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THE REGULATION BY CELL DENSITY OF AMINO ACID TRANSPORT SYSTEM L IN SV40 3T3 CELLS

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The rate of transport of phenylalanine by System L has been measured in SV40 3T3 cells at various cell densities. When the activity of the L system was determined before any cell depletion of intracellular amino acids, a density-dependent increase in transport paralleled the decrease in cell density. This regulation was lost after cell depletion but reappeared after reloading the cells with pertinent substrates of System L. The phenylalanine transport activity modulated by cell density appeared to be related to the internal level of amino acids capable of exchange upto a definite concentration, beyond which transport activity by System L did not parallel a further increase of internal substrate level. Analysis of the relationship between influx and substrate concentration suggested that two saturable components contribute to entry of phenylalanine and leucine in depleted and in reloaded cells: a low-affinity and a high-affinity component. Both kinetic parameters of the high-affinity component appeared to be modulated by the loading treatment, but only V changed markedly. Activation energies for the high-affinity component of the amino acid transport reaction were calculated from an Arrhenius plot in reloaded cells, and were found to be different for low- and high-density cultures. This result is consistent with the interpretation that cell density modulated the rates at which the amino acid-carrier complex can move within the cell membrane.

Introduction

In a recent paper [1] we have shown that the amino acid transport activity of the Na^+-dependent systems A and ASC decreased markedly with the increase of cell density in 3T3 and SV40 3T3 cells, whereas the activity of the Na^+-independent systems L and Ly^+ remained substantially unchanged when assayed under appropriate conditions to avoid interference from trans-effects, i.e., after extensive cell depletion. However, when the activity of the L system, as assayed by leucine uptake in a Na^+-free medium, is measured before any cell depletion of intracellular amino acid pool, a density-dependent increase in transport paralleled the decrease of cell density in SV40 3T3 cells [2]. The amino acid transport system L shows reactivity towards amino acids with branches or rings on the side-chain and is known to be an Na^+-independent agency endowed with strong exchanging properties [3]; furthermore, it has been reported that its activity is closely related to the cellular levels of those amino acids that participate in exchange [4]. Therefore the higher level of System L transport activity seen in sparse SV40 3T3 cultures before depletion could well be ascribed to a higher internal level of the amino acid pool producing a stronger trans-stimu-

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Abbreviation: BCH, 2-aminobicyclo(2,2,2)heptane-2-carboxylic acid.

lation of the uptake. This interpretation, however, is in contrast with the results of Oxender et al. [4,5] showing that in sparse animal cells the levels of endogenous amino acids are lower than in confluent cells. This observation is related to the activity of System L which appeared to increase with increasing cell density. The apparent contrast between our results [2] and those of Oxender et al. [4,5] prompted us to investigate in detail the activity of System L in SV40 3T3 cells as a function of cell density, depletion of the intracellular amino acid and reloading with pertinent substrates.

In this paper we report that the activity of System L, when measured under appropriate and controlled conditions, is regulated by cell density; this regulation does not appear to be always related to the internal level of amino acids capable of exchange. Moreover, analysis of kinetic parameters of the high-affinity component of System L determined at different temperatures suggests that a change in membrane fluidity may be responsible for the cell density effect.

Experimental

Materials

1-[4,5-^{3}H]Leucine and 1-phenyl[2,3-^{3}H]alanine were obtained from the Amersham International, U.K. Unlabelled amino acids were purchased from Sigma, St. Louis, MO, U.S.A. except for 2-methylaminoisobutyric acid obtained from Aldrich-Europe, Beerse, Belgium, and 2-aminobicyclo (2,2,1)heptane-2-carboxylic acid (isomeric form b($\pm$)) which came from Calbiochem-Behring, La Jolla, CA, U.S.A. Media, salt mixtures, fetal calf serum and antibiotics for cell culturing were obtained from GIBCO, Grand Island, NY. U.S.A.

Cell culture

Starter cultures of simian virus-40 (SV40) transformed Balb/c 3T3 cells (SV40 3T3) were kindly provided by Dr. Paul Black (Boston) and obtained through Dr. Salvatore Ruggieri (Florence). The cells were maintained in Dulbecco's modified Eagle medium containing 100 units penicillin per ml, 100 μg streptomycin per ml and supplemented with 5% fetal calf serum. All cultures were kept in incubators at 37°C in a water-saturated 5% CO_2 atmosphere in air. Cells were passaged twice a week.

Uptake assay

The measurements of amino acid uptake by cells still attached to the substratum were essentially as described previously [1,6] with minor modifications. Cells were seeded into 9 cm^2 wells of disposable multiwell trays (Costar) to give the desired cell density, and allowed to incubate for 24 h in complete growth medium at 37°C. Following medium withdrawal by aspiration, the wells were rinsed twice with Earle basal salt solution containing 0.1% glucose. In some experiments the cells were incubated for 60 min at 37°C in Earle solution with 2% dialyzed fetal calf serum prior to measurement of transport activity in order to reduce the intracellular levels of amino acids (depletion phase). In other experiments, cell monolayers were preincubated, after the depletion phase, at 37°C in Earle solution with 2% dialyzed fetal calf serum in the presence of the amino acid under study (reaccumulation phase) and at the end of this phase the medium was aspirated. Cell monolayers were rinsed in a cold Na^+-free medium (in which choline replaced Na^+ in the sodium salts of the Earle's mixture) and incubated for 1 min at 37°C in 0.5 ml of Na^+-free medium containing the labelled amino acid under study. The incubations were terminated by rapidly rinsing the cells four times with ice-cold Na^+-free medium. Acid-soluble pools were then extracted with 0.5 ml cold 10% trichloroacetic acid, and an aliquot was counted in a scintillation spectrometer. The cells were then dissolved in 0.5 M NaOH and an appropriate aliquot was taken for protein determination following the method of Lowry et al. [7] using bovine serum albumin as standard.

Calculations

Rates of amino acid uptake were expressed as μmol per ml intracellular water $\pm$ S.D. of the mean as previously described [1]. When kinetic parameters of amino acid transport were to be calculated, high concentrations of substrate in the medium were used to determine, in each experiment, the rate constant for the non-saturable component of transport by extrapolation to infinite concentration; net uptake velocity was then corrected for

the non-saturable component. The results relating substrate concentration to velocity and corrected for the non-saturable component were analyzed by the Eadie-Hofstee method. When curvilinear plots were obtained, the assumption was made that two independent Michaelis-Menten components contributed to transport [8]. The best fitting values of kinetic parameters were obtained using computer analysis. The method for parameter fitting developed by Feldman [9] was used for this analysis.

Results

Cell density effect on amino acid transport activity by System L

Phenylalanine was chosen as neutral amino acid substrate with a rather high affinity for the rapidly exchanging L system in several types of mammalian cells [3]. Recently we provided evidence that in SV40 3T3 cells phenylalanine enters mainly by a BCH-inhibitable transport system, as expected for a L-site-reactive substrate [1]. Because of the properties of this mediating system, such as Na^+-independence and flux by transtimulation, uptake of phenylalanine approaching initial velocity was measured before and after extensive cell depletion and after a reloading phase during which accumulation of unlabelled phenylalanine took place. The results presented in Fig. 1 show that when the activity of the system is measured before any cell depletion of the intracellular amino acids, a density-dependent increase in transport paralleled the decrease in cell density. In contrast, after the depletion phase, cell density did not affect the transport of phenylalanine. In the same figure the effect of reloading the depleted cells with phenylalanine prior to determining the transport activity is presented: the density-dependent regulation of the activity of System L, lost after depletion, reappeared after a reaccumulation phase. It should also be noted that the profiles of phenylalanine transport activity, described by a complex double-exponential curve, and measured either before

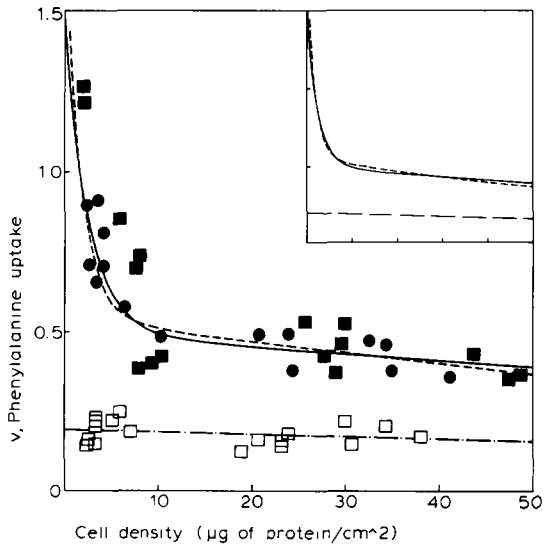

Fig. 1. Phenylalanine transport as a function of cell density, depletion and reaccumulation phase. SV40 3T3 cells seeded over a range of densities were assayed for amino acid uptake 24 h later. Initial rates (1 min assay) of uptake of 0.05 mM phenyl[^{3}H]alanine (1 µCi/ml) were performed at 37°C in a Na^+-free medium as described in the experimental section. Computer-drawn curves represent the best fitting of the experimental points as obtained by linear regression analysis for 90 min depleted cells (□) or according to a double-exponential equation of the type $y = Ae^{-k_1 t} + Be^{-k_2 t}$, with a 90% confidence limit for the final estimate of parameters, for untreated (■) or depleted and reloaded (●) cells. Details of the depletion phase are reported in the experimental section. The reaccumulation phase lasted 4 min and the concentration of phenylalanine during this period was 2 mM. Inset: solid line represents untreated cells, dotted line depleted cells and broken line cells depleted and reloaded with phenylalanine.

TABLE I

EFFECT OF BCH ON PHENYLALANINE UPTAKE

SV40 3T3 cells were seeded at two densities 24 h before uptake measurements. Initial rates of phenylalanine entry were determined at the end of the reaccumulation phase as described in the legend of Fig. 1 in the absence (control) and in the presence of increasing concentrations of BCH, which was added simultaneously with the tracer, in sparse (11.6 ± 2.9 µg protein/cm^2) and dense (66.7 ± 8.3 µg protein/cm^2) cell cultures. The values are shown with the standard deviation for three independent determinations.

[BCH] (mM)	*V*, phenylalanine uptake	
	Low density	High density
0	0.825 ± 0.128	0.458 ± 0.013
0.025	0.545 ± 0.085	0.409 ± 0.066
0.1	0.471 ± 0.050	0.423 ± 0.032
0.5	0.213 ± 0.016	0.186 ± 0.031
2	0.069 ± 0.004	0.068 ± 0.025
5	0.041 ± 0.003	0.037 ± 0.005

depletion or after reloading, were strictly comparable, suggesting that the reaccumulation phase restored the cell-density control of phenylalanine transport. Similar results were also obtained with leucine, another preferred substrate of transport System L.

BCH inhibition of phenylalanine transport

Since our previous discrimination on the activity of System L was carried out on depleted cells, in the experiment described in Table I the inhibition of phenylalanine transport in reloaded cells by 2-aminobicycloheptane-2-carboxylic acid (BCH) (an analogue known to be specifically transported by System L [10]) was tested. Uptake was measured in an Na^+-free medium to minimize the contribution to transport by Na^+-dependent routes. The results indicate that the largest fraction of the Na^+-independent uptake (approx. 90%) was inhibited by BCH, as expected for a System L-mediated uptake.

Time-course of phenylalanine uptake (reaccumulation phase)

The time-course of phenylalanine uptake was determined in order to investigate whether low-density cultures have a higher transport activity than high-density cultures. As shown in Fig. 2, phenylalanine entry is more rapid and the steady-state distribution is higher in sparse than in crowded cells. Because the reloading phase took place in an Na^+-containing medium, the higher values of phenylalanine accumulated by low-density cultures could be ascribed to a higher level of intracellular amino acid, participating in exchange. This higher level could be reached by the contribution of the Na^+-dependent systems (it is known that Na^+-dependent systems are more active in sparse cells). The tendency for as many as two or three distinct systems of mediation to contribute to the uptake of a single amino acid is well known [3]. Therefore the involvement of other systems of transport besides System L in phenylalanine uptake has been investigated in detail during the reaccumulation phase. Using 2-methylaminoisobutyric acid as a model substrate with specificity for the A System [11] and serine to prevent uptake by the ASC system in these cells [1] the activity of System L for the uptake of phenylalanine can be

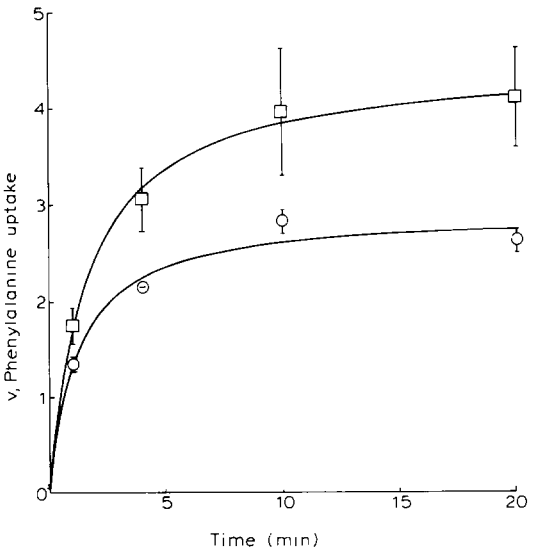

Fig. 2. Time-course of phenylalanine uptake in the presence of Na^+. SV40 3T3 cells were seeded 24 h before uptake measurements. Phenylalanine uptakes were determined after a 60 min depletion phase in sparse (12.1 ± 0.1 μg protein/cm^2, □) and dense (103 ± 11.9 μg protein/cm^2, ○) cultures at 37°C and at the indicated times in an Na^+-containing medium in the presence of the labelled amino acid, at 2 mM final concentration. Values are the average of three independent determinations ±S.D.

TABLE II

THE EFFECT OF INHIBITORY AMINO ACIDS ON THE PHENYLALANINE UPTAKE DURING THE REACCUMULATION PHASE

Cells were seeded 24 h before uptake measurements. Phenylalanine initial entry was determined as described in the legend of Fig. 1 at the end of the reaccumulation phase, in the absence and in the presence of the inhibitory amino acid at a 20 mM final concentration in sparse (8.9 ± 0.9 μg protein/cm^2) and dense (129 ± 16.5 μg protein/cm^2) cell cultures. The values are the means of three independent determinations ±S.D. MeAIB, methylaminoisobutyric acid.

Inhibitor	*V*, phenylalanine uptake	
	Low density	High density
None	7.00 ± 0.42	5.85 ± 0.64
MeAIB(20 mM)	4.64 ± 0.24	2.72 ± 0.22
Serine(20 mM)	4.10 ± 0.43	2.74 ± 0.54
MeAIB(20 mM)+ Serine(20 mM)	3.96 ± 0.75	2.50 ± 0.01

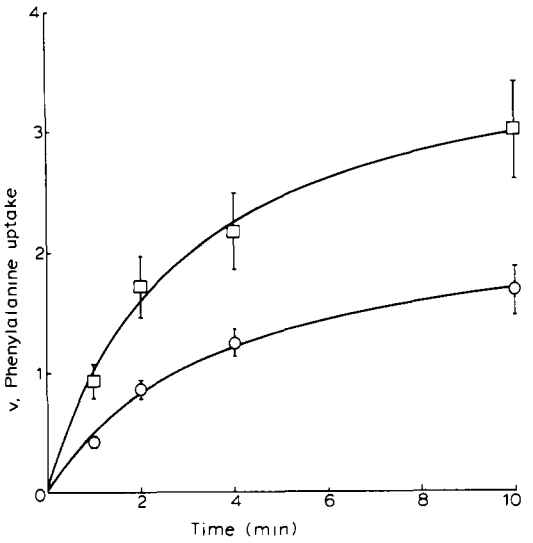

Fig. 3. Time-course of phenylalanine uptake in the absence of Na^+. Details of the experiment were identical of those described in the legend of Fig. 2 with the exception of the uptake measurement, which was performed in a Na^+-free medium for both low-density (6.9 ± 1.3 μg protein/cm^2, □) and high-density (78.6 ± 7.8 μg protein/cm^2, ○) cultures. The values are the means of three independent determinations ± S.D.

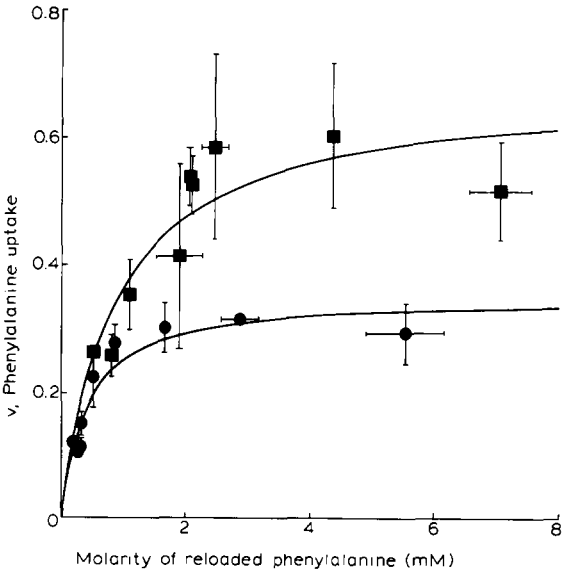

Fig. 4. Transport of phenylalanine as a function of the molarity of the reloaded substrate. The levels for the reloaded phenylalanine were determined at the end of a 10 min reaccumulation phase following a 60 min depletion time in sparse (7.1 ± 6.3 μg protein/cm^2, ■) and dense cultures (81 ± 16.2 μg protein/cm^2, ●) 24 h after seeding. Initial rates of phenylalanine uptake were determined on sister cultures following a reaccumulation phase as described in the legend of Fig. 1. The values are the means of three independent determinations ± S.D.

operationally discriminated even in the presence of Na^+. Table II shows that 2-methylaminoisobutyric acid and serine both reduce phenylalanine transport in low-density as well in high-density cultures, although the difference between uptakes by low- and high-density cultures still persists. This result suggests that a different contribution of the Na^+-dependent systems to the total uptake of phenylalanine in low versus high-density cultures does not explain the higher rates of transport observed in sparse cells. If the accumulation phase of phenylalanine uptake is measured in the absence of Na^+ (see Fig. 3), a difference between low- and high-density cultures is still present either in the initial rate of entry or in the steady-state level attained. Therefore the higher values of accumulated phenylalanine may be ascribed to the intrinsic properties of transport System L observed in sparse cells.

Effect of the initial phenylalanine level on the substrate initial entry

The intracellular phenylalanine levels of sparse cells were determined at the end of the reaccumulation phase and compared to those of crowded cultures. Low-density cultures accumulated phenylalanine at higher levels than dense cultures at all the substrate concentrations tested (data not presented). In Fig. 4 is presented the initial entry of phenylalanine as a function of the apparent molarity of reloaded phenylalanine. The data presented in this figure and described by rectangular hyperbolae show that the activity of transport System L correlated almost linearly with the level of intracellular phenylalanine until the carrier was saturated on the cytoplasmic side. The same figure emphasizes that reloaded low-density cultures display a higher initial entry for phenylalanine than high-density cultures. Similar results, not presented here, have been obtained with leucine, which also undergoes strong exchange properties. These observations suggest that the internal substrate level, even though permissive for the trans-stimulatory effect, does not correlate with the initial veloc-

ity of substrate entry once the carrier has been fully loaded. Therefore the higher activity of phenylalanine transport seen with sparse cells in comparison to dense cultures should not be ascribed to a more active trans-stimulatory effect responding to the higher amino acid level.

Initial rate kinetics

Initial velocities of phenylalanine transport as a function of substrate concentrations were measured in depleted and reloaded cells over a broad range of substrate concentrations. The Eadie-Hofstee plot of the initial velocity of transport, v, against $v/[S]$ was curvilinear. This can be explained either by cooperative interactions or by the contribution of two or more independent families of carriers with different affinities. In this case the assumption was made that two components participate in transport. Fig. 5 shows the plot of phenylalanine entry in low- and high-density cultures after the depletion phase with the resolution of the experimental curve into two linear components: a high-affinity, low-capacity and a low-affinity, high-capacity component. It should be noted that the K_m and the V values of the high-affinity component are almost identical for both sparse and dense cultures: a result that correlates well with the uptake rates as function of cell density in depleted cells (see Fig. 1). In reloaded cells (Fig. 6) the V in the high-affinity component

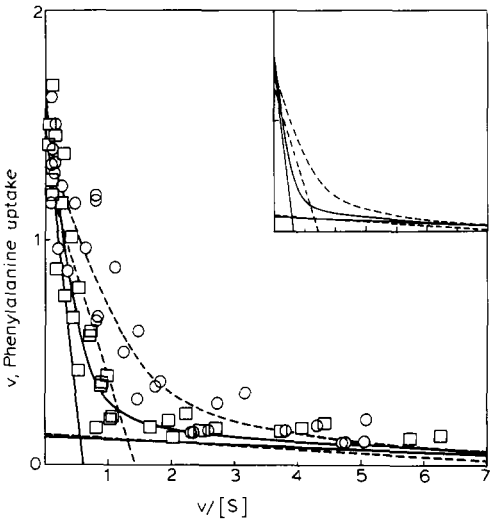

Fig. 5. Kinetic analysis of phenylalanine uptake in depleted SV40 3T3 cells. Initial rates of phenylalanine uptake corrected for the non-saturable component as described in the 'calculation' paragraph were determined in sparse (10.8 ± 1.8 μg protein/cm^2, □) and dense (71.5 ± 7.4 μg protein/cm^2, ○) cultures 24 h after seeding and following 60 min of depletion time. Data were analyzed by the Eadie-Hofstee method. The range of phenylalanine concentrations tested was 0.01 to 40 mM. Lines relating the variation of initial velocity of phenylalanine transport to the ratio of velocity to substrate concentration ($v/[S]$) were drawn according to the fitting of the data obtained by computer analysis (see the Experimental section). Inset: two Michaelis-Menten components obtained after resolution of the curvilinear plot by computer analysis were presented. For both curvilinear and linear plots, solid lines represent sparse and broken lines dense cells.

TABLE III

KINETIC PARAMETERS FOR AMINO ACID TRANSPORT BY SYSTEM L IN SV40 3T3 CELLS

Apparent K_m and V values of the high-affinity component are expressed in micromolar concentrations and μmol per ml intracellular water per min, respectively. The depletion phase lasted 60 min, and the reaccumulation phase, when performed, 4 min in the presence of amino acid at 2 mM final concentration.

	V		K_m	
	Sparse	Dense	Sparse	Dense
Phenylalanine				
Depleted cells	0.12	0.13	12	17
Cells reloaded with phenylalanine	1.35	0.47	54	32
Leucine				
Depleted cells	0.12	0.14	12	19
Cell reloaded with leucine	1.47	0.59	51	35

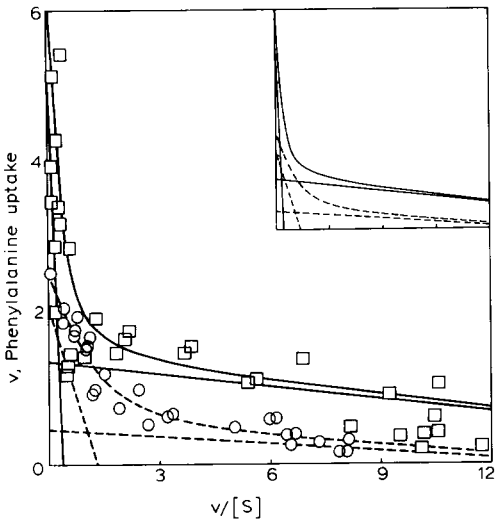

Fig. 6. Kinetic analysis of phenylalanine uptake in reloaded SV40 3T3 cells. Initial rates of phenylalanine uptake were determined in sparse (8 ± 1.1 μg protein/cm^2, □) and dense cultures (63.3 ± 8.1 μg protein/cm^2, ○) 24 h after seeding, following a 60 min depletion time and a 4 min reaccumulation phase during which phenylalanine was present at 2 mM final concentration. Experimental details and calculations are identical to those presented in the legend of Fig. 5. Inset: the two Michaelis-Menten components obtained after resolution of the curvilinear plot by computer analysis were presented. Solid lines represent phenylalanine uptake at low cell density and broken lines represent uptake at high cell density.

is clearly different in the low- and the high-density cultures, whereas the K_m seems substantially similar. The kinetic parameters for the high-affinity component of the phenylalanine entry in sparse and dense cultures, either depleted or reloaded, are presented in Table III. Depleted cells have identical V and similar K_m values. In contrast, after reloading cells with an approximately similar level of phenylalanine, a dramatic increase (one order of magnitude) appeared for the V value of sparse cells, whereas the V value of the high-density cultures increased only 3-times. The changes in the K_m are much less marked. Taken together, the results presented in this section suggest that the affinity of the phenylalanine transport component decreases slightly as cells accumulate substrate, but the capacity of this system of mediation increases several times; this outcome correlates well with the higher level of transport seen in reloaded cells in comparison with depleted cells for both densities tested and might explain the difference observed in reloaded cells between low- and high-density cultures as regards phenylalanine transport activity.

Arrhenius plot

If the mobility of an amino acid-carrier complex within the cell membrane is the rate-limiting parameter for solute transport, a culture condition such as cell density, which modulates the kinetic parameters of transport, e.g., an increase of V, might change the number of effective carrier molecules or their mobility within the cell membrane. To test which of these two hypotheses may be correct, the effect of temperature on the rate of amino acid transport was studied by calculating the kinetic parameters of the high-affinity component from an Eadie-Hofstee plot at three different temperatures. The V for transport increased, with

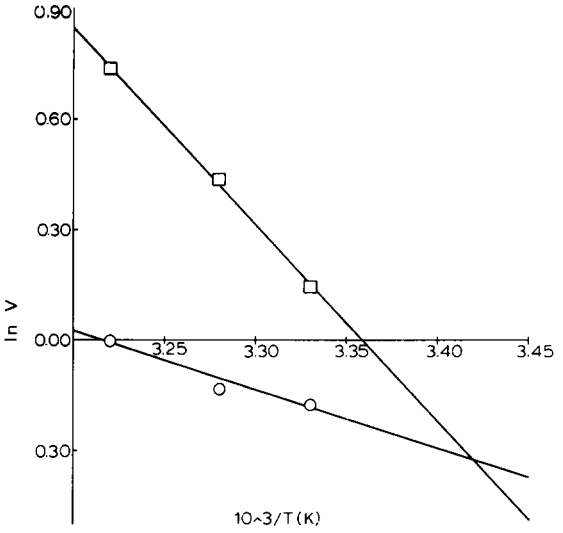

Fig. 7. The temperature-dependence of the kinetic parameter, V, for phenylalanine transport in SV40 3T3 cells. Eadie-Hofstee plots of the initial rates of 0.01–0.4 mM phenylalanine transport at 27, 32 and 37°C were used to calculate the values of kinetic parameter V of the high-affinity component. The assay for phenylalanine transport was performed as described in the experimental section in low-density (17.7 ± 6 μg protein/cm^2, □) and high-density (154 ± 18.9 μg protein/cm^2, ○) cell cultures, 24 h after seeding following a 60 min depletion time and a 4 min reaccumulation phase during which phenylalanine was present at 2 mM final concentration. The values are expressed as μmol per ml intracellular water per min.

a Q_{10} of about 3.9 for the low-density cultures and of about 1.5 for the high-density cultures. The K_m values increased with temperature. The values were analyzed by an Arrhenius plot (Fig. 7), and values of 24.3 and 7 cal/mol for the activation energy were calculated for sparse and dense cultures, respectively.

Discussion

The results presented in this paper show that the rate of transport of phenylalanine in SV40 3T3 cells decreased with increasing cell density or that the activity of the Na^+-independent System L was sensitive to the density of the culture. This conclusion is by no means in contrast with our previous observation that the activity of System L remained substantially unchanged with increasing cell density [1]; as a matter of fact, the amino acid uptake was measured in our previous work in depleted cells to avoid interference by trans-effects. But under physiological conditions the activity of System L, which displays strong exchanging properties, may be modulated by the endogenous amino acid levels. Indeed, modulation of the activity of System L by feed-back mechanisms which respond to the endogenous amino acid concentrations has been proposed as a method of balancing nutrient supply with cell growth requirements [12]. Therefore the present study was designed to investigate the basis for the regulation of the transport System L as function of cell density in non-depleted cells or in cells reloaded after depletion with L-site reactive substrates. The observations presented indicate clearly that in SV40 3T3 cells a density-dependent increase of transport paralleled the decrease in cell density. This regulation of amino acid transport, seen before cell depletion, was lost following an extensive depletion of intracellular substrates and may be restored after reloading cells with pertinent amino acids mainly transported by System L.

The difference in phenylalanine entry by System L seen in sparse vs. dense cultures persisted even at an intracellular molarity of substrate sufficient to saturate the carrier under both culturing conditions. Moreover, at similar concentrations of intracellular phenylalanine, low-density cultures always exhibited higher levels of transport activity than high-density cultures. Analysis of the relation between initial velocity of phenylalanine transport and substrate concentration suggested that two saturable transport components contribute to total phenylalanine and leucine uptake in depleted as well in reloaded cells. A recent paper by Rosenberg et al. [13] provided evidence for a second Na^+-independent component of System L involving particularly tryptophan uptake in human red blood cell. Furthermore, during preparation of this manuscript, Kilberg and co-workers [14] published data on the presence and differential regulation of a low-affinity and high-affinity component of System L in rat hepatocyte. In our studies, the high-affinity component appeared to be regulated only in cells which had been reloaded; under these conditions cell density modulated the kinetic parameters: V changed dramatically as cell density decreased, suggesting that an increase in the number of functional carriers or a higher mobility of the substrate-carrier complex in the cell membrane occurred. Moreover, a decrease in the affinity of the carrier for its substrate was evident when depleted cells were reloaded with phenylalanine; these results, the increase of V and K_m, taken together, confirm that the movement of a loaded carrier is more rapid than that of an empty carrier [15] and suggest that the affinity of the carrier for the substrate decreases when shifted from an unloaded to a loaded condition. This change in K_m, which paralleled alteration in the nutritional cell supply, is reminiscent of a previous observation by Otsuka and Moskovitz [16] which postulated that subconfluent rapidly growing 3T3 cells utilize a high-affinity, low-K_m system for leucine transport, but switch over to a higher-K_m system when the culture becomes dense. The experimental results presented in the Arrhenius plot show that the difference in the rate of phenylalanine transport between sparse and crowded cultures decreases with decreasing temperature. Furthermore, it should be calculated by extrapolation that the rate of phenylalanine transport becomes similar in sparse and dense cultures at around 20°C, a temperature at which a lipid phase transition is known to occur in mammalian membranes, thus abolishing any difference inherent in the viscosity of the cell membrane. These findings support the interpretation that cell density modulated the rates at

which the amino acid carrier complex can move within the cell membrane. This calculation may be strengthened if higher degrees of membrane fluidity were present in low-density cultures. Indeed, our interpretation of the Arrhenius plot correlates well with the report of Inbar et al. [17] that the membrane microviscosity of SV40 3T3 cells was low under sparse cultures but high in dense cultures.

It remains to be explained why there is a contrast between our results on the density-dependent regulation of System L in SV3T3 cells and those of Oxender and coworkers [4] on the increased activity of System L as 3T3 cells approach confluency, unless we grant that viral transformation may considerably alter the activity of the transport system.

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