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Exit Characteristics of ^{14}C-Labeled Leucine from Ehrlich Ascites Tumor Cells

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The present investigation was undertaken to examine the exit mechanisms of D- and L-leucine compared with those of D- and L-alanine. The exit processes of D- and L-amino acids appear to consist mainly of an unsaturable process under physiological conditions. The exit velocities of D-leucine and D-alanine were slower than those of the corresponding L-amino acid under various conditions. A marked increase in the exit velocity of L-leucine was observed in the presence of gramicidin D. This is assumed to be due to the operation of an Na^+-dependent carrier process. On the other hand, the exit of D-leucine was not accelerated, suggesting that the exit of D-leucine is little mediated by an Na^+-dependent process, though the influx of D-leucine was well as that of L-leucine was partly mediated by an Na^+-dependent process.

Keywords——D-leucine transport; D-amino acid transport; gramicidin D upon transport; exit process of membrane transport; Ehrlich ascites tumor cells; amino acid transport

Introduction

In a previous paper on the *in vitro* uptake processes of ^{14}C-labeled D- and L-leucine in Ehrlich ascites tumor cells,[1] we reported that the distribution ratios of ^{14}C-labeled D- and L-leucine were almost the same in a steady state, while the initial velocity of D-leucine uptake was slower than that of the L-isomer. This suggests that the exit mechanism of D-leucine is different from that of the L-isomer. Oxender[2] also assumed that the mediation by which amino acids escaped from Ehrlich cells was more stereospecific than their mediated entry because a similarity in the steady state distributions reached by D- and L-isomers of alanine and valine was observed despite large differences in their influxes. Furthermore, we showed that the exit rates of D-leucine[3] and D-alanine[4] were slower than those of the L-isomers.

In order to acquire further data on the asymmetry between the uptake and exit of D- and L-amino acids in membrane transport, the present investigation was undertaken to examine in detail the mechanisms of exit of D- and L-leucine compared with those of D- and L-alanine.

Experimental

Chemicals——Radioactive amino acids used were the same products as described in previous papers.[1,4] They were checked for radiochemical purity before use. Other chemicals (inhibitors, nonradioactive amino acids, *etc.*) were of analytical grade.

Mouse Ehrlich Ascites Tumor Cells——The tumor cells were prepared as described previously.[1] The cells were suspended in a modified Krebs–Ringer phosphate buffer (mdKRP), pH 7.2, containing 0.64 mM $CaCl_2$, and were used in final amounts of 0.13 ml of packed cell volume per ml of incubation mixture.

Uptake Experiment——The cell suspension (0.5 ml) was preincubated with 0.1 mM cycloheximide at 37° for 5 min, then incubated in the presence of gramicidin D (7 μg/ml) for 1 min with ^{14}C-labeled amino acid dissolved in 0.5 ml of 0.1 mM cycloheximide-containing mdKRP (mdKRP-CH). After five volumes of ice-cold mdKRP-CH had been added to the mixture, it was immediately centrifuged at $650 \times g$ for 1 min and then the cells were treated as described previously for the determination of the uptake and distribution ratio.[1] The effect of Na^+ concentration on the influx was determined by replacement of sodium chloride with choline chloride.

Exit Experiment——The intracellular amino acid-minimized and ^{14}C-labeled amino acid-preloaded tumor cells were prepared as described previously.[3,4] The cells, resuspended in 1 ml of ice-cold mdKRP-CH

or Na^+-free mdKRP-CH, were transferred into 25 ml of mdKRP-CH or Na^+-free mdKRP-CH in the presence or absence of gramicidin D (7 μg/ml) and incubated. The solution had previously been warmed to the same temperature as that used for incubation. An aliquot (5 ml) of the incubation mixture was pipeted off at a given time and chilled in an ice-bath. Afterwards, the cells were treated as described previously.[4]

Results

Effects of Intracellular Leucine or Alanine Concentration on Its Exit Velocity

The fact that intracellular amino acids are rapidly lost introduces difficulties in determining initial velocities except at extremely early times. However, taking $[S]_i$ as the initial intracellular amino acid concentration and $[S]_t$ as the concentration remaining at time t, a plot of $\ln([S]_t/[S]_i)$ against t was approximately linear during incubation, and the first-order rate coefficient for exit, $k_e=\ln([S]_t/[S]_i)/t$, could be calculated.[5] Therefore, the initial velocity of exit was estimated as a product of $[S]_i$ and k_e. The initial velocities were plotted against initial concentrations of cellular amino acid (Fig. 1). The exit velocities appear to show little saturation with rising intracellular amino acid concentrations, and the relationship is nearly linear. This suggests that the exits of leucine and alanine in active transport occur mainly by a nonsaturable process rather than a saturable process. The exit velocities for D-leucine and D-alanine are slower than those for L-leucine and L-alanine, respectively.

Temperature Dependence of K_e for Leucine Exit

Figure 2 shows k_e values for the exits of D- and L-leucine at various temperatures. The initial concentrations of D- and L-leucine were 1.67±0.05 mM and 2.30±0.04 mM, respectively. The exit rate of L-leucine was faster than that of the D-form in the range of 0° to 37°. The k_e values of both D- and L-leucine decreased with a fall in temperature. The activation energies for exit of D- and L-leucine were obtained from Arrhenius plots as 9.6 and 10.6 kcal/mol, respectively. The values corresponded to the lower limit of the range of values, about 8 to 16 or 20 kcal/mol, for typical enzyme-catalyzed reac-

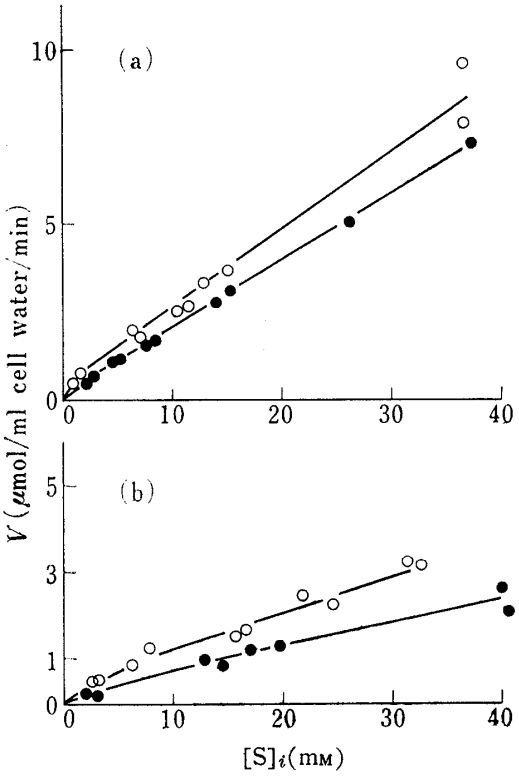

Fig. 1. Exit of Leucine and Alanine as a Function of Intracellular Concentration

The cells were preloaded, at 37° for 30 min, with various concentrations of ^{14}C-labeled amino acid (1.25 μCi/μmol) in mdKRP-CH and incubated in 25 ml of amino acid-free mdKRP-CH at 37° for leucine and at 20° for alanine, then treated as described in "Experimental."
(a): leucine; (b): alanine, ○: L-form, ●: D-form, V: initial velocity of efflux.

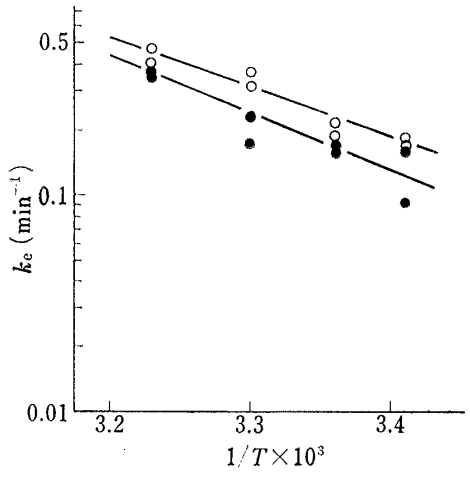

Fig. 2. Temperature Dependence of K_e for Leucine Exit

The procedure was as described in "Experimental" except that cells preloaded with 2.0 mM leucine-^{14}C (1.2 μCi/μmol) were incubated at various temperatures.
○: L-leucine, ●: D-leucine

tions.[6] This suggests that a mediated process was involved at least in part in the exit of these amino acids.

Effects of Gramicidin D on the Uptake and Exit Rates of D- and L-Leucine or -Alanine

Although inhibitors such 2,4-dinitrophenol, 2-deoxyglucose, iodoacetate, sodium azide, and sodium cyanide had little effect on the exits of D- or L-leucine, gramicidin D at a concentration of 7 μg/ml markedly inhibited the uptakes of D- or L-form of leucine and alanine (Fig. 3).

The initial velocities for exit of D- and L- forms of leucine and alanine were determined in the presence of gramicidin D with or without Na^+ ions at various concentrations of intracellular amino acids (Fig. 4). The results show that the exits of L-leucine and L-alanine from

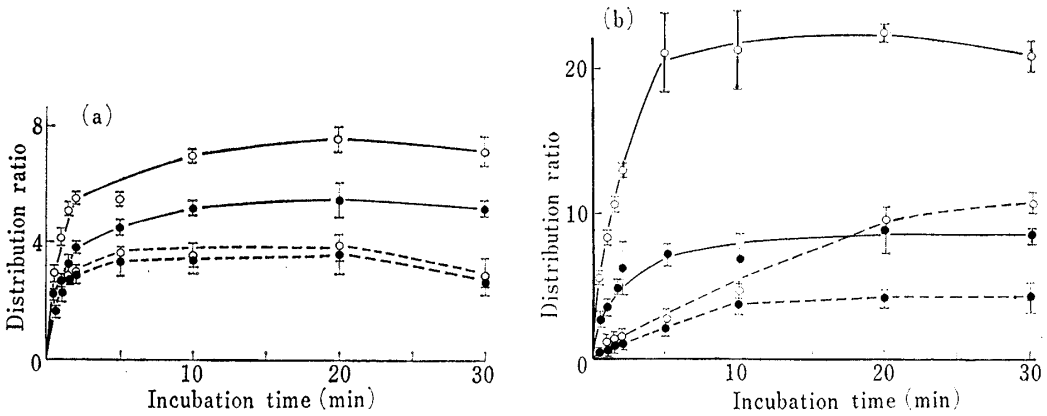

Fig. 3. Effects of Gramicidin D on the Uptake of Leucine and Alanine

The procedure was as described in "Experimental."
(a): leucine, (b) alanine, ○: L-form, ●: D-form, ——: in the absence of gramicidin D, ----: in the presence of the gramicidin.

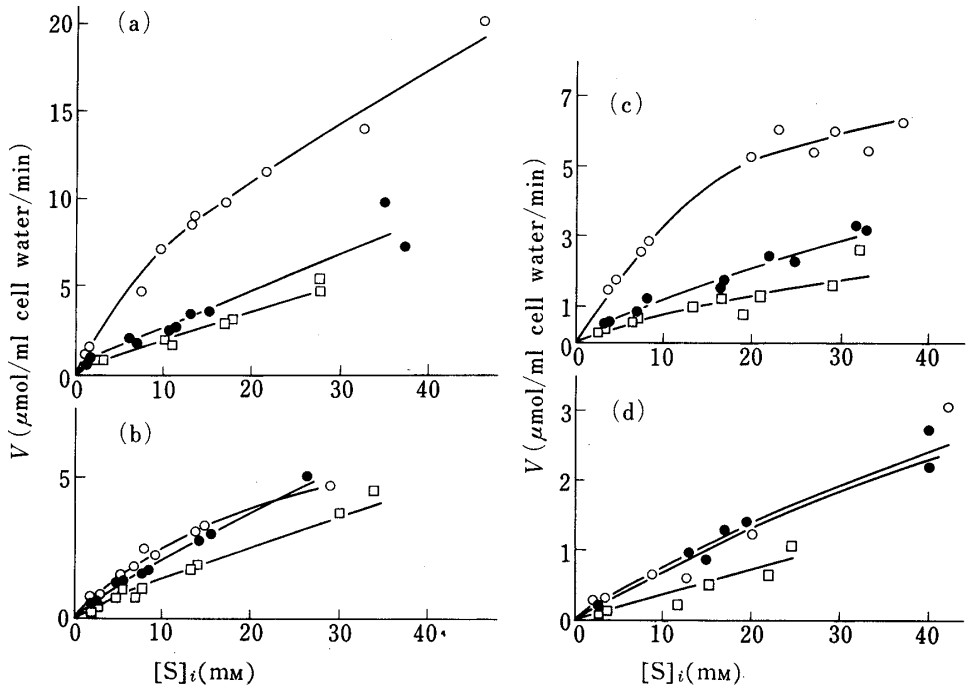

Fig. 4. Exits of Leucine and Alanine as a Function of Intracellular Concentration with or without Na^+ Ions in the Presence of Gramicidin D

The procedure was as described in the legent to Fig. 1, except that the preloaded cells were incubated in amino acid-free mdKRP-CH with or without Na^+ in the presence of gramicidin D. The Na^+-free medium was prepared by replacing NaCl with choline chloride.
(a): L-leucine, (b): D-leucine, (c): L-alanine. (d): D-alanine. ●: control, ○: with Na^+ in the presence of gramicidin D, □: without Na^+ in the presence of gramicidin D.

Ehrlich ascites cells were markedly increased by gramicidin D at various internal concentrations of the amino acids, whereas the exits of D-lecucine and D-alanine were hardly affected by the antibiotic. The exit rates of these L-amino acids in the presence of gramicidin D were at least two times greater than those of the control and appeared to become saturated as the intracellular amino acid concentration was increased, while the control exit velocities gave a linear relationship as described above (Fig. 1). However, little effect of gramicidin D was found on the exits of L-leucine and L-alanine when Na^+ ions were replaced by choline chloride.

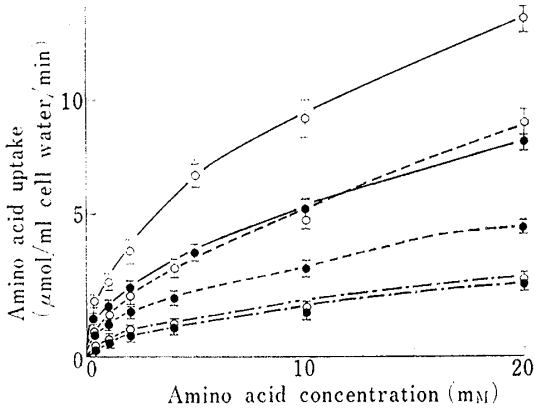

Fig. 5. Effects of Na^+ Ions on the Uptakes of D- and L-Leucine

The procedure was as described in "Experimental," except that the cells were incubated in the absence of gramicidin D.
○: L-form, ●: D-form, ——: control, - - -: Na^+ ion conc., 60 mM, -·-·-: Na^+ ion conc., 0 mM.

Effect of Gramicidin D on Exchange Diffusion of D- and L-Leucine

This effect was examined as described by Johnston for glycine transport.[7] The results showed that exchange efflux of D- and L-leucine was less influenced by gramicidin D.

Effects of Na^+ ions on the Uptake of D- and L-Leucine

The uptakes of D- and L-leucine were inhibited as the Na^+ concentration of the medium was decreased (Fig. 5). Although leucine is known to be mainly incorporated by a sodium-independent system (L-system),[8] the results show that the uptake of D-leucine as well as that of L-leucine was mediated by both sodium-dependent and -independent systems.

Discussion

The initial velocities of exit for the D- and L-forms of leucine and alanine are linearly related to the initial concentration of amino acid (Fig. 1). Therefore, the exit appears to consist mainly of an unsaturable process under physiological conditions, in contrast to the influx. Such a difference between the exit and influx processes has already been reported by Schaffer and Williams[9] for the transport of α-aminoisobutyric acid in Ehrlich cells. These findings support the view that an asymmetry in the influx and exit of various substrates is requisite for active transport systems.[10]

Figure 1 shows clearly that the exit velocities of D-lecucine and D-alanine are slower than those of the corresponding L-amino acids. This may account, at least in part, for a finding that the radioactivity of D-amino acids was accumulated more highly into various tumor cells *in vivo* than that of the L-isomers.[11]

A marked increase in the exit velocities of L-leucine and L-alanine was observed in the presence of gramicidin D, while the exits of D-leucine and D-alanine were not accelerated by it. This is particularly interesting, because the influxes of D- and L-leucine and -alanine were markedly inhibited in the presence of gramicidin. This marked increase of the exit of L-amino acids is not due simply to elevated passage of these amino acids through the gramicidine pore because, if so, the exit of both the D- and L-amino acids should be accelerated by gramicidin D.[7] Gramicidin D had no influence on the exit of L-amino acids in Na^+-free medium. Since gramicidin D is known to cause an elevation of cellular Na^+,[5,7] it seems possible to account for the observed increased in exit mainly in terms of the effect of Na^+ ions. Little effect of gramicidin D was found on the exchange efflux of D- and L-leucine in an experiment performed to confirm whether gramicidin affects all transport systems or just the Na^+-dependent systems, since exchange diffusion is known to operate in the absence of Na^+ or

ATP[12], and this result supports the view that gramicidin D affects mainly the Na^+-dependent system for leucine and alanine. Furthermore, when the exit experiment was carried out in a medium containing Na^+ but not K^+, the exit pattern of the L-amino acids was almost the same as that in a medium containing both K^+ and Na^+ ions (unpublished data). Johnstone[5] reported that variable cellular K^+ concentrations had no apparent effect on glycine exit. Therefore, it is suggested that the elevated exit velocities of L-leucine and -alanine in the presence of gramicidin D are not due to the altered cellular K^+ levels.

The exit velocities in the presence of gramicidin D appear to show saturation with rising cellular concentration of L-alanine and -leucine. This suggests that an elevation of cellular Na^+ by gramicidin D appears to make a carrier, which is normally not mediating the exit, available for exit, in agreement with Schaffer's suggestion.[9] Exit of L-alanine, transported by an Na^+-dependent route, more clearly exhibited a carrier-mediated process in the presence of gramicidin D than did the exit of L-leucine, which is transported partly by an Na^+-dependent route.

Several reports have already appeared on the effect of gramicidin D upon the exit velocity of amino acids, as seen in the case of glycine. α-Aminoisobutyric acid, phenylalanine, and histidine are all transported, at least in part, by an Na^+-dependent pathway.[7] Johnstone[5] described that the increased exit of glycine in the presence of gramicidin D was due to the operation of the Na^+-dependent carrier mechanism, and furthermore that the effluxes of ethanolamine and 3-O-methylglucose not transported by an Na^+-dependent route in Ehrlich cells are not affected by gramicidin D.[7] That the exits of D-leucine and -alanine from Ehrlich cells were not accelerated by gramicidin D in our experiments suggests that the exits of these D-amino acids were not mediated by an Na^+-dependent process. This is of interest in view of our result that the influx of D-leucine was inhibited by gramicidin D and was partly Na^+-dependent. The absence of any effect of gramicidin D upon the exit of D-leucine or D-alanine suggests that these exit mechanisms lack a binding site for Na^+ on the internal surface of the cell membrane. Also, it appears that the influx and exit processes of D-leucine and -alanine exhibit an extreme asymmetry toward gramicidin D.

In conclusion, it is suggested that the exit system of L-leucine is partly Na^+-dependent while that of D-leucine is almost Na^+-independent, though the influx systems of both amino acids are partly Na^+-dependent.

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