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Characteristics of D- and L-Alanine Transport into Mouse Ehrlich Ascites Tumor Cells

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The present investigation was undertaken to examine the temperature dependence of the kinetic parameters for the uptake and exit of D- and L-alanine in Ehrlich ascites tumor cells, as one of a series of studies to account for the high *in vivo* uptake of radioactivity of ^{14}C-labeled D-amino acids in various tumor cells. The V_{max} values for D- and L-alanine uptake decreased with a fall in temperature. The activation energies for both isomers obtained from plots of ln V_{max} against $1/T$ suggested that these isomers were transported through a mediated process. On the other hand, K_m for D-alanine uptake was only slightly dependent on temperature, whereas K_m for L-alanine decreased with a fall in temperature. These temperature dependences of K_m are discussed in comparison with those of K_m for D- and L-leucine uptakes. Furthermore, it was observed that the exit rate of D-alanine was considerably slower than that of the L-isomer. The activation energies of the isomers obtained from plots of ln k_e against $1/T$ (k_e: the first-order rate constant for exit) suggested that free diffusion is predominant in the exit process of D-alanine, in contrast to that of the L-form. This slow exit of D-alanine may account in part for the high *in vivo* uptake of radioactivity of D-alanine-^{14}C into Ehrlich tumor cells.

Keywords——D-alanine transport; amino acid transport; Ehrlich ascites tumor cells; temperature dependence of alanine transport; D- and L-alanine exit; kinetic parameters for alanine uptake; biological transport of amino acid

In previous papers,[2] we reported that, after subcutaneous injection of ^{14}C-labeled D-amino acids into tumor-bearing animals, higher levels of radioactivity were found in the cells of several tumors tested than in the cases of the corresponding L-forms, and we suggested that this preferential uptake was in part due to a difference in the transport systems of the isomers.

In the analysis of transport, the significance of V_{max}, the maximum rate of the saturable component, and K_m, the Michaelis constant, for substrate transport in intact cells is not yet fully understood.[3] Although the temperature dependence of V_{max} and K_m for amino acid transport has been studied in various cells,[3a,3c,3f,4] the kinetics of the transport process have not been interpreted in detail.

In the preceding paper,[2b] we reported the transport of D- and L-leucine in mouse Ehrlich ascites tumor cells, and described the kinetic parameters for both isomers at various temperatures in terms of equation [1],[3c]

$$v = V_{max}/(1+K_m/S) + k_u S \qquad [1]$$

where v is the rate of initial uptake, S is the substrate concentration in the medium, and k_u is the first-order rate constant for the nonsaturable component. We also tried to interpret the

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2) *a*) O. Tamemasa, R. Goto, and T. Suzuki, *Gann*, **69**, 517 (1978); *b*) R. Goto, T. Suzuki, and O. Tamemasa, *J. Biochem.*, **86**, 363 (1979).

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temperature dependence of V_{max}, K_m, and k_u for D- and L-leucine by obtaining an "energy of activation" from Arrhenius plots. In contrast to L-leucine, the temperature dependence of K_m of D-leucine uptake could not be interpreted in terms of this activation energy because of its negative value. However, this difference in temperature dependence of K_m for D- and L-leucine suggested that the bulky hydrocarbon side chains of the two isomers were bound to different sites of the carrier protein,[2b] assuming that the α-amino and α-carboxylate groups of the two isomers had common binding sites. In order to clarify this, the present investigation was undertaken to examine the temperature dependence of the uptake of D- and L-alanine, which have a smaller side chain than leucine.

In a previous paper,[5] we also pointed out that the slower exit of D-leucine, compared with L-leucine, seemed to be one of some factors leading to the *in vivo* preferential uptake of radioactivity of D-leucine. In this paper, the characteristics of exit of D- and L-alanine from the cells were also investigated as an aid to the interpretation of the *in vivo* preferential uptake of radioactivity of D-alanine.

Experimental

Chemicals——D- and L-alanine [-U-^{14}C] were products of the Radiochemical Centre, U.K. The purity of the radioactive amino acids was checked by paper radiochromatography before use. Other chemicals (nonradioactive D- and L-alanine, cycloheximide, *etc.*) were of analytical grade.

Mouse Ehrlich Ascites Tumor Cells——The tumor cells were prepared as described previously.[2b] 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 Experiments——The cell suspension (0.5 ml) was preincubated with 0.1 mM cycloheximide at various temperatures for 5 min then incubated with ^{14}C-labeled D- or L-alanine at various concentrations dissolved in 0.5 ml of 0.1 mM cycloheximide-containing mdKRP (mdKRP-CH) at various temperatures. After adding five volumes of ice-cold mdKRP-CH to the mixture, it was immediately centrifuged at $650 \times g$ for 1 min and then the cells were treated as described previously to determine the uptake and distribution ratio.[2b]

Exit Experiments——In order to minimize endogenous free L-alanine, the tumor cell suspension (0.5 ml) was mixed with 2 ml of mdKRP and incubated at 37° for 5 min. After the incubation, the cells were pelleted by centrifugation and suspended in 2 ml of mdKRP. This treatment was repeated four times. After the treatment, the amount of free L-alanine remaining in the cells was about 1—2 nmol per 0.5 ml of cell suspension, which was negligible in relation to the amount of L-alanine taken up. These amino acid-minimized cells were preincubated in 0.37 ml of mdKRP containing cycloheximide at a final concentration of 0.1 mM, at 37° for 5 min and then further incubated with 0.5 ml of 2 mM D- or L-alanine-^{14}C (specific activity: 0.125 μCi/μmol) dissolved in mdKRP-CH, at 37° for 30 min. After the addition of 5 ml of ice-cold mdKRP-CH, the cells were separated from the mixture by centrifugation for 1 min at $650 \times g$ and washed once with 2 ml of ice-cold mdKRP-CH. The cells were resuspended in 1 ml of ice-cold mdKRP-CH, transferred into 25 ml of mdKRP-CH previously warmed to various temperatures and incubated at the same temperature. An aliquot (5 ml) of the incubation mixture taken after a given time was pipetted off and chilled. The cells were rapidly separated from the medium by centrifugation. Residual fluid in the tubes was taken up with a fine paper strip, then the cells were suspended in 0.5 ml of 5% trichloroacetic acid. The radioactivity of the suspension was counted with an Aloka type 661 scintillation counter.

Results and Discussion

Kinetic Analysis of D- and L-Alanine Uptake

D-Alanine, compared with the L-isomer, was taken up slowly in Ehrlich ascites tumor cells in the presence of 0.1 mM cycloheximide, in agreement with an observation of Oxender[6] (Fig. 1). Since the uptakes of both isomers of alanine increased almost linearly for at least 1.5 min, it seems reasonable to consider the uptake for 1 min in determining the initial rate of influx. Figure 2 shows the inital rate of uptake for D- or L-alanine as a function of substrate concentra-

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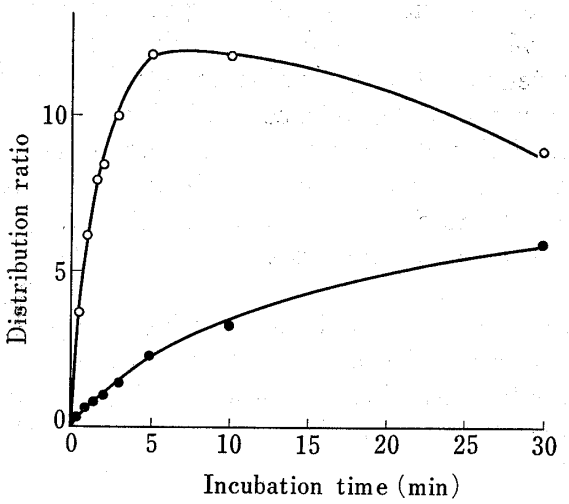

Fig. 1. Time Course of Alanine Uptake

An Ehrlich tumor cell suspension (0.5 ml) was pre-incubated with 0.1 mM cycloheximide at 37° for 5 min, then incubated with 2 mM D- or L-alanine-^{14}C (0.5 ml, specific activity: 0.05 μCi/μmol) containing 0.1 mM cycloheximide at 37° and subsequently treated as described in "Experimental."
○—○: L-alanine, ●—●: D-alanine.

tion at various temperatures. From these plots, K_m, V_{max}, and k_u for the uptake of both isomers were calculated according to equation [1]. First, the k_u values were estimated by two methods, which gave essentially identical results. The ordinate intercept of a plot of velocity divided by substrate concentration against reciprocal substrate concentration can be used to estimate k_u (Method I).[7] The other method for estimating k_u is an iterative procedure which seeks a value for k_u which give the least errors of fit to a straight line for the derived saturable influx in a double-reciprocal plot (Method II).[8] In this investigation, the values for L-alanine were estimated by Method II. The double-reciprocal plots of saturable influx (V-$k_u S$) of alanine against substrate concentration at 37° are shown in Fig. 3.

Oxender[6] stated that the K_m for D-alanine uptake could not be readily estimated in the usual way because too much of it entered by a nonsaturable process, and that K_i for D-alanine acting as an inhibitor of L-alanine uptake was close to the K_m of D-alanine. In the study of Schultz *et al.*[9] on the stereospecificity of amino acid uptake in the brush border of rabbit ileum, the K_m values for D-amino acids were estimated in the range of substrate concentrations below K_m without considering k_u.

We tried to estimate the K_m of D-alanine in the usual way by measuring the initial rate over a wide concentration range (1—200 mM), as shown in Fig. 2b. The best fit according to

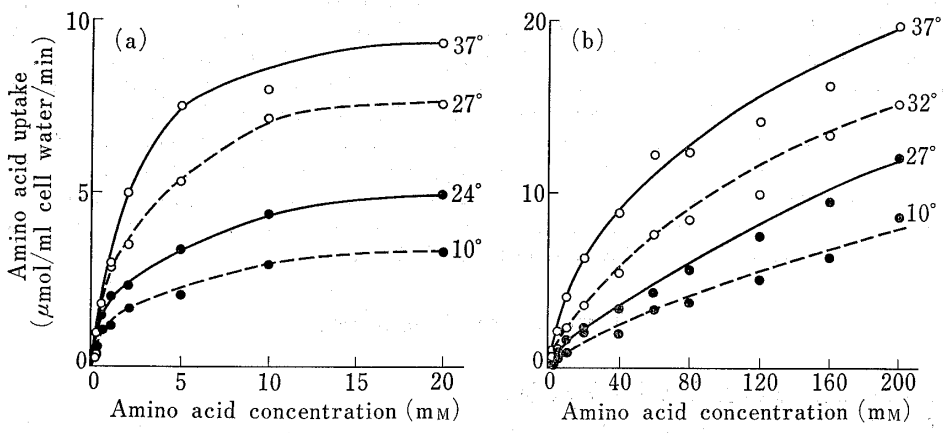

Fig. 2. Alanine Uptake at Various Temperatures

The procedure was as described in the legend to Fig. 1, except that the cells were incubated with D- or L-alanine-^{14}C at various concentrations for 1 min.
(a): L-alanine, (b): D-alanine.

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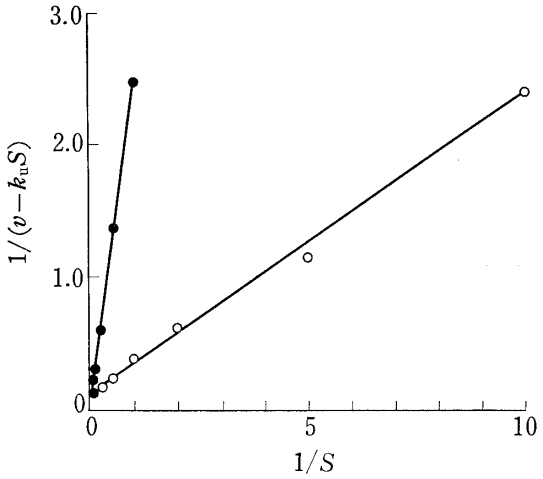

Fig. 3. Double-Reciprocal Plot of the Initial Rate of Alanine Transport against Substrate Concentration at 37°

The procedure was as described in the legend to Fig. 2. Observed velocities (v) were corrected for k_uS as described in the text.
○—○: L-alanine, ●—●: D-alanine.

Table I. Kinetic Parameters for Alanine Uptake at Various Temperatures

	Temp. (°)	K_m (mM)	V_{max} (μmol/ml/min)	k_u (min^{-1})
L-Alanine	37	2.236	9.988	0.033
	27	1.016	5.650	0.100
	24	0.669	3.358	0.084
	20	0.541	1.926	0.075
D-Alanine	37	19.50	8.318	0.065
	32	22.21	3.869	0.065
	27	19.12	3.407	0.037
	20	23.32	1.253	0.033

Method II for estimating k_u was obtained for $k_u=0$. However, since the curves of D-alanine uptake did not have a sufficiently flat plateau at high concentrations, it is not appropriate to neglect the nonsaturable process. Accordingly, Method I was applied. The correlation coefficients for the double-reciprocal plots of saturable influx were more than 0.98 up to the k_u value obtained by Method I and rapidly decreased beyond this value. Double-reciprocal plots of the saturable influxes of D-alanine against substrate concentration corrected for k_u at 37° are shown in Fig. 3. The k_u values for D-alanine uptake at various temperatures were estimated in a similar manner and double-reciprocal plots of saturable influx were obtained. The plots gave reasonably straight lines. The K_m value of D-alanine (19.5 mM) at 37° obtained by this method was in fair agreement with the K_i for D-alanine (about 20 mM) mentioned above. The kinetic parameters of D- and L-alanine at various temperatures are given in Table I. The results show that there is a strict stereospecificity for alanine.

Figure 4a shows plots of ln V_{max} against $1/T$, where T is the absolute temperature. It can be seen that the V_{max} values for D- and L-alanine decreased with a decrease in temperature. Since V_{max} is a function of the rate of translocation for the amino acid-carrier complex, the "energy of activation" was calculated for V_{max}. The activation energies for V_{max} are 18.9 kcal/mol for D-alanine and 17.1 kcal/mol for L-alanine. These results are compatible with Oxender's conclusion[6] that D- and L-alanine were transported through a mediated process, on the basis of mutual inhibition and the pH-dependences of uptake of both isomers.

A simplified kinetic scheme is presented below, where S refers to the substrate and C to the carrier.[3d]

$$S + C \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} SC \xrightarrow{k_2} S + C$$

k_1 is the rate constant for the association of amino acid with carrier, k_{-1} for the dissociation reaction, and k_2 for the movement of the substrate-carrier complex (SC) across the membrane. The Michaelis constant for transport is expressed as $K_m=(k_{-1}+k_2)/k_1$. On the basis of this assumption that the K_m value is a ratio which contains the three rate constants, the energy of activation can be determined for K_m. However, Jacquez *et al.*[3a] poined out that it was difficult to interpret the temperature dependence of K_m in amino acid transport in terms of the "energy of activation" because the temperature dependence of K_m showed considerable

variation from one amino acid to another. We also reported[2b] that, while K_m for L-leucine decreased with a fall in temperature, that for D-leucine increased, as shown in Fig. 4b. Figure 4b shows that K_m for L-alanine decreased with a fall in temperature while that for D-alanine had little dependence on temperature.

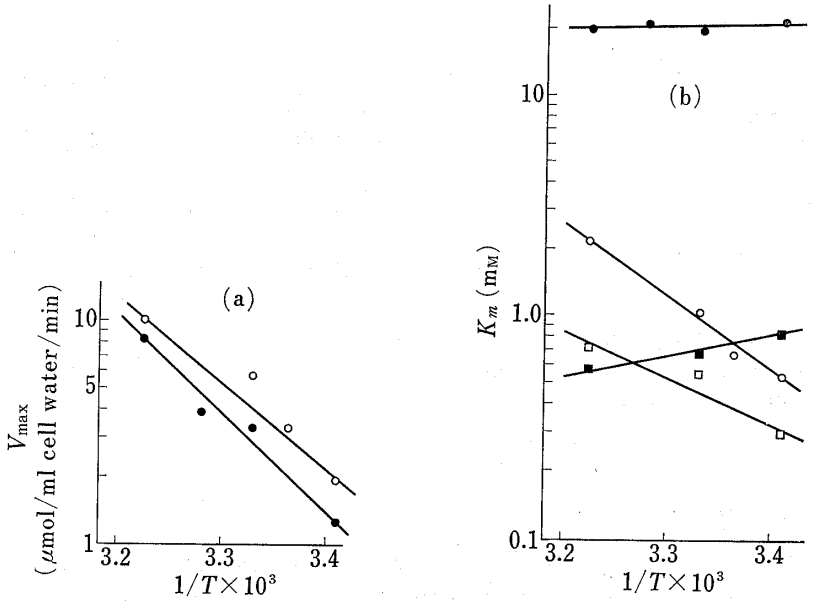

Fig. 4. Temperature Dependence of V_{max} (a) and K_m (b) for Alanine Uptake

The procedure was as described in the legend to Fig. 2. For comparison, this figure also includes K_m values for D- and L-leucine uptake, taken from a previous paper.[2c]
○—○: L-alanine, ●—●: D-alanine, □—□: L-leucine, ■—■: D-leucine.

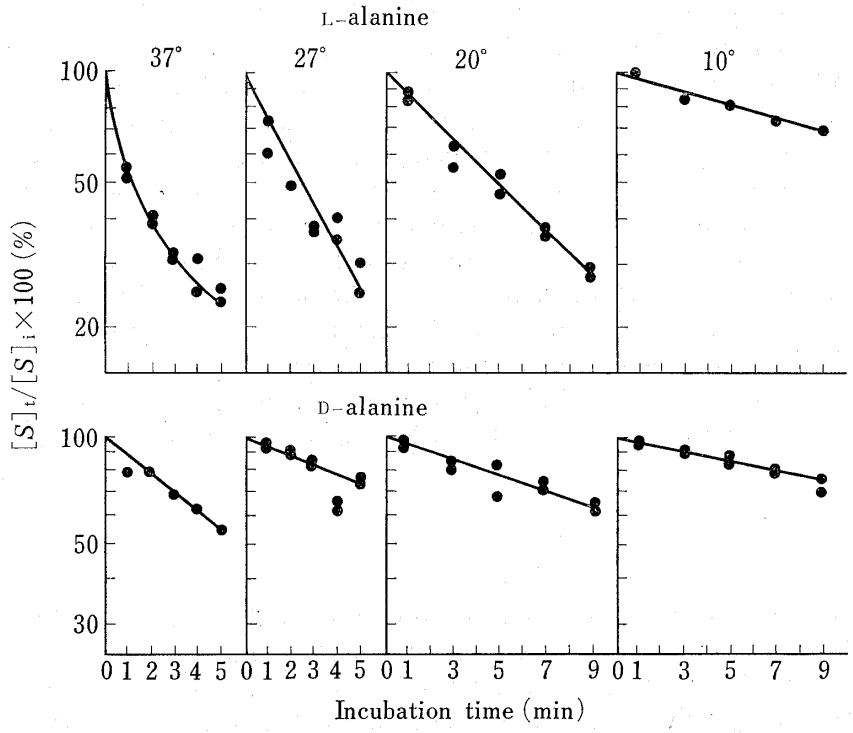

Fig. 5. Time Course of the Exit of Alanine at Various Temperatures

The procedure was as described in "Experimental."
$[S]_t$: intracellular amino acid concentration remaining at time t; $[S]_i$: initial intracellular amino acid concentration.

If the rate of dissociation is much more rapid than that of movement (*i.e.* $k_{-1} \gg k_2$), the reciprocal of K_m may approximate the binding constant for the carrier-amino acid complex. It should be possible to examine semiquantitatively the nature of the interaction of D- and L-amino acids with a carrier, because the plots of ln K_m against $1/T$ are nearly straight (Fig. 4b). By applying the relationship $\Delta G = -\boldsymbol{R}T\ln[1/K_m]$, ΔG for the binding of an amino acid to a carrier is obtained,[10] where ΔG is the free energy of the binding process and $\boldsymbol{R}$ is the gas constant. The enthalpy of the binding reaction was obtained according to the following equation, $(\Delta H)1/K_m = \boldsymbol{R}\delta\ln K_m/\delta(1/T)$. Furthermore, the entropy, ΔS, is given by the Gibbs-Helmholtz equation [2],

$$\Delta G = \Delta H - T\Delta S \qquad [2]$$

ΔH values for L-alanine and L-leucine were −15.6 and −10.1 kcal/mol, respectively, both being negative. This means that the attachment of the L-amino acids to a binding site is an exothermic process. This seems to favor the binding of the L-amino acids to a carrier in agreement with Oxender's assumption[6] that the transport of L-amino acids probably involves three points of attachment to a binding site. In contrast to L-amino acids, ΔH values for D-alanine and D-leucine were not negative: they were 1.4 and 4.9 kcal/mol, respectively. This means that the binding process of these D-amino acids to a carrier is not an exothermic process, and is compatible with Oxender's speculation[6] that a D-amino acid is forced to use only two of the three points of attachment utilized by the L-isomers. In other words, it is suggested that the interaction of a D-amino acid with the carrier will be less stable than that of the L-amino acid.

It is of interest to compare the $T\Delta S$ values of D-amino acids with those of L-amino acids: $T\Delta S$ values for L-alanine and L-leucine at 37° were −11.8 and −5.6 kcal/mol, respectively, and those for D-alanine and D-leucine at 37° were 3.8 and 9.5 kcal/mol, respectively. The existence of entropy-increasing factors can therefore be assumed, such as the destruction of iceberg structure around hydrophobic groups[11] and/or an increase of unfolding of the amino acid-carrier complex during the binding process.[12] The values of entropy are compatible with the assumption that the binding process of D-leucine with a carrier involves a hydrophobic interaction, because D-leucine has a larger alkyl chain than D-alanine. On the other hand, the slower rate of D-alanine uptake compared with the L-form seems to be a result of limited participation of hydrophobic interaction in the binding of D-alanine to the carrier. This interpretation of the temperature dependence of K_m in intact cells requires further study, preferably using a purified carrier and amino acid. It is not clear why the k_u value for L-alanine uptake at 37° is smaller than those at lower temperatures.

Exit of D- and L-Alanine

Figure 1 shows that the distribution ratio of D-alanine gradually approached that of L-alanine in the steady state despite large differences in their initial influxes. This suggests that the exit of D-alanine is slower than of that L-alanine. Figure 5 shows the time course of exit of intracellular alanine at various temperatures. The exit of alanine was determined in a volume 200 times the cellular volume in order to minimize recapture. Taking $[S]_i$ as the initial intracellular amino acid concentration and $[S]_t$ as the concentration remaining at time t, ln $([S]_t/[S]_i)$ was approximately linear during the incubation, and the first-order rate coefficient for exit, $k_e = 1n([S]_t/[S]_i)/t$, was accordingly calculated.[13] The initial intracellular

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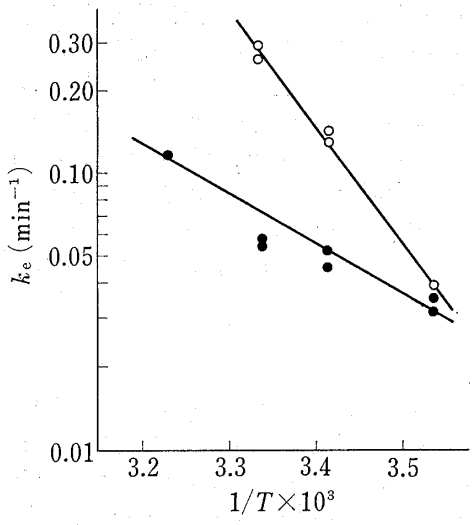

Fig. 6. Temperature Dependence of k_e for Alanine Exit

The procedure was as described in "Experimental."
○—○: L-alanine, ●—●: D-alanine.

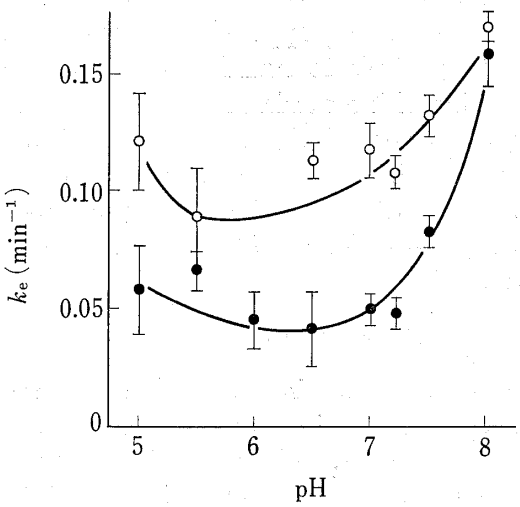

Fig. 7. pH Dependence of k_e for Alanine Exit

The procedure was as described in "Experimental," except that cells preloaded with alanine-^{14}C at pH 7.2 at 37° were incubated at various pHs at 20°.
○—○: L-alanine, ●—●: D-alanine.

concentrations were 2.98±0.41 mM (S.D.) for L-alanine and 3.28±0.31 mM (S.D.) for D-alanine. The rate of D-alanine exit from Ehrlich cells at 37° was found to be considerably slower than that of L-alanine.

Furthermore, Fig. 6 shows that the k_e for L-alanine was remarkably dependent on temperature, while that for D-alanine was less dependent on temperature. The activation energy for exit of L-alanine was obtained as 19.2 kcal/mol. The results correspond fairly well to those for the exit and influx of D-glucose in human red blood cells.[14] The k_e for exit of D- and L-alanine was dependent on the pH of the medium (Fig. 7). The activation energy and pH-dependence for L-alanine exit suggest that the exit process is a mediated process. On the other hand, k_e for the exit of D-alanine was less dependent on temperature, and the exit process was pH-dependent. The activation energy for k_e of D-alanine is less than half that for k_e of L-alanine. The value, 7.0 kcal/mol, for D-alanine is at the lowest limit of the range of values, about 8 to 16 or 20 kcal/mol, for typical enzyme-catalyzed reactions.[3f] Thus, it may be assumed that free diffusion is a predominant process in the exit of the D-isomer, although a mediated process may also be involved in part, in view of the pH-dependence of exit.

Finally, the very slow exit of D-alanine may account, at least in part, for the finding[2a] that the radioactivity of D-alanine is strongly taken up into Ehrlich cells *in vivo*, compared with that of the L-isomer.

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