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PHENYLALANINE AND METHIONINE TRANSPORT IN *TETRAHYMENA PYRIFORMIS*

CHARACTERISTICS OF A CONCENTRATING, INDUCIBLE TRANSPORT SYSTEM

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SUMMARY

1. *Tetrahymena pyriformis* can take up L-phenylalanine against a concentration gradient.

2. The influx of L-phenylalanine and L-methionine shows saturation kinetics.

3. L-Methionine inhibits uptake of L-phenylalanine. The interaction may be described as a partially competitive type of inhibition.

4. L-Phenylalanine inhibits uptake of L-methionine. The interaction resembles a non-competitive type of inhibition.

5. The transport sites show stereospecificity.

6. A counter-transport of L-phenylalanine which is dependent on the extracellular concentration of the amino acid can be demonstrated.

7. Preloading of the cells with L-phenylalanine leads to an accelerated influx of of this amino acid. This is shown not to be a result of an accelerated exchange diffusion.

8. Induction of food vacuoles does not alter the influx of L-methionine significantly.

9. These results indicate that there is a concentrative uptake of L-phenylalanine mediated by a carrier which, in part, is shared by L-methionine. Furthermore, the uptake is inducible and independent of formation of food vacuoles, under our experimental conditions.

INTRODUCTION

STEPHENS AND KERR[1] have reported results which indicate a membrane transport mechanism for uptake of L-phenylalanine in *Tetrahymena pyriformis*. Furthermore, several workers have found that a number of amino acids interfere with each other, with respect to uptake or utilization in this organism[2–4]. Still lacking, however, are thorough descriptions of amino acid influx and studies attempting to localize the

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uptake mechanism, with respect to morphological structures. In the present report we describe the uptake of L-phenylalanine and L-methionine, singly and in combination, and we attempt to elucidate the possible contribution of the food vacuoles in the transport of L-methionine. A preliminary report on this work has been published previously[5].

METHODS

Cultures

Tetrahymena pyriformis strain GL was grown under sterile conditions in a proteose peptone–liver extract broth and synchronous cell divisions were induced in the cell populations by eight heat shocks[6] (see ref. 7 for details). The cells were transferred to an inorganic suspension fluid[8] at the end of the sixth shock by three washings in a refrigerated (6–8°) centrifuge and were kept under starving conditions in that suspension medium at an initial population density of about 100 000 cells/ml for the last two heat shocks, as well as for the remainder of the experiment. The time point of the end of the last heat shock is designated EH.

Determinations of internal amino acids

The live cells were separated from the external medium by differential centrifugation[9], carried out as described in a previous report[4], but the following variations were introduced: the size of the samples was 25 ml; the cells were centrifuged through 1 ml of the oil mixture; the pellet of cells was resuspended with 0.5 ml redistilled water; and macromolecules were precipitated with 2.5 ml 1 % picric acid. The precipitate was separated from the suspension fluid by centrifugation (10 min, 2000 $\times g$) and the supernatants of eight identical samples were pooled. Excess picric acid was removed as described earlier[10], but on resin beds (Dowex, 2-X8, mesh 200–400) of 1 cm $\times$ 2 cm. Amino acid analysis was carried out on a Beckman Spinco Automatic Amino Acid analyzer, Model 120[11]. Extracellular space in the pellet under "oil" amounts to 9.7 $\pm$ 2.7 % ($n = 17$)[4], and this value has been used to correct the measured amounts of amino acids for contamination with extracellular fluid. Intracellular concentrations of amino acids were calculated from cell volumes, determined as "packed volume" after correction for extracellular space[12].

Determination of external radioactivities

The cells and the suspension fluid were separated by filtration[4]. Samples of extracellular medium were applied to glass filters and counted in a Beckman Scintillation Counter.

Calculation of initial transport rates

Since steady-state conditions are not applicable for our purpose (see DISCUSSION) we have chosen to measure the initial transport rates, in the non-steady state, after addition of a radioactive amino acid. Radioactivity of the extracellular fluid was followed as a function of time and the labelled amino acid was added in known concentrations and specific activities at EH + 100 min. In the present study we have not been able to handle the extensive model which describes the depletion of amino acids from the external medium. Such a model would have to take in account both reflux and

time-dependent rate constants for both influx and outflux (caused by induction of carrier sites and changed trans-concentrations of phenylalanine and other amino acids which interfere with the transport of phenylalanine, see p. 213 and Fig. 2). Instead we have tried to work within a short time interval, in which reflux still seems to be insignificant and in which the rate "constant" for influx, k_i, can be considered unchanged. We assume that these conditions are fulfilled as long as we observe an exponential depletion of the radioactivity from the external fluid, *i.e.* for an interval of 8–10 min after addition of the radioactive amino acid (see Fig. 1). From plots which are analogous to Fig. 1, with necessary corrections for logarithmic transformations[13], we found the constants of the best single exponential function by the method of least squares. The number of measurements which are included in the determination of the straight line are the maximal number which lead to ever-decreasing values of the variance around the respective calculated lines; however, five points were chosen as the minimal number in any calculation. The rate constant for influx, k_i was found from the exponential function. Subsequently, the initial transport rate per cell was calculated from k_i and from the known values of the external concentration, the specific activity and the population density.

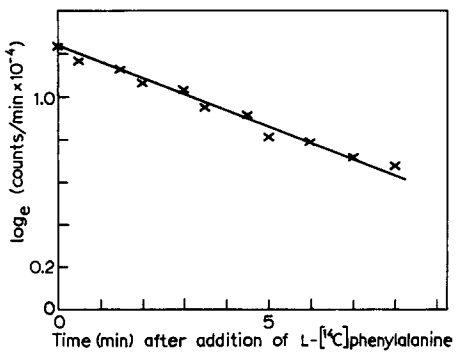

Fig. 1. Log$_e$ of the radioactivity in 0.2 ml of the extracellular fluid as a function of time after addition of L-[^{14}C]phenylalanine (concn. 0.02 mM, spec. act. 5 mC/mmole) to the incubation medium. The straight line has been calculated as described in the text.

RESULTS

The pool of the cellular picric acid-soluble, unconjugated amino acids is very sensitive to environmental changes in starving Tetrahymena cells. We have studied variations in the composition of this amino acid pool as a result of externally added phenylalanine in the period from EH + 5 min to EH + 105 min, *i.e.* in the interval from 2 to 3.75 h after starvation was initiated. The concentration of free amino acids (the sum of 17 individually determined amino acids) in the untreated controls is about 35 mmoles per l cells. Previously, CHRISTENSSON[14] and SCHERBAUM, JAMES AND JAHN[15], working with synchronized cells in organic medium, found about 100 mmoles and 36 mmoles, respectively, and STONER AND DUNHAM[16], studying non-synchronized cells in inorganic medium, reported 55.6 mmoles of free amino acids per kg cells. Alanine, glycine and glutamic acid constitute about 60 % of this pool in our system (*cf.* refs. 14–16), and phenylalanine and methionine contribute less than 1 %. Addition

of L-phenylalanine to the culture (final concentration 0.24 mM) results in the changes shown in Fig. 2. The concentration of L-phenylalanine increases in 17 min from 0.16 to 2.31 mmoles per l cells (Fig. 2A). At this point the phenylalanine pool concentration exceeds the external concentration by a factor of about 10. Later the internal free phenylalanine concentration is again reduced. This is in agreement with previous

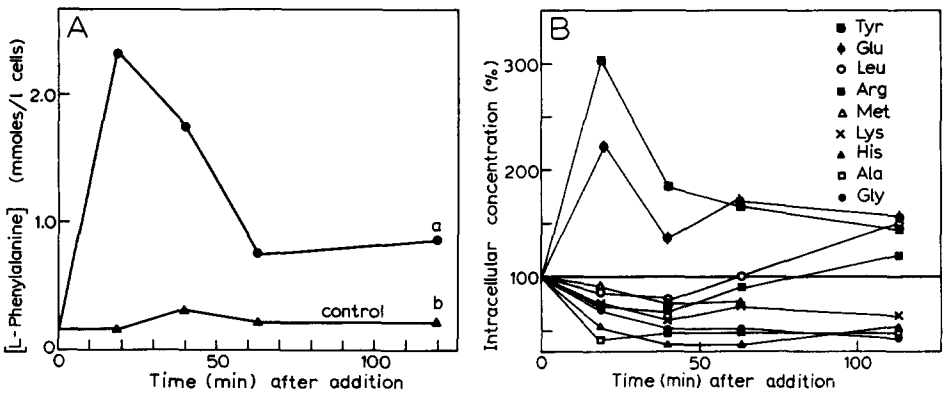

Fig. 2. (A) Intracellular concentration of free phenylalanine with (Curve a) and without (Curve b) addition of L-phenylalanine (0.24 mM) to the suspension fluid at time zero. (B) Intracellular concentration of free amino acids after addition of L-phenylalanine (0.24 mM) at time zero, expressed as percentage of a control culture to which no L-phenylalanine was added.

findings where radioactivity in the trichloroacetic acid-soluble cell fraction was followed after addition of L-[^{14}C]phenylalanine to the external medium, showing that the phenylalanine which appears in the cell is taken up from the medium[4]. Fig. 2B shows the percentage variation in the intracellular concentrations of those amino acids, except phenylalanine, which vary by more than $\pm$ 20 % from the control values. It may be seen that addition of L-phenylalanine greatly affects the internal concentration of other amino acids. The only quantitatively important changes in intracellular concentrations 17 min after addition of L-phenylalanine are seen in alanine (*minus* 7.9 mmoles per l cells), glycine (*minus* 2.5 mmoles), glutamic acid (*plus* 3.3 mmoles) and phenylalanine (*plus* 2.2 mmoles). The total amount of free amino acids decreases by 4.3 mmoles per l cells and the intracellular sodium ion concentration increases from 7 to 10 mmoles per l cells[17]. In conclusion, Tetrahymena cells take up added L-phenylalanine against a concentration gradient and large changes in the internal concentrations of other amino acids accompany such an uptake. A description of this uptake of phenylalanine and of the interaction between the transport of phenylalanine and methionine is given in detail below.

Uptake of L-phenylalanine and L-methionine in Tetrahymena shows saturation kinetics. Fig. 3 presents the initial influx rates of the two amino acids as functions of their concentration in the extracellular fluid. Both curves are similar to the saturation curve of an enzyme reaction where the initial rates of the reactions are plotted against the concentrations of the substrate. We have estimated the apparent values of K_m and V (see Table I) and the variances and covariances from direct fits to data like those in Fig. 3, assuming that the curves can be described by the Michaelis–Menten equation (see DISCUSSION). The curve with the best fit was calculated by an iterative non-

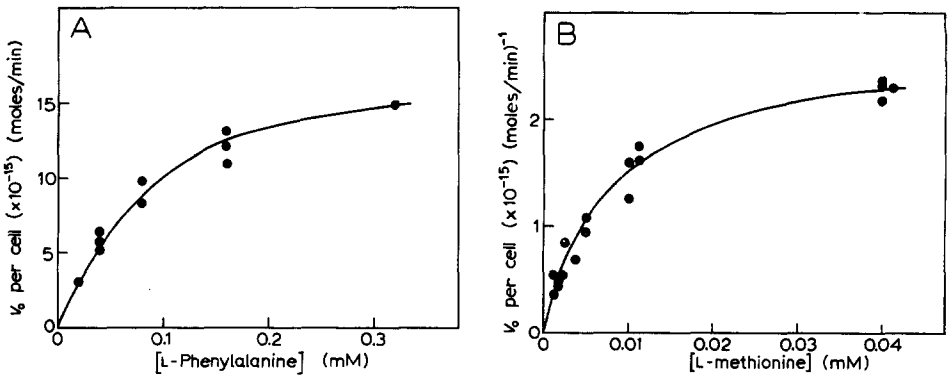

Fig. 3. The initial influx rates (v_0) of L-phenylalanine (A) and L-methionine (B) as functions of their respective external concentrations are given as the saturation curves, $y = V/(1 + K_m/x)$, the constants of which are calculated as described in the text. For L-phenylalanine $y = 19.45/(1 + 0.094/x)$ and for L-methionine $y = 2.72/(1 + 0.0078/x)$.

TABLE I

VALUES OF K_m AND V FOR THE INITIAL INFLUX RATES OF L-PHENYLALANINE AND L-METHIONINE

The values are estimated from direct fits to pooled data by a digital computer. n equals the number of pooled experiments.

	$K_m \pm$ S.D. (μM)	$V \pm$ S.D. *per cell* ($\times 10^{-15}$) (*moles/min*)
L-Phenylalanine	93.6 ± 15.3 ($n = 4$)	19.45 ± 1.37 ($n = 4$)
L-Methionine	7.78 ± 1.02 ($n = 5$)	2.72 ± 0.126 ($n = 5$)

linear unweighted least-square analysis[18]. It may be noted that the saturation concentration and the maximal rate of uptake are much higher for L-phenylalanine than for L-methionine.

L-Methionine inhibits uptake of L-phenylalanine. In order to visualize the type of competition (Fig. 4A) we have used double reciprocal plots[19] of the initial rates of influx of L-phenylalanine as a function of its external concentration with and without addition of 0.16 mM L-methionine, Curves b and a, respectively. Curve b has a higher $1/v_0$ value than Curve a, thus the influx of L-phenylalanine is inhibited by L-methionine. In order to test if the apparent K_m and the V values are different with and without addition of L-methionine we have found the respective values and their variances, as described for Table I, and used a Student's "t" test. The V values ar not significantly different ($0.8 > P > 0.6$) while the apparent K_m values appear to be significantly different ($P < 0.01$). Thus, the interaction of L-methionine with L-phenylalanine uptake is not incompatible with a competitive inhibition.

The inhibitory effect of L-methionine on L-phenylalanine influxes is concentration dependent, although it does not increase strictly in proportion to the concentration of L-methionine. Fig. 4B shows the reciprocals of the initial influxes of L-phenylalanine as a function of the concentration of L-methionine, as proposed by DIXON[20]. A fully competitive inhibition would have given a straight line relationship between the two parameters. This does not seem to be the case in Fig. 4B. In analogy

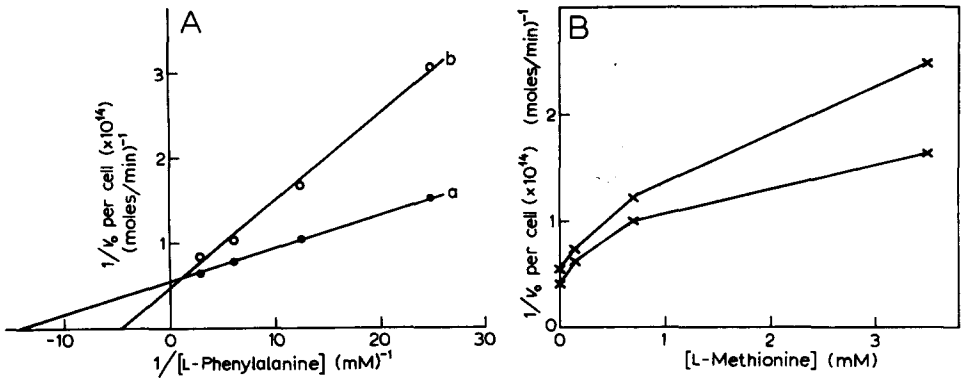

Fig. 4. (A) Double reciprocal plots of the initial transport rates for L-phenylalanine as a function of the external L-phenylalanine concentrations in the absence (Curve a) and in the presence (Curve b) of 0.16 mM L-methionine. The calculated straight lines, $y = (b \pm S_b)\ x + (A \pm S_A)$, have the following equations: Curve a: $y = (0.038 \pm 0.0014)\ x + (0.556 \pm 0.02)$, and Curve b: $y = (0.107 \pm 0.0066)\ x + (0.402 \pm 0.086)$. (B) The reciprocal values of initial transport rate of L-phenylalanine (0.16 mM) as a function of the external L-methionine concentration in two different experiments.

with the concepts of enzyme kinetics, the curves of Figs. 4A and 4B remind us of a partially competitive inhibition[21].

L-Phenylalanine inhibits influx of L-methionine. Fig. 5A is a plot analogous to that of Fig. 4A, showing initial influx rates of L-methionine with and without addition of 0.16 mM L-phenylalanine, Curves b and a, respectively. The data show that L-phenylalanine lowers the uptake of L-methionine. In this case, the two V values are found to be significantly different ($P < 0.001$) while the apparent K_m values are not significantly different at the 5 % level ($0.02 < P < 0.05$). Thus not even high concentrations of L-methionine can release the inhibition fully, but affinity does not seem to be affected. Furthermore, the inhibitory effect of L-phenylalanine increases strictly in proportion to its concentration. This is seen in Fig. 5B, which shows a

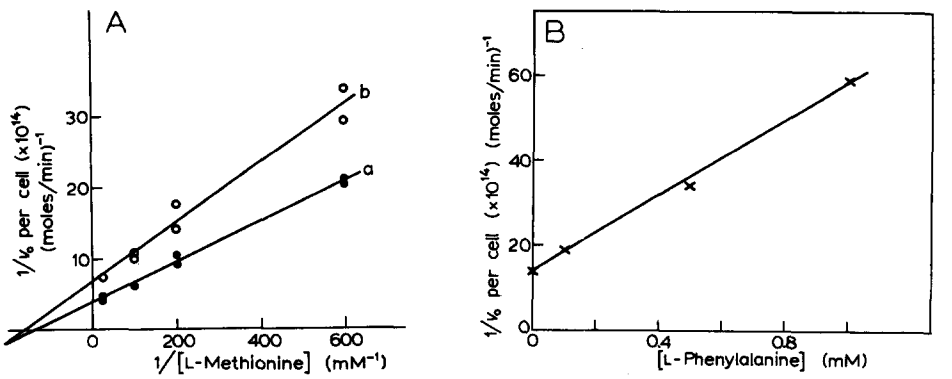

Fig. 5. (A) Double reciprocal plots of the initial transport rate for L-methionine as a function of the external L-phenylalanine concentration in the absence (Curve a) and in the presence (Curve b) of 0.16 mM L-phenylalanine. The calculated straight lines have the following equations: Curve a: $y = (0.0026 \pm 0.0001)\ x + (0.406 \pm 0.039)$ and Curve b: $y = (0.0041 \pm 0.0004)\ x + (0.691 \pm 0.123)$. (B) The reciprocal values of the initial transport rate of L-methionine ($4 \cdot 10^{-3}$ mM) as a function of the external L-phenylalanine concentration.

Dixon diagram analogous to Fig. 4B. In this case a straight line seems to fit the points very well. Thus, the effect of L-phenylalanine on the influx of L-methionine is different from the effect of L-methionine on influx of L-phenylalanine.

The average rates of uptake of 0.0108 mM L-methionine were found to be 1.5 ($\pm$ 0.02)·10^{-15} moles/min per cell and for D-methionine at the same concentration 0.16 ($\pm$ 0.2)·10^{-15} moles/min per cell. The transport system for L-methionine is, therefore, stereospecific.

Tetrahymena shows a mutual dependence of Na^+ movements and amino acid transport[17], a phenomenon well known from many other systems[22]. In some of these it is demonstrated that inhibitors of the Na^+ pump (g-strophantin, ethacrynic acid[23]) also inhibit amino acid transport[22,24]. In our system, g-strophantin does not interfere with Na^+ transport[25], nor (at 0.1–1.0 mM concentrations) with the transport of phenylalanine. Ethacrynic acid (0.3–0.8 mM), however, promotes an increase in the Na^+ concentration in Tetrahymena (E. K. Hoffmann and B. Kramhøft, unpublished experiments), and, accordingly, we found that ethacrynic acid (0.4 and 0.6 mM) added 20 min prior to the amino acid, reduced the initial influx of phenylalanine (concn. 0.2 μM, spec.act. 475 mC/mmole) to 30 % and 23 % of the control values, respectively. However, low concentrations of ethacrynic acid (below 0.3 mM) bring about a marked decrease in intracellular sodium concentration (E. K. Hoffmann and B. Kramhøft, unpublished experiments), but 0.2 mM ethacrynic acid, nevertheless, reduced the phenylalanine influx to 78 % of the control (same conditions as above). Moreover, 0.2, 0.4 and 0.6 mM ethacrynic acid reduced phenylalanine influx to 95, 50 and 48 % of the control values, respectively, in a Na^+-free medium (NaCl replaced by Tris–HCl). This means that the effect of ethacrynic acid on amino acid transport is not a simple consequence of its effects on the Na^+ gradient.

Counter-transport of L-phenylalanine may be induced by addition of a number of amino acids[4]. Fig. 6 shows the results of an experiment in which the effect of different L-phenylalanine concentrations has been tested. Cells suspended in the inorganic suspension fluid were incubated with L-[^{14}C]phenylalanine (concn. 4·10^{-2} mM; spec. act. 2.5 mC/mmole) for 1 h and then transferred to a non-radioactive suspension medium for an additional 60-min period. The culture was then divided into five

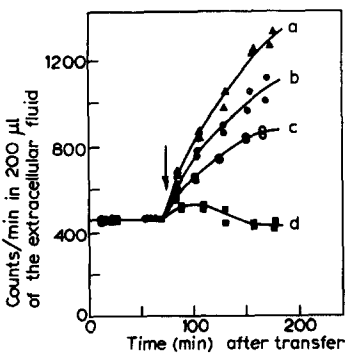

Fig. 6. The effect of various concentrations of L-phenylalanine on the radioactivity of the extracellular fluid of a culture in which the cells had been preincubated with L-[^{14}C]phenylalanine for 2 h and then transferred to fresh, non-radioactive inorganic medium (at time zero). At the time indicated by an arrow unlabelled L-phenylalanine, in the concentrations 3.2 mM, 0.32 mM, 0.16 mM and 0.04 mM, was added to Subcultures a, b, c and d, respectively.

subcultures and a different concentration of phenylalanine was added to each. The radioactivity of the extracellular fluids was measured at periodic intervals. It appeared that the efflux increased with increasing external phenylalanine concentrations and the process was saturated below 3.2 mM. The efflux occurred against a concentration gradient when the external concentrations exceeded 0.24 mM. (Paper chromatographic separation experiments show that more than 75 % of the radioactivity of the components of the extracellular medium after addition of L-phenylalanine moved with an R_F value similar to that of phenylalanine.)

Counter transport phenomena show stereospecificity. Thus we found that the initial rates for increase of extracellular radioactivity in cultures preincubated with L-[^{14}C]phenylalanine (same condition as those experiments depicted in Fig. 6) were 4.3 times higher after addition of L-phenylalanine than after addition of D-phenylalanine. Similarly, the effect of L-methionine was 2.9 times greater than that of the D-isomer.

Some effects of preincubation with L-phenylalanine were investigated. The cells were preincubated for 100 min in inorganic medium to which was added L-phenylalanine (0.24 mM) at EH + 5 min. The preincubation was terminated and the cells were transferred to fresh inorganic medium by 3 centrifugal washes. The influx rates were assessed in the presence of 0.02 mM L-[^{14}C]phenylalanine (spec.act. 10 mC/mmole) from the decrease in radioactivity of the extracellular medium. The initial flux rate in the preincubated cells was 18.7 ($\pm$ 0.7)$\cdot 10^{-15}$ moles/min per cell compared to 4.1 ($\pm$ 0.2)$\cdot 10^{-15}$ moles/min per cell in the non-preincubated cells, comparable results have been obtained with 0.04 and 0.12 mM L-[^{14}C]phenylalanine. The intracellular concentrations of phenylalanine after the three washings are rather similar in cells with and without preincubation (0.28 $\pm$ 0.02 *versus* 0.26 $\pm$ 0.01 mmoles/l cells, respectively), although the intracellular concentrations of phenylalanine differed by a factor of 3.4 just before washing (see Fig. 2A). The increased influxes after preincubation are thus not examples of the accelerative exchange diffusion observed in many transport systems and first reported for influx of glycine into Ehrlich cells[26]. Therefore increased influx must reflect some kind of induction resulting from the previously described pool changes during the period of preincubation. We have also observed that preincubation leads to a faster increase in the L-[^{14}C]phenylalanine content of the intracellular pool, as compared to that of the controls, supporting the view that preincubation results in higher influx.

Food vacuoles are implicated in uptake of nutrients in ciliates. Although we have used several different experimental approaches we have found no significant increase in the measured uptake of amino acids by induction of food vacuoles in Tetrahymena. In a typical experiment, the rate of food vacuole formation was increased from 1.2 to 8.9 vacuoles per cell in 30 min by addition of SP–Sephadex beads, with a diameter of 1 μm to cells in sterile filtered inorganic medium. After 15 min incubation with Sephadex beads L-[^{14}C]methionine (0.001 mM, spec.act. 55.1 mC/mmole) was added to two cultures, one to which SP-Sephadex beads had been added, the other serving as a control. Removal of L-[^{14}C]methionine from the external fluid was followed in the two cultures. The initial influx rates were 0.41$\cdot 10^{-15}$ mole/min per cell in both subcultures. Thus, the uptake of L-methionine is not significantly increased by induction of formation of food vacuoles.

DISCUSSION

We have demonstrated a number of properties characteristic of facilitated diffusion for transport of phenylalanine and methionine in Tetrahymena.

Saturation kinetics

It is evident from Fig. 3 that the uptake systems for L-phenylalanine and L-methionine can be saturated. Whether or not it is reasonable to describe this hyperbolic curve by the Michaelis–Menten equation depends on whether the general conditions for Michaelis–Menten kinetics are fulfilled in our system. We know this is the case at the start of each experiment. On the other hand, we also know that during the experiments the various concentrations of the added phenylalanine and methionine affect the distribution of both Na^+ and amino acids between the internal and external phases. Moreover, the transport capacity of the carrier becomes grossly different in the various experimental cultures. It follows from these considerations that comparisons between cultures which have reached the "steady state" under different conditions are meaningless, unless we account for all the changed parameters. Therefore, we use initial transport rates in the non-steady state and our interpretations then depend on whether we have found the true initial transport rates for influx, *i.e.* whether or not we have worked within a time period in which the reflux is negligible (see METHODS).

Competition between related compounds

The two amino acids each affect the uptake of the other, but in different ways. L-Methionine inhibits influx of L-phenyalanine in a way resembling partially competitive inhibition[21], whereas L-phenylalanine inhibits influx of L-methionine in a way resembling non-competitive inhibition. We can propose a hypothesis which may account for the observed results. According to this hypothesis, L-methionine and L-phenylalanine can attach themselves to the same carrier at different sites. However, the binding of one type of molecule affects the subsequent binding of the other but in a different manner. The prior binding of L-methionine by the carrier reduces (but does not eliminate) the affinity of the carrier for L-phenylalanine, without affecting the other characteristics of L-phenylalanine transport. If L-phenylalanine is bound first, the carrier cannot bind L-methionine. The behaviour of such a system would be consistent with the experimental observations.

Stereospecificity

At least some of the amino acid transport systems in Tetrahymena are stereospecific. STEPHENS AND KERR[1] have given indirect evidence supporting the idea that the affinity of L-phenylalanine for the transport system exceeds that of D-phenylalanine. We have observed that the influx of D-methionine is only 11 % of that of L-methionine, and that the D-isomers of phenylalanine and methionine have far smaller effects on the counter transport of accumulated L-phenylalanine than the L-isomers.

These results lead to the suggestion that amino acid transport in Tetrahymena is carrier mediated. Our results indicate that L-phenylalanine and L-methionine share this carrier which is probably also shared by a number of other amino acids[4].

Uphill transport

In this and in a previous study, we have shown that the carrier system is able to exhibit an uphill transport driven by counter-flow, "counter-transport"[4]. Accor-

ding to WILBRANDT AND ROSENBERG[27], such a phenomenon implies a movable binding site. Our results do not permit us to draw any conclusions as to whether this countertransport results from an accelerated exchange diffusion or from a competitive exchange diffusion[28] since we have not measured the true unidirectional efflux. Moreover, we think that *T. pyriformis* is able to achieve an active transport of L-phenylalanine. Thus, we have found accumulation against a concentration gradient of 10-fold. This value has to be considered in light of the fact that the electrochemical gradient must be even higher, since more than 99.99 % of the molecules of L-phenylalanine at the pH used (7.3) are negatively charged and that the (few) measurements of membrane potentials which have been determined in protozoa all gave negative values[25]. It is understood that these considerations are valid only if the amino acid concentrations determined in this study represent truly free amino acids. It has previously been shown in our laboratory that at least part of the L-phenylalanine transport depends on simultaneous transport of Na^+(ref. 17).

Cells preloaded with L-phenylalanine were shown to have a higher influx of L-[^{14}C]phenylalanine than non-preloaded cells. This increase was observed even though the excess internal phenylalanine concentration was removed by several centrifugal washings in the cold. The accelerated influx in the preloaded cells is thus not a result of an accelerated exchange diffusion. These results are not conclusive in themselves, but they do support the postulate of SCHAEFFER AND DUNHAM[29] of an inducible transport system for amino acids in Tetrahymena.

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