of the amino acids in the expectation that in the subsequent exchange diffusion experiment, the carrier would be near saturation in the intracellular amino acid. From the work of CHRISTENSEN AND RIGGS[10] and CHRISTENSEN *et al.*[11] one would expect that the intracellular levels attained with practically all of the amino acids used would be considerably higher than 20 mM. The incubation was terminated by chilling the suspension

by agitating the vessel in an ice bath. The cells were collected by centrifuging in the cold and were resuspended in fresh, cold KRB, centrifuged once more and resuspended in sufficient cold KRB to give a suspension with a cytocrit of 0.20–0.22. As shown in previous experiments with azaserine, an amino acid analog, and tryptophan[6], high intracellular levels are maintained during this washing in the cold. 3 ml of this suspension was placed in one arm of a Heinicke reaction vessel and 3 ml of KRB containing 5.0 mM L-tryptophan was placed in the other arm. This gave initial extracellular concentrations of L-tryptophan of 2.70–2.77 mM. This concentration was chosen because it was above the steeply rising portion of the curve of initial flux against extracellular concentration[6] and because at much lower concentrations there was a large drop in extracellular concentration if the uptake was markedly stimulated by exchange diffusion. The vessels were gassed with 5% CO_2 in air and stoppered. The vessels were immersed in a water bath at 37.0–37.2° for 1 min, the contents of the two arms were mixed and the incubation continued, with shaking, for one more minute. The vessels were plunged into ice water and agitated to hasten temperature equilibration. 2 ml of the suspension was pipetted into a 100 × 13 mm test tube, and centrifuged at 3500 rev./min in a Precision Vari-Hi-Speed Centricone centrifuge for 20 min, in the cold. The pellet and 1 ml of the supernatant solution were extracted and analyzed for L-tryptophan. The remainder of the suspension was used to obtain cell counts, cytocrit, and wet and dry weights, as described in a previous paper[6].

"Competition" experiments

4 ml of an ascites cell suspension (cytocrit 0.20–0.22) was pipetted into one arm of a Heinicke reaction vessel and 4 ml of KRB containing L-tryptophan or L-tryptophan plus another amino acid was pipetted into the other arm. The vessels were gassed with 5% CO_2 in air and stoppered. After an incubation of 2 min at 37.0–37.2°, the contents of the two arms were mixed and the incubation continued for another minute. The remainder of the procedure was the same as for the exchange experiments with the addition that in some experiments the pH of the extracellular fluid was determined at 37° with a Beckman GS pH meter with use of a Beckman No. 40299 constant temperature blood electrode assembly.

The extraction procedures and methods of analysis have been described in detail[6].

Calculations

The quantities which were actually measured on each sample were the extracellular concentration of L-tryptophan, the total L-tryptophan in the pellet derived from 2 ml of suspension, pellet dry weight and total pellet water. The extracellular water retained in the pellets was assumed to equal the sucrose space of the pellets prepared by our procedure. The sucrose space was not measured in the experiments but was read from a calibration curve relating pellet extracellular water and pellet volume which was determined beforehand. The intracellular concentration was calculated with the assumption that the pellet tryptophan, corrected for that in the pellet extracellular space, was uniformly distributed in the intracellular water.

Source of materials

All amino acids used were the best commercially available L forms.

RESULTS

Exchange diffusion

Table I shows the results of a typical experiment. As indicated in this table all variations were run in duplicate. The 1-min fluxes and flux coefficients were calculated in each experiment but these offered no advantages over the 1-min distribution ratios for comparative purposes and had the disadvantage that the cell count, our least accurate procedure, was used in their calculation. The variation in 1-min distribution ratio is greater in "between experiment" comparisons than in "within experiment" comparisons. In seven independent repeats of duplicate determinations of the 1-min distribution ratio for L-tryptophan, the standard error of the means of the duplicates

TABLE I

TRANSPORT AND EXCHANGE DIFFUSION OF L-TRYPTOPHAN

After the first incubation, cells were washed with cold KRB and resuspended in cold KRB; 3 ml suspension was placed in one arm of a Heinicke reaction vessel, and 3 ml of 5.0 mM L-tryptophan in KRB was pipetted into the other arm. After 1 min of temperature equilibration, the contents of the two arms were mixed and incubated 1 min longer before being chilled and analyzed.

	L-Tryptophan found after second incubation – 1 min		
First incubation (30 min)	*Extracellular concentration* (c_e) (*nmoles/kg H_2O*)	*Intracellular concentration* (c_i) (*nmoles/kg H_2O*)	*Distribution ratio* (c_i/c_e)
KRB	2.51	2.61	1.04
	2.48	2.85	1.15
L-Isoleucine	1.95	10.48	5.37
20 mM	1.96	9.94	5.07
L-Glutamine	2.43	3.46	1.42
20 mM	2.42	3.25	1.34
L-Glutamic acid	2.45	3.22	1.31
20 mM	2.48	2.99	1.21

(variation between experiments) was 0.122 whereas the standard error of duplicates (variation within experiments) was 0.029. For this reason the average distribution ratio for L-tryptophan for cells which had not been loaded with another amino acid was set equal to one in each experiment and the relative distribution ratio for the preloaded cells was determined. The means of the duplicates and standard errors of the mean for all amino acids tested are given in Table II. The values grouped around a relative distribution ratio of one (L-glutamic to L-lysine) do not differ significantly from 1.00 or from each other. A large number of repeats will be required to determine whether the small deviations from 1.00 are significant. We have previously reported[6] on an experiment in which the cells were preloaded for 30 min with glycine at 15 mM extracellular concentration; the initial extracellular concentration of L-tryptophan was 3.4 mM. In this experiment the one-minute uptake of L-tryptophan by the glycine-loaded cells (triplicate determinations) was 86 % of that in the cells not loaded with glycine and the relative distribution ratio was 0.84. If these data can be combined with those presented in Table II, we obtain a mean relative distribution ratio for tryptophan of 0.92 for glycine-loaded cells with a standard error of the mean of 0.053 (4 degrees of freedom).

TABLE II

EXCHANGE DIFFUSION EXPERIMENTS, RELATIVE DISTRIBUTION RATIO FOR L-TRYPTOPHAN AFTER PRELOADING CELLS WITH DIFFERENT AMINO ACIDS

Preloaded amino acid	*L-Tryptophan relative distribution ratio**	
	Mean	*Standard error of mean*
L-Methionine	6.44	0.085
L-Histidine	5.77	0.042
L-Leucine	5.68	0.043
L-Norleucine	5.49	0.014
L-Isoleucine	4.77	0.136
L-Valine	3.95	0.018
L-Norvaline	3.84	0.035
L-α-Amino-*n*-butyric acid	1.87	0.022
L-Threonine	1.72	0.024
L-Citrulline	1.63	0.064
L-Glutamine	1.26	0.038
L-Cysteine	1.26	0.012
L-Glutamic acid	1.15	0.046
L-Arginine	1.04	0.051
L-Alanine	1.03	0.001
Glycine	1.03	0.112
L-2,4-Diaminobutyric acid	1.00	0.004
L-Ornithine	0.98	0.004
L-Serine	0.98	0.005
L-Lysine	0.96	0.032
L-Proline	0.74	0.017

* Incubation time, 1 min. Initial extracellular concentration of L-tryptophan was 2.70–2.77 mM. The distribution ratio is given relative to that for L-tryptophan for cells which had no other amino acid present in the preliminary incubation.

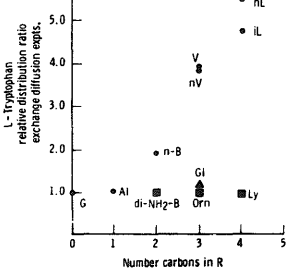

Fig. 1. Effect of side chain of preloaded amino acid on relative distribution ratio for L-tryptophan in exchange diffusion experiments. Incubation time 1 min. Abbreviations: G, glycine; Al, L-alanine; n-B, L-α-amino-*n*-butyric acid; nV, L-norvaline; nL, L-norleucine; Me, L-methionine; V, L-valine; L, L-leucine; iL, L-isoleucine; diNH_2-B, L-2,4-diaminobutyric acid; Orn., L-ornithine; Ly, L-lysine; Gl, L-glutamic acid.

Examination of Table II suggests a correlation between the lipid solubility properties of the side chain of the loaded amino acid and its effect in stimulating the initial flux of L-tryptophan by exchange diffusion. This is shown in Fig. 1 in which the relative distribution ratio for L-tryptophan is plotted against the number of carbons in the side chain for the amino acid which have a straight or branched side chain. Methionine is included in the graph since it is a straight chain with sulfur replacing a carbon. The covalent single-bond radii for carbon and sulfur are 0.77 and 1.04 Å respectively[12]. Methionine was placed at 4.25 carbon atoms to correspond to measurements made on molecular models, using atomic models according to Stuart and Briegleb (LaPine Scientific Co.). There is a strong correlation between the number of carbons in the side chain and the exchange diffusion effect. The value for isoleucine suggests that there may be a superimposed steric effect from the branching; this is brought out more clearly in the competition experiments. The lipid solubility argument is strengthened when we examine the effect of adding a charged group to the side chain as in diaminobutyric, ornithine, lysine and glutamic acid. In each case the enhanced uptake of L-tryptophan due to exchange diffusion is abolished. Of course this may mean that these amino acids use a different transport system. On the other hand, perhaps the combination of carrier with amino acid can cross the membrane at a significant rate only when the ionizable group on the side chain is nonionized. If we take lysine, for example, with a pK_a of 10.5 for the ε-amino group and assume an intracellular pH of 6.8, we find that only 0.02 % of the intracellular lysine would have the ε-amino group nonionized; even if the initial intracellular concentration of lysine were 50 mM in these exchange experiments this would give an effective concentration of only 0.01 mM. Since histidine markedly stimulated the exchange uptake

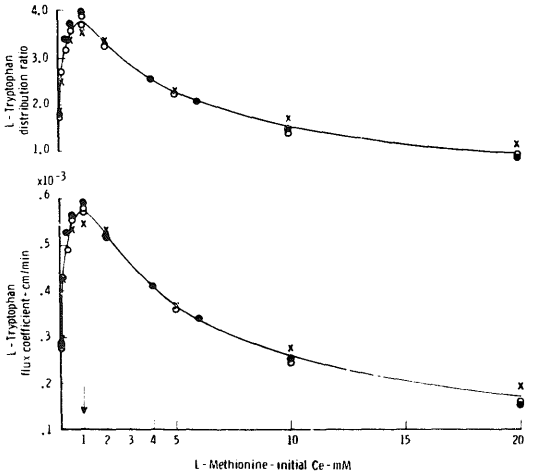

Fig. 2. Effect of extracellular L-methionine on initial uptake of L-tryptophan. Initial extracellular concentration of L-tryptophan, 1 mM. Incubation time, 1 min. c_e, extracellular concentration.

of tryptophan it is instructive to look at it from this viewpoint. The imidazole group of histidine has a pK_a of 6.1. At an intracellular pH of 6.8, 83% of the imidazole groups would be nonionized.

Competition experiments

In a previous paper[6], we reported that the uptake of L-tryptophan in a 2-min incubation was inhibited by many of the other amino acids but that azaserine, 2,4-diaminobutyric acid, histidine and leucine stimulated when present in roughly

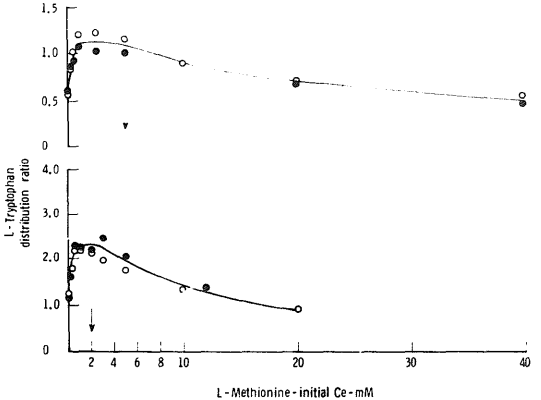

Fig. 3. Effect of extracellular L-methionine on initial uptake of L-tryptophan. Initial extracellular concentrations of L-tryptophan, 2 mM for lower curve; 5 mM for upper curve. Incubation, 1 min.

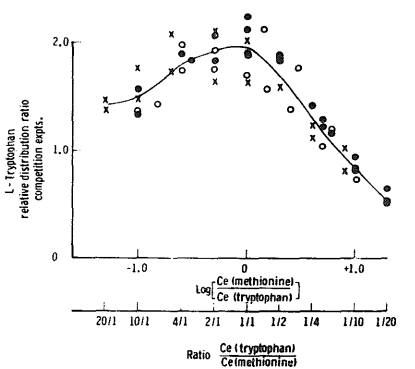

Fig. 4. Relative distribution ratio for L-tryptophan as function of the initial ratio of L-tryptophan to L-methionine in the extracellular fluid. Initial extracellular L-tryptophan: ●—●, 1 mM; ○—○, 2 mM; ×—×, 5 mM. Incubation, 1 min.

equimolar amounts and inhibited when present at five fold higher concentrations. SCHOLEFIELD[13] has also reported significant stimulation of uptake of DL-leucine in the presence of a few other amino acids but for incubation times of 45 min. These findings require more detailed examination. All of the amino acids tested in the exchange diffusion experiments were therefore tested in competition type experiments.

In Fig. 2 are plotted the results of three independent experiments on the 1-min uptake of L-tryptophan, initially at 1 mM extracellular concentration, at different initial extracellular concentrations of L-methionine. The data are given in terms of distribution ratio and flux coefficient. The flux coefficient was calculated by dividing the 1-min flux by the average of the initial and final extracellular concentrations of L-tryptophan, the latter being expressed in mmoles/ml. At 1 mM L-methione there is slightly more than a two-fold stimulation of L-tryptophan flux and inhibition does not appear until the L-methionine concentration is greater than 8–9 mM. Furthermore, there is a marked stimulation when the L-methionine is only 0.1 mM. CHRISTENSEN *et al.*[11] have reported a marked effect of pH on the concentrative uptake of glycine and histidine. The findings reported here cannot be attributed to a pH effect; there was no significant difference in extracellular pH at the end of the incubations, for the entire range of L-methionine concentrations used. Also, there were no significant differences in pellet volume at the various concentrations of L-methionine. Fig. 3 gives the results of similar experiments conducted at initial extracellular concentrations of L-tryptophan of 2 and 5 mM respectively. In Fig. 4 all of the data on competition experiments with L-methionine are given in terms of the relative distribution ratio for L-tryptophan and the tryptophan–methionine ratio in the extracellular fluid. Apparently the relative effect is dependent primarily on the tryptophan to methionine ratio. However, it is also apparent from examination of the curves in Figs. 2 and 3 that the peak tends to broaden and that there is a small shift in the L-tryptophan to L-methionine ratio at the peak as the concentration of L-tryptophan increases.

In contrast to the results with methionine, alanine, which gave no enhanced uptake of L-tryptophan in the exchange diffusion experiments gave only inhibition in competition experiments, the inhibition increasing with the concentration of alanine (Fig. 5).

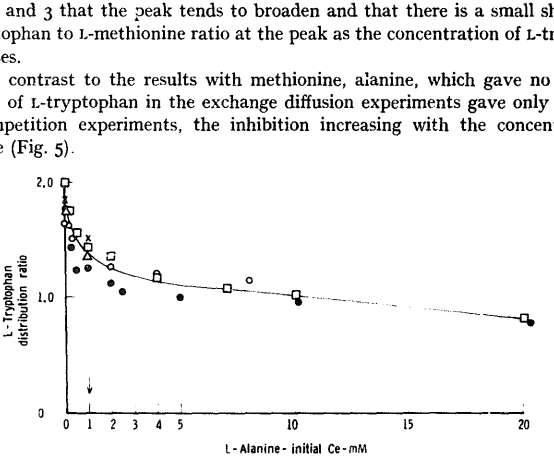

Fig. 5. Effect of L-alanine on uptake of L-tryptophan. Initial extracellular concentration of L-tryptophan, 1 mM. Incubation, 1 min.

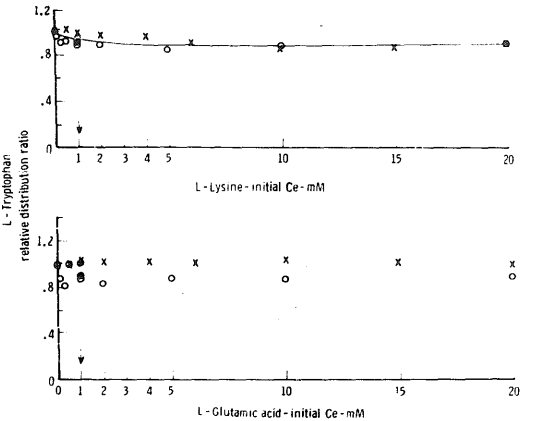

Fig. 6. Relative distribution ratio for L-tryptophan in presence of various concentrations of L-lysine and L-glutamic acid. Incubation, 1 min. Initial extracellular concentration of L-tryptophan, 1 mM.

TABLE III

COMPETITION EXPERIMENTS

EFFECT OF OTHER AMINO ACIDS ON 1-MIN DISTRIBUTION RATIO OF L-TRYPTOPHAN

Other amino acid	*No. of determinations*	*L-Tryptophan* relative distribution ratio*	
		Mean	*Standard error of mean*
L-Methionine	5	2.09	0.06
L-Norleucine	4	1.93	0.08
L-Norvaline	4	1.49	0.08
L-Histidine	5	1.41	0.07
L-Leucine	4	1.25	0.02
L-Cysteine	4	1.25	0.02
L-α-amino-*n*-butyric acid	4	1.11	0.03
L-Valine	4	1.06	0.02
L-Ornithine	5	1.03	0.02
L-Arginine	5	1.01	0.03
L-Citrulline	5	1.00	0.03
L-Glutamic acid	4	0.96	0.04
L-Threonine	4	0.94	0.02
L-Lysine	4	0.94	0.02
L-2,4-Diaminobutyric acid	4	0.93	0.03
Glycine	4	0.91	0.04
L-Serine	4	0.91	0.03
L-Glutamine	5	0.88	0.03
L-Proline	4	0.88	0.06
L-Isoleucine	5	0.80	0.04
L-Alanine	5	0.78	0.03.

* L-tryptophan and the other amino acid were present at initial extracellular concentrations of 1 mM. The distribution ratio after a 1-min incubation is given relative to that for L-tryptophan in the absence of any other amino acid.

Because of the findings in the exchange diffusion experiments, it was important to do concentration-dependence studies with glutamic acid and lysine. As shown in Fig. 6 no evidence for any stimulatory or inhibitory effect was found with glutamic acid. In one of the experiments with glutamic acid all points but those at zero and

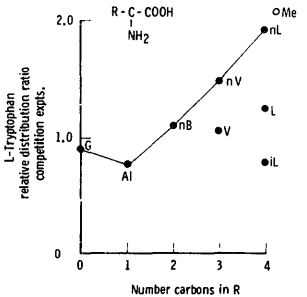

Fig. 7. Effect of side chain of "competing" amino acid on relative distribution ratio for L-tryptophan in competition experiments. Incubation, 1 min. Abbreviations as for Fig. 1.

0.5 mM glutamic acid were slightly low, but no concentration dependence was evident. With lysine, however, a small inhibitory effect was obtained, increasing up to about 5 mM lysine and then remaining constant up to 20 mM.

All of the amino acids used in the exchange diffusion experiments were tested in "competition" experiments with the initial extracellular concentrations of L-tryptophan and the other amino acid both at 1 mM. The results are summarized in Table III. Because the stimulatory effects were not nearly so large as in the exchange diffusion

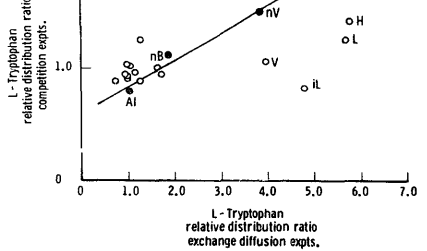

Fig. 8. Relative distribution ratios for L-tryptophan for incubation time of 1 min. Correlation between results of "competition" experiments and exchange diffusion experiments. The straight line was drawn by eye to fit the points for the straight-chain amino acids indicated by the darkened circles. Abbreviations as for Fig. 1; H, L-histidine. The unlabeled points are for the other amino acids given in Tables II and III.

experiments, 4 or 5 independent experiments were carried out with each amino acid. The stimulatory effects of leucine and histidine were again found but our previous report of a stimulatory effect with 2,4-diaminobutyric acid[6] could not be substantiated in the present more detailed investigation.

In Fig. 7 the results for the amino acids with aliphatic side chains are plotted against the number of carbons in the side chain. Methionine is included as in the corresponding graph of the exchange diffusion data. Starting with alanine, which inhibits, there is a progressive increase in the stimulatory effect for the straight-chain compounds, but branching the chain decreases this effect and isoleucine actually inhibits. The results obtained in the "competition" experiments are plotted against those from the exchange diffusion experiments in Fig. 8. For the straight aliphatic side-chain amino acids there is a close correlation between the results obtained in the "competition" and in the "exchange diffusion" experiments with L-tryptophan.

THEORY

In theoretical discussions of carrier active transport the assumption has usually been made that the carrier combines with one molecule of substrate[8,9,14,15]. Thus HEINZ AND PATLAK'S[14] calculation of the energy expenditure for active transport of glycine in Ehrlich asocites cells is based on a model which assumes a 1:1 combination of carrier with amino acid. Recently, STEIN[16] has suggested that facilitated diffusion of glycerol in the erythrocyte could be explained with the assumption that dimerization occurs at a binding site on the membrane followed by dissociation of the dimer and crossing of the membrane. This comes close to an assumption of a ternary complex involving a carrier, however, STEIN specifically discards a movable carrier as part of his hypothesis. In a previous paper[9] we have shown that a number of carrier models, all of which involve a 1:1 carrier – substrate complex, can predict the stimulation of initial flux by exchange diffusion. In the simplest of these, the linkage to cellular metabolism is via reactions whose effect is to speed up the movement of free carrier from the inner to the outer surface of the cell membrane. In order to explain the increase in initial flux of amino acid "a" after preloading with amino acid "b" this model requires that the rate of regeneration of free carrier at the outer surface of the cell membrane by movement as the complex Cb be higher than the rate of regeneration by the active transport system. This implies that the rate of regeneration of free carrier by active transport is the primary rate-limiting step in the uptake of an amino acid like L-tryptophan. To prove this, assume the contrary, that is assume that the rate of formation of Ca and the rate at which Ca crosses the membrane is rate-limiting. But then an increase in the delivery of free carrier to the outer surface of the cell membrane by exchange diffusion could have little effect on the initial flux of L-tryptophan. Furthermore, preloading the cells with L-tryptophan could not stimulate uptake of another amino acid by exchange diffusion. However, we have shown that preloading with either tryptophan or with azaserine markedly stimulates the uptake of the other amino acid[6].

With this as a background it is easy to show that a model which assumes a 1:1 carrier – substrate combination cannot explain the results of the competition experiments. To be specific, consider the experiments with tryptophan and methionine. Assume that the carrier – amino acid complex is 1:1. Then so long as we assume that methionine and tryptophan utilize the same carrier and that the total amount of

carrier is constant during the 1-min incubations, we must expect competition between the two amino acids. At first glance, one might assume that the methionine can enter much more rapidly than the tryptophan and then increase the tryptophan flux by exchange diffusion. However, application of the above conservation conditions makes this untenable. No matter how much carrier is made available at the outer surface of the cell membrane by outward movement as a complex with methionine, this same amount of carrier had to be used to get the methionine into the intracellular phase in the first place, a self-cancelling process. Furthermore, the amount of methionine which could have entered the cells by free diffusion would be negligible compared to the carrier-borne methionine at the low concentrations which gave marked stimulation of the initial flux of L-tryptophan.

Let us consider then whether a model in which one molecule of carrier can combine with two molecules of amino acid can explain the experimental findings on amino acid transport. To simplify matters we assume an obligatory 2:1 amino acid to carrier ratio. Whatever new features are introduced by the assumption of the 2:1 ratio should appear in this model. However, it should be kept in mind that this is a simplification and if 2:1 amino acid – carrier complexes can be formed then it is possible that 1:1 complexes may also be able to cross the cell membrane at an appreciable rate.

A ternary complex model of carrier active transport

The basic model is shown schematically in Fig. 9 for the case in which only one amino acid is present. The following simplifying assumptions are made: (a) The combination of carrier with amino acid is so rapid in comparison to the rate constants

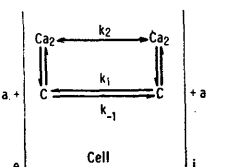

Fig. 9. Schematic diagram of a transport model with a 2:1 ratio of amino acid to carrier.

for the crossing of the membrane that we can use equilibrium constants. This simplifies the model considerably and cannot be far off because if the opposite were true it would be difficult to explain the stimulation of initial flux by exchange diffusion. (b) The dissociation constants are the same for the two sides of the membrane. (c) The complex Ca moves so slowly in comparison to Ca_2 that its effect may be neglected. This is the assumption of an obligatory ternary complex. (d) The "active transport" is introduced in a highly simplified form by assuming that the effect of the linkage to cellular metabolism is equivalent to increasing the rate constant for outward movement of free carrier, *i/e.* $k_1 \gg k_{-1}$.

Notation

Assuming that two amino acids "a" and "b" are present, the following notation will be used.

C_0 = total amount of carrier in μmoles/cm^2 surface area; x_1, x_2 = concentrations of free carrier at outer and inner surface of cell membrane, in μmoles/cm^2. c_{ea}, c_{ia} = concentration of amino acid "a" in the extracellular and intracellular phases respectively in μmoles/cm^3; c_{eb}, c_{ib} = concentration of amino acid "b" in the extracellular and intracellular phase respectively; k_{pa}, k_{pb} = permeability constants for free amino acids, cm/min; k_3, k_4 = rate constants for movement of complexes Cb_2 and Cab across cell membrane: k_2, k_1 and k_{-1} are indicated in Fig. 9; A = surface area of cells in cm^2; V_e = volume of extracellular phase in cm^3; V_i = volume of intracellular water in cm^3; A, V_e and V_i are assumed to be constant; K_1, K_2 = association constants for Ca_2 and Cb_2, K_1 = [Ca$_2$]/[C] [a]^2; K_3 = association constant for Cab, K_3 = [Cab]/[C] [a] [b].

The association constants have units (μmoles)$^{-2}$ cm^{+6}. These association constants are different from the usual association constants since the process involved is the crossing of an interface between a bulk aqueous phase and the membrane lipid phase by the amino acid and the formation of the complex with the carrier.

Mathematical formulation

The model is completely described by the initial conditions, three conservation equations (1–3) and three differential equations (4–6).

$$C_0 = x_1[1 + K_1c_{ea}^2 + K_2c_{e0}^2 + K_3c_{ea}c_{eb}] + x_2[1 + K_1c_{ia}^2 + K_2c_{ib}^2 + K_3c_{ia}c_{ib}] \quad (1)$$

$$S_a = V_ec_{ea} + V_ic_{ia} + A[2K_1x_1c_{ea}^2 + K_3x_1c_{ea}c_{eb} + 2K_1x_2c_{ia}^2 + K_3x_2c_{ia}c_{ib}] \quad (2)$$

$$S_b = V_ec_{eb} + V_ic_{ib} + A[2K_2x_1c_{eb}^2 + K_3x_1c_{ea}c_{eb} + 2K_2x_2c_{ib}^2 + K_3x_2c_{ia}c_{ib}] \quad (3)$$

S_a, S_b are the amounts of amino acids "a" and "b" in the system, assumed constant. If one can assume that the extracellular concentrations are constant, Eqns. 2 and 3, are not needed.

$$\frac{d}{dt}\left\{x_2[1 + K_1c_{ia}^2 + K_2c_{ib}^2 + K_3c_{ia}c_{ib}]\right\} = x_1[k_{-1} + k_2K_1c_{ea}^2 + k_3K_2c_{eb}^2 + k_4K_3c_{ea}c_{eb}] - x_2[k_1 + k_2K_1c_{ia}^2 + k_3K_2c_{ib}^2 + k_4K_3c_{ia}c_{ib}] \quad (4)$$

$$\frac{d}{dt}\left\{\frac{V_i}{A}c_{ia} + 2K_1x_2c_{ia}^2 + K_3x_2c_{ia}c_{ib}\right\} = k_{pa}(c_{ea} - c_{ia}) + x_1[2k_2K_1c_{ea}^2 + k_4K_3c_{ea}c_{eb}] - x_2[2k_2K_1c_{ia}^2 + k_4K_3c_{ia}c_{ib}] \quad (5)$$

$$\frac{d}{dt}\left\{\frac{V_i}{A}c_{ib} + 2K_1x_2c_{ib}^2 + K_3x_2c_{ia}c_{ib}\right\} = k_{pb}(c_{eb} - c_{ib}) + x_1[2k_3K_2c_{eb}^2 + k_4K_3c_{ea}c_{eb}] - x_2[2k_3K_2c_{ib}^2 + k_4K_3c_{ia}c_{ib}] \quad (6)$$

Simple analytic solutions of these equations cannot be obtained. However, we can find the stationary state fluxes corresponding to the intial conditions of exchange diffusion and competition experiments. This should tell us whether this model could give the type of results found experimentally.

Exchange diffusion

In order to see whether this model can predict an increase in the carrier flux of "a" after the cells have been preloaded with "b" we solve for the stationary state flux under the conditions c_{ea} and c_{ib} constant and $c_{eb} = 0$, $c_{ia} = 0$. We neglect simple

permeability. Using F_x (a) to denote the flux of "a" in the exchange diffusion type of experiment, we obtain Eqn. 7.

$$F_x(a) = \frac{2k_2K_1C_0c^2_{ea}}{\Delta_2}[k_1 + k_3K_2c^2_{ib}] \tag{7}$$

where

$$\Delta_2 = [k_1 + k_{-1} + K_1(k_1 + k_2)c^2_{ea} + K_2(k_{-1} + k_3)c^2_{ib} + K_1K_2(k_2 + k_3)c^2_{ea}c^2_{ib}] \tag{8}$$

If we also set $c_{ib} = 0$, we obtain the transport flux of "a", F_T(a), when the cells have not been preloaded.

$$F_T(a) = \frac{2k_1k_2K_1C_0c^2_{ea}}{\Delta_1} \tag{9}$$

where

$$\Delta_1 = k_1 + k_{-1} + K_1(k_1 + k_2)c^2_{ea} \tag{10}$$

Note that F_T(a) shows a saturation effect[6]. The difference $F_x(a) - F_T(a)$ is given by

$$F_x(a) - F_T(a) = \frac{2k_2K_1K_2C_0}{\Delta_1\Delta_2}c^2_{ea}c^2_{ib}(k_{-1} + k_2K_1c^2_{ea})(k_3 - k_1) \tag{11}$$

Since all the rate constants and association constants are positive by definition, Δ_1 and Δ_2 are positive and $F_x - F_T > 0$ if $k_3 > k_1$. This then is the necessary condition for the carrier-mediated flux to be greater in an exchange diffusion experiment than in a transport experiment. Furthermore, if $k_3 < k_1$ this model predicts that preloading the cells with "b" will decrease the flux of "a". This is fairly obvious physically for in this case the intracellular amino acid competes for carrier with the transport system but delivers carrier to the outer surface of the cell membrane at a rate lower than the rate of regeneration of carrier by active transport. L-Proline may be an example of this (Table II); however, the experiments with L-proline should be repeated to clearly establish the significance of the value 0.74 for the relative distribution ratio.

The present model clearly predicts the stimulation of influx of one amino acid by preloading with another amino acid.

Competition

To check whether the initial flux of "a" could be increased when both "a" and "b" are present initially only in the extracellular phase, Eqns. 1–6 were solved for the stationary state fluxes under the assumptions $c_{ia} = 0$, $c_{ib} = 0$, and c_{ea} and c_{eb} constant, these being the conditions at the start of the competition experiments. If F_C(a) represents the stationary state flux of "a" in such a hypothetical competition experiment, the solution obtained is given by Eqn. 12.

$$F_C(a) = \frac{k_1C_0}{\Delta_3}[2k_2K_1c^2_{ea} + k_4K_3c_{ea}c_{eb}] \tag{12}$$

$$\Delta_3 = [k_1 + k_{-1} + K_1(k_1 + k_2)c^2_{ea} + K_2(k_1 + k_3)c^2_{eb} + K_3(k_1 + k_4)c_{ea}c_{eb}] \tag{13}$$

As expected Eqn. 12 reduces to Eqn. 9 if $c_{eb} = 0$. Eqn. 12 may be rewritten

$$F_C(a) = \frac{c^2_{ea} + Ec_{ea}c_{eb}}{A + Bc^2_{ea} + Cc_{ea}c_{eb} + Dc^2_{eb}} \tag{14}$$

An attempt was made to fit the curves of tryptophan uptake in the presence of methionine with Eqn. 14, but a good fit could not be obtained under the constraint that all rate constants and association constants are positive. This was not unexpected because Eqn. 14 is derived with the assumption that the intracellular concentrations remain zero over the time of measurement of the flux. In the experiments, the intracellular concentration of L-tryptophan attained after 1 min of incubation was in most cases higher than the extracellular. Thus even if one had good estimates for the constants A, B, C, D and E, Eqn. 14 should give fluxes higher than the measured initial fluxes. However, it is important to determine whether any choice of positive constants can give the type of curve for the flux coefficient shown in Fig. 2. To this end, the constants A and B were estimated from the initial flux data on L-tryptophan in the absence of L-methionine; for the reasons given above these are probably overestimated. The constants C, D and E were then estimated from the tryptophan–methionine competition experiments by assuming that even if Eqn. 14 gives a poor quantitative fit, the concentration of L-methionine giving maximal stimulation for a given concentration of L-tryptophan will not differ appreciably from that predicted by Eqn. 14. Carrying out this program we obtain a range of positive values for A, B, C, D, E which give reasonable values for the fluxes when substituted into Eqn. 14. Choosing a particular set: A, $1 \cdot 10^3$ (μmoles) cm^{-4} min; B, $2 \cdot 10^3$(μmoles)$^{-1}$ cm^2 min; C, $2 \cdot 10^3$ (μmoles)$^{-1}$ cm^2 min; D, $3 \cdot 10^3$ (μmoles)$^{-1}$ cm^2 min; and E, 10 (dimensionless constant), and calculating the flux, $F_C(a)$, for "a" at a constant extracellular concentration of 1 mM one obtains the graph shown in Fig. 10. This curve has practically the same shape as does the experimental curve but as expected the predicted fluxes are considerably higher than the experimental. Furthermore, retaining the values for A and B, one can fit the curve for inhibition of tryptophan uptake by alanine by a new choice of C, D and E and the data on glutamic acid and lysine can be fitted by choosing very low values for the association constants K_2 and K_3 and for the rate constants k_3 and k_4.

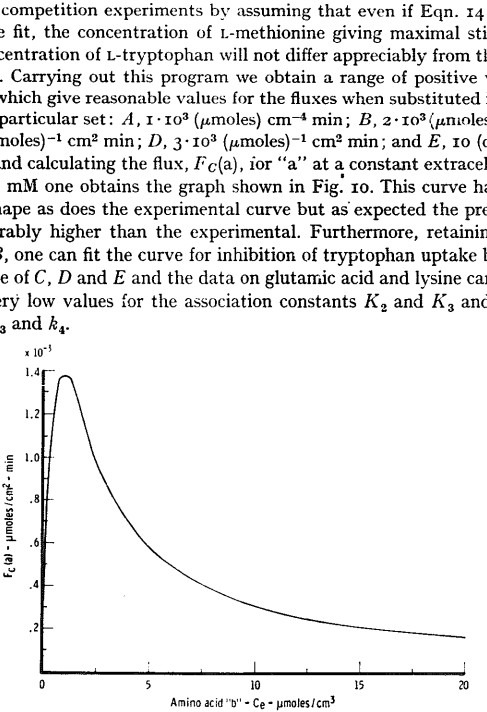

Fig. 10. Stationary state flux, $F_c(a)$, calculated from Eqn. 14 for different extracellular concentrations of amino acid "b". The constants appearing in Eqn. 14 were chosen to be: A, 10^3; B, $2 \cdot 10^3$; C, $2 \cdot 10^3$; D, $3 \cdot 10^3$; E, 10. The units are given in the text.

Considering the approximations made in the derivation, this model adequately predicts all of the results of the experiments on competition between tryptophan and other amino acids.

The steady state

One can solve, without any approximations, for the steady state relation between intracellular and extracellular concentrations if one amino acid is present. Doing this one obtains Eqn. 15.

$$(c_e - c_i) = \frac{2k_2K_1C_0}{\kappa_p} \frac{[k_1c^2_e - k_{-1}c^2_i]}{[k_1 + k_{-1} + K_1(k_1 + k_2)c^2_e + K_1(k_{-1} + k_2)c^2_i + 2K_1{}^2k_2c^2_ec^2_i]} \qquad (15)$$

Comparison of initial fluxes and steady state concentrations

CHRISTENSEN *et al.*[5] have pointed out that among the neutral amino acids one can find all combinations of values for initial flux and steady state distribution ratio, that is, one can find both high and low steady state distribution ratios in combination with high and low initial fluxes. They have suggested that this might be explained by the existence of a second carrier for the amino acid, this second carrier being either entirely distinct from the active transport carrier or an altered form of the active transport carrier. The alteration in the latter case could be an essential part of the active process which moves the carrier outwards. The present model is also consistent with these findings. If one arbitrarily picks values for C_0, k_1, k_{-1}, k_2, K_1 which give correct order of magnitude values for the steady state distribution ratios for L-tryptophan, then by varying k_2 and K_1 one can obtain either high or low initial fluxes from Eqn. 14 combined with either high or low steady state distribution ratios as calculated from Eqn. 15.

In summary, a simplified model of carrier active transport, based on the assumption of an obligatory 1:2 carrier – amino acid combination, at least qualitatively fits the experimental findings on amino acid transport. In particular, this model predicts (a) saturation kinetics of transport (b) the possibility of an increase or decrease of initial flux in an exchange diffusion experiment (c) stimulation or inhibition in competition experiments and the correct concentration dependence of these effects, and (d) the occurrence of high or low steady state distribution ratios in combination with high or low initial fluxes for different amino acids.

There remains one experimental finding which is not explained by previous models or by the model with an obligatory 2:1 amino acid – carrier ratio. In a previous paper[6] we have reported on the 1-min uptakes of L-tryptophan at initial extracellular concentrations of 3.3–3.4 mM. The intracellular concentrations attained were 12.15 mmoles/kg water for cells which had been loaded with azaserine and 2.6 for cells which had not been loaded with azaserine. For the cells loaded with azaserine, the intracellular azaserine concentration dropped 6 mmoles/kg water more in the cells incubated with tryptophan than in those incubated in KRB. Corrections for simple diffusion, made with the assumption that the permeability constants are 10^{-5} cm/min, changed these figures by insignificant amounts. This allows us to calculate upper and lower bounds for the tryptophan–azaserine exchange ratio. To obtain a lower limit, assume that the active transport was unchanged in the cells undergoing exchange diffusion, then L-tryptophan and azaserine exchanged in the ratio 1.59:1. The assumption that the

active transport was abolished in the exchange diffusion situation gives an upper limit for the exchange ratio of 2.03:1. If active transport and exchange diffusion compete for available carrier one would expect the true value to lie inside these limits. For the reverse experiment in which the cells were first loaded with tryptophan. the limits of the exchange ratio for azaserine:tryptophan were 1.4:1 to 1:2.06. JOHNSTONE AND SCHOLEFIELD[3] have reported exchange ratios for methionine and ethionine. Recalculating lower and upper bounds for the exchange ratios from their data, one obtains the bounds 1:1.23 to 1:1.35 for the methionine:ethionine ratio when the cells have been preloaded with ethionine. For the reverse experiment, in which the cells were preloaded with methionine, one obtains ethionine:methionine exchange ratios of 1:1.16 to 1:1.43. Some reservations should be made about the experiments with azaserine because this amino acid is toxic. However even if one assumes that the cells were damaged and that the permeability of the cell membrane was changed one cannot explain the exchange ratio obtained in the experiment in which the cells were loaded with azaserine. Obviously the stoichiometry of amino acid heteroexchange must be re-examined in extenso. For the moment, it should be pointed out that the above observations can fit into the present model if we relax the restriction on an obligatory 2:1 amino acid to carrier ratio. For the sake of argument, assume that the relative rate constants and ease of formation of 1:1 and 2:1 complexes can vary with different amino acids; the size, orientation and polar character of the amino acid side chain might be significant factors involved in this. Then with this addition, the present model predicts that the exchange ratios for different pairs of amino acids should fall in the range 1:1 to 2:1 in exchange diffusion experiments. This is obviously a critical test of this added assumption and should be tested with a number of different pairs of amino acids. Finally, it should be observed that exchange ratios other than 1:1 are not excluded on the basis of energetics; the exchange diffusion or counter flow experiments are carried out under conditions which are far from an equilibrium or stationary state.

DISCUSSION

The data presented relate amino acid transport to the lipid phase of the cell membrane. The involvement of lipids in ion transport has frequently been suggested[17–24] and recently lipid–amino acid complexes[25–27] have been reported which could conceivably be involved in amino acid transport. However the data on exchange diffusion put constraints on the complexes admissable for this role. Since active transport fluxes are inhibited but exchange diffusion fluxes are practically unaffected by inhibitors such as dinitrophenol[4,28], it is unlikely that cellular metabolism is required for formation of the amino acid–carrier complex. Thus this binding must be readily reversible, suggesting that we seek salt linkages or coordination linkages between amino acid and carrier. Because the α-amino and -carboxyl groups are involved in the binding[29], a zwitterionic membrane constituent such as phosphatidyl ethanolamine or choline[30] would seem a likely candidate. Some time ago, CHRISTENSEN *et al.*[31–33] proposed a specific coordination hypothesis but recently CHRISTENSEN[29] has questioned its validity.

The amino acids alanine, α-amino-*n*-butyric acid, *n*-valine and *n*-leucine are a structural series with increasing length of a straight, aliphatic side chain. Methionine

fits into this series if we accord it a position corresponding to the increase in chain length obtained by substituting sulfur for one carbon. Their effect on the initial flux of L-tryptophan, both in exchange diffusion and competition experiments, increases progressively with the length of the side chain and there is a good correlation between the effects obtained in the two types of experiments. This series suggests a lipid solubility effect. In terms of a carrier model there are two basic processes to consider. One is the formation of the carrier–amino acid complex, the other is the crossing of the membrane by this complex. The former involves not only binding of the α-amino and -carboxyl groups to the carrier but also the ease with which an amino acid can cross from the aqueous phase into the surface layer. The latter should be markedly affected by the length of the aliphatic side chain. PANKHURST[34] has shown that the penetration of water-soluble aliphatic alcohols and acids into monolayers of higher fatty acids, of cholesterol and of coprostenone, increases 4–5-fold for each additional CH_2 group in the penetrating molecule. We would expect to find a similar effect of side-chain length on the penetration of amino acids into the cell membrane, particularly since the cell membrane may have an ordered structure[35] similar to that found in monolayers of lipids on water. The length of the side chain may also affect the rate of crossing the membrane, depending on the ease of fit into the membrane structure. Both the ease of penetration of the membrane and the rate of crossing the membrane might be decreased in the branched-chain analogs because of a bulk effect of the side chain on the case of fitting into the membrane structure. There might also be a more specific steric effect on the binding to the carrier. This effect of branching of the chain is quite obvious in the competition experiments, less so in the exchange diffusion experiments. Perhaps saturation of the carrier by the high intracellular concentrations in the exchange diffusion experiments partly masked the effect of the branching of the side chain. In terms of the model of an obligatory 2:1 amino acid–carrier complex the correlation between the fluxes in exchange diffusion and competition experiments implies a correlation between the association constants K_2 and K_3 and also between the rate constants k_3 and k_4.

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