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L- and D-Alanine Transport in Brush Border Membrane Vesicles from Lepidopteran Midgut: Evidence for Two Transport Systems

Giorgio M. Hanozet, Barbara Giordana, Paolo Parenti, and Andrea Guerritore
Dipartimento di Fisiologia e Biochimica generali, Università di Milano, via Celoria 26, 20133 Milan, Italy

Summary. In brush border membrane vesicles from the midgut of *Philosamia cynthia* larvae (Lepidoptera) the L- and D-alanine uptake is dependent on a potassium gradient and on transmembrane electrical potential difference. Each isomer inhibits the uptake of the other form: inhibition of L-alanine uptake by D-alanine is competitive, whereas inhibition of D-alanine uptake by L-alanine is noncompetitive. Transstimulation experiments as well as the different pattern of specificity to cations suggest the existence of two transport systems. Kinetic parameters for the two transporters have been calculated both when $K_{out} > K_{in}$ and $K_{out} = K_{in}$. D-alanine is actively transported also by the whole midgut, but it is not metabolized by the intestinal tissue.

Key Words lepidopteran midgut · alanine transport · amino acid transport · K-cotransport · brush border membrane vesicles

Introduction

Vesicles from brush border membranes of lepidopteran midgut, a K^+-transporting epithelium, have proved to be a useful tool to study the characteristics of amino acid absorption. The midgut epithelium is constituted of two different kinds of cells with specific functional differentations. The so-called goblet cells, whose morphological features have been described in detail (Cioffi, 1979), are most likely the site of the electrogenic K^+ pump, which has not yet been biochemically characterized, associated with the ionic homeostasis of the animal (Harvey & Nedergaard, 1964; Zerahn, 1977; Wolfersberger, Harvey & Cioffi, 1982; Harvey, Cioffi & Wolfersberger, 1983). The absorption of nutrients takes place in columnar cells, which have the typical structure of absorptive cells (Cioffi, 1979). From these cells, brush border membrane vesicles (BBMV) have been prepared by Ca^{2+} precipitation and assayed for purity by means of marker enzymes. On the basis of the experimental evidence obtained with this preparation, as well as with the isolated midgut, a model for amino acid absorption has been proposed (Giordana, Sacchi & Hanozet, 1982). According to this model, the driving force for amino acid absorption is supplied by the K^+ electrochemical gradient generated by the luminally directed K^+ pump.

To further test this model, a study of the transport characteristics of L-alanine, a nonessential amino acid for Lepidoptera, was undertaken and the results are reported here. During the course of this study, the existence of a carrier-mediated D-alanine transport emerged, with characteristics somewhat different from the amino acid transport thus far described in Lepidoptera.

Materials and Methods

Experimental Animals

Larvae in the fifth instar of *Philosamia cynthia* were used. The larvae were fed on *Ailanthus glandulosa* leaves. The midgut was dissected from the larvae as a cylinder, and the peritrophic membrane with enclosed intestinal content was removed.

Flux Measurements

The midgut excised from the larvae was mounted as a cylinder on an apparatus similar to that described by Nedergaard and Harvey (1968). Flux measurements were performed as previously described (Sacchi, Hanozet & Giordana, 1984).

BBMV Preparation and Transport Experiments

BBMV[1] from *P. cynthia* midgut were prepared by means of Ca^{2+} precipitation, following the procedure of Schmitz et al. (1973),

[1] Abbreviations used: BBMV, brush border membrane vesicles; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethansulfonic acid; MES, 2-(N-morpholino)-ethansulfonic acid; Tris, *tris*-(hydroxymethyl)aminomethane.

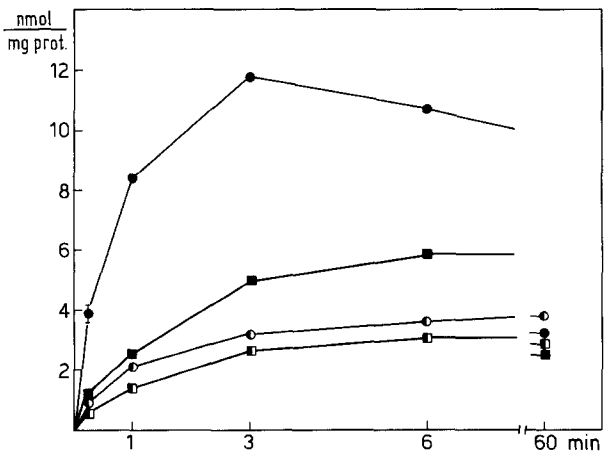

Fig. 1. Effect of valinomycin on L- and D-alanine uptake. BBMV, resuspended in 100 mM mannitol and 10 mM HEPES-Tris, pH 7.4, and preincubated 10 min in the absence (filled symbols) and in the presence (half filled symbols) of 3 μg/mg protein of valinomycin, were incubated in a medium of the following final composition: 100 mM mannitol, 10 mM HEPES-Tris, pH 7.4, 100 mM KSCN, 1 mM L-^{14}C-alanine (●), 1 mM D-^{14}C-alanine (■), 1 mM L-^{14}C-alanine + 3 μg/mg protein of valinomycin (◐), 1 mM D-^{14}C-alanine + 3 μg/mg protein of valinomycin (◧). Each point represents the mean ± SE of a typical experiment carried out in triplicate. When not given, SE bars were smaller than the symbols used

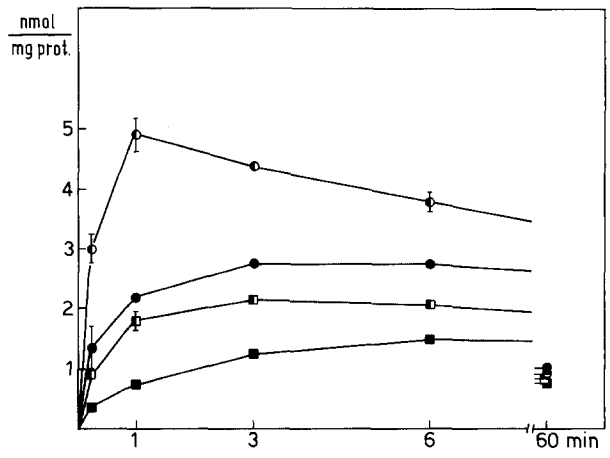

Fig. 2. Effect of FCCP and proton gradient on L- and D-alanine uptake. BBMV, resuspended in 193 mM mannitol, 90 mM MES and 17 mM Tris, pH 5.5, were incubated in a medium of the following final composition: 166 mM mannitol, 18 mM MES, 72 mM HEPES, 39 mM Tris, pH 7.21, 40 mM K_2SO_4, 1 mM L-^{14}C-alanine (●), 1 mM L-^{14}C-alanine + 80 μM FCCP (◐), 1 mM D-^{14}C-alanine (■), 1 mM D-^{14}C-alanine + 80 μM FCCP (◧). Each point represents the mean ± SE of a typical experiment carried out in triplicate. When not given, SE bars were smaller than the symbol used

modified by Kessler et al. (1978), as described in a previous paper (Giordana et al., 1982). When preloading of the vesicles was necessary, the pellet from the second centrifugation step and the final pellet were resuspended in an appropriate medium with the composition reported in the legends of the figures. Transport experiments were performed in triplicate or quadruplicate by the rapid filtration technique, as described by Hanozet, Giordana and Sacchi (1980). The uptake was linear almost up to 15 sec. Radioactivity associated with the filters was measured with a Packard scintillation counter, Tri Carb 300C. Ionophores, when present, were added from ethanol stocks, so that ethanol concentration in the incubation mixture did not exceed 0.5%. The final membrane pellet was resuspended at a protein concentration of 5–10 mg/ml, as determined according to Bradford (1976), with a Bio-Rad kit, using bovine serum albumin as a standard. All calculations as well as statistical and linear regression analysis were performed with an Apple personal computer.

Enzyme Assays

Glutamic pyruvic transaminase activity was assayed according to Bergmeyer, Gawehn and Grassl (1974); alanine racemase activity was similarly determined with D-alanine as substrate instead of L-alanine. D-alanine dehydrogenase activity was measured spectrophotometrically at 600 nm with dichlorophenolindophenol as acceptor dye, as described by Franklin and Venables (1976). D-amino acid oxidase was assayed with D-alanine as substrate either with a spectrophotometric method at 243 nm, according to Fonda and Anderson (1967) or with an oxygraf, according to Simonetta, Vanoni and Curti (1982). All enzyme assays were carried out at 30°C.

L- and D-Alanine Metabolism

Tissue extracts were obtained from midguts mounted as indicated before and incubated for 1 hr with a perfusion saline containing 1 mM L- or D- ^{14}C-alanine. The exposed tissue was then removed, blotted on filter paper, and crushed. Distilled water (5 vol) was added, the suspension was frozen, thawed, and centrifuged in a Beckman Microfuge at 10,000 × g for 3 min. Vesicles were also incubated for 1 hr with the labeled amino acid and centrifuged as above. Analysis of the samples (0.75 μl) of each supernatant was carried out by single dimensional thin-layer chromatography on HPTLC cellulose plates (Merck n.5787), with a solvent system composed of n-butanol/acetic acid/water (12 : 3 : 5). The labeled amino acids were used as standards. For autoradiography, the developed and dried plates were exposed to Kodak X-ray films (X-Omat film SO-282) for 8 days at −80°C.

Materials

L-(U-^{14}C)-Alanine, 165 mCi/mmol, and D-(U-^{14}C)-alanine, 40 mCi/mmol, were obtained from Radiochemical Centre (Amersham, UK); valinomycin from Boehringer (Mannheim, FRG); FCCP from Sigma (St. Louis, MO). All other reagents were analytical grade products from Merck (Darmstadt, FRG).

Results

L-alanine uptake into BBMV in the presence of an initial gradient in KSCN shows the overshoot typi-

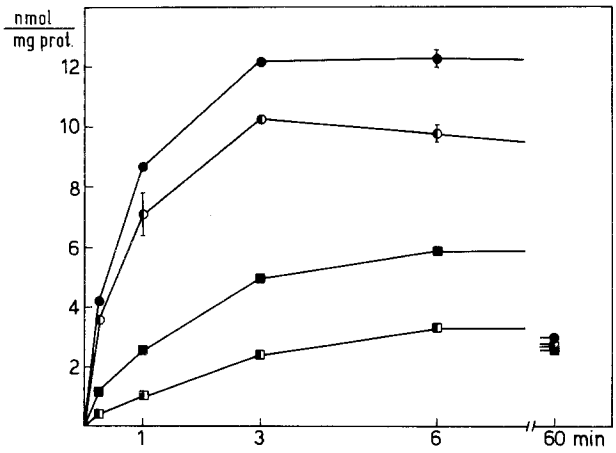

Fig. 3. Time course of L-alanine and D-alanine uptake. BBMV, resuspended in 100 mM mannitol and 10 mM HEPES-Tris, pH 7.4, were incubated in a medium of the following final composition: 100 mM mannitol, 10 mM HEPES-Tris, pH 7.4, 100 mM KSCN, 1 mM L-^{14}C-alanine (●), 1 mM L-^{14}C-alanine + 1 mM D-alanine (◐), 1 mM D-^{14}C-alanine (■), 1 mM D-^{14}C-alanine + 1 mM L-alanine (◧). Each point represents the mean ± SE of a typical experiment carried out in triplicate. When not given, SE bars were smaller than the symbol used

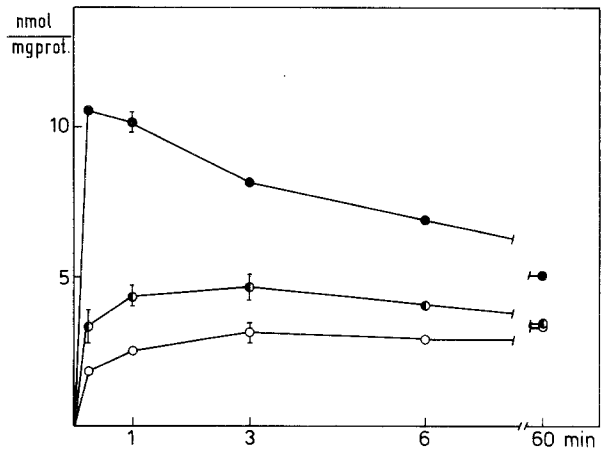

Fig. 4. Transstimulation of L-alanine uptake. BBMV, resuspended in 100 mM mannitol, 10 mM HEPES-Tris, pH 7.4, 50 mM K_2SO_4 (○), 50 mM K_2SO_4 + 40 mM L-alanine (●), 50 mM K_2SO_4 + 40 mM D-alanine (◐) and preincubated 10 min with 8 μg/mg protein of valinomycin, were incubated in a medium of the following final composition: 100 mM mannitol, 10 mM HEPES-Tris, pH 7.4, 50 mM K_2SO_4, 8 μg/mg protein of valinomycin, 2 mM L-^{14}C-alanine. Each point represents the mean ± SE of a typical experiment carried out in triplicate. When not given, SE bars were smaller than the symbol used

cal of a symport. In the same conditions, the D isomer is also transiently accumulated within BBMV, although with an initial rate and accumulation ratio much smaller than those of L-alanine. The concentrative uptake of L- and D-alanine appears strictly dependent on potassium gradient: the potassium ionophore, valinomycin, induces the complete disappearance of the overshoot of both amino acids and an inhibition up to 75% of the initial rate of L-alanine uptake (Fig. 1). This result is similar to that obtained with potassium equilibrated vesicles (Hanozet et al., 1980).

When vesicles are preloaded with a buffer at pH 5.5 and diluted 1 : 5 in a medium at pH 7.2, a strong stimulation of the uptake of both L- and D-alanine is obtained, if the proton ionophore FCCP is added. In these conditions, the proton flux down its electrochemical gradient generates a transmembrane electrical potential difference that accelerates amino acid uptake into the vesicles (Fig. 2).

To ascertain if L- and D- isomers are taken up via the same agency, inhibition tests were performed in which a ^{14}C labeled form was present in the incubation mixture together with the same concentration of cold corresponding enantiomer. As is shown in Fig. 3, in both cases an inhibitory effect was apparent, quantitatively much more evident when the effect of L-alanine on D-alanine uptake was examined, since in this case the concentrative D-alanine transport became merely equilibrative.

The existence of two distinct carrier-mediated systems was suggested by transstimulation experiments. For these experiments, vesicles were preloaded with 40 mM L- or D-alanine, and then diluted 20-fold in a medium containing one or the other labeled amino acid. Valinomycin and 50 mM K_2SO_4 were present on both sides of the vesicles, in order to short circuit any potential difference across the membrane due to the electrogenic carrier-mediated transfer of the unlabeled amino acid from the vesicle. Under these conditions the initial rate of L- ^{14}C-alanine in vesicles preloaded with cold L-alanine was some six times larger than that observed in vesicles preloaded with cold D-alanine. Furthermore, counterflow accumulation was found when the elicitor was L-alanine, not when it was D-alanine (Fig. 4). Likewise, only unlabeled D-alanine, and not L-alanine, transtimulated D-^{14}C-alanine uptake: the initial rate was, in this case, 2.6-fold the control rate (Fig. 5).

Carrier-mediated transports of L- and D-alanine were also different as far as cation specificity is concerned. The uptake of L-alanine was maximal with potassium and sodium, lower with lithium and similar to the control with rubidium and cesium (Table 1). A completely different pattern of specificity was shown by D-alanine uptake. In this case, sodium was more effective than potassium, whereas lithium, rubidium, and cesium were practically ineffective. In contrast, the anion dependence of alanine

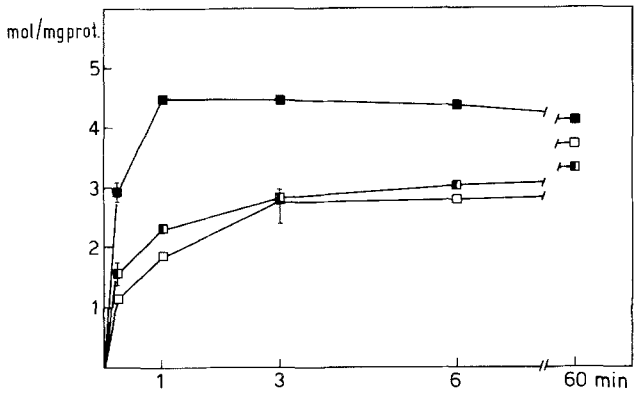

Fig. 5. Transstimulation of D-alanine uptake. BBMV, resuspended in 100 mM mannitol, 10 mM HEPES-Tris, pH 7.4, 50 mM K_2SO_4 (□), 50 mM K_2SO_4 + 40 mM D-alanine (■), 50 mM K_2SO_4 + 40 mM L-alanine (◧) and preincubated 10 min with 8 μg/mg protein of valinomycin, were incubated in a medium of the following final composition: 100 mM mannitol, 10 mM HEPES-Tris, pH 7.4, 50 mM K_2SO_4, 8 μg/mg protein of valinomycin, 2.25 mM D-^{14}C-alanine. Each point represents the mean ± SE of a typical experiments carried out in triplicate. When not given, SE bars were smaller than the symbol used

Table 1. Effect of monovalent cations on L- and D-alanine uptake[a]

Salt added	L-alanine uptake (pmol/3 min/mg protein)	D-alanine uptake (pmol/3 min/mg protein)
—	1824 ± 35	1335 ± 70
LiCl	3496 ± 25	850 ± 63
NaCl	8367 ± 151	6828 ± 457
KCl	8532 ± 361	3406 ± 277
RbCl	1377 ± 16	818 ± 37
CsCl	1144 ± 50	1529 ± 357

[a] Membrane vesicles, resuspended in 100 mM mannitol, 10 mM HEPES-Tris, pH 7.4, were incubated in a medium of the following final composition: 100 mM mannitol, 10 mM HEPES-Tris, pH 7.4, 1 mM L-^{14}C-alanine or D-^{14}C-alanine, 100 mM of the indicated salt. Mean ± SE of three experiments.

Table 2. Effect of anions on L- and D-alanine uptake[a]

Salt added	mM	L-alanine uptake (pmol/3 min/mg protein)	D-alanine uptake (pmol/3 min/mg protein)
—	—	1824 ± 35	1335 ± 70
KSCN	100	11446 ± 264	5169 ± 105
KCl	100	8532 ± 361	3406 ± 277
K_2SO_4	50	7537 ± 407	2789 ± 101
K gluconate	100	7221 ± 306	2516 ± 17

[a] Membrane vesicles, resuspended in 100 mM mannitol, 10 mM HEPES-Tris, pH 7.4, were incubated in a medium of the following final composition: 100 mM mannitol, 10 mM HEPES-Tris, pH 7.4, 1 mM L-^{14}C-alanine or D-^{14}C-alanine and the indicated salt. Means ± SE of three experiments.

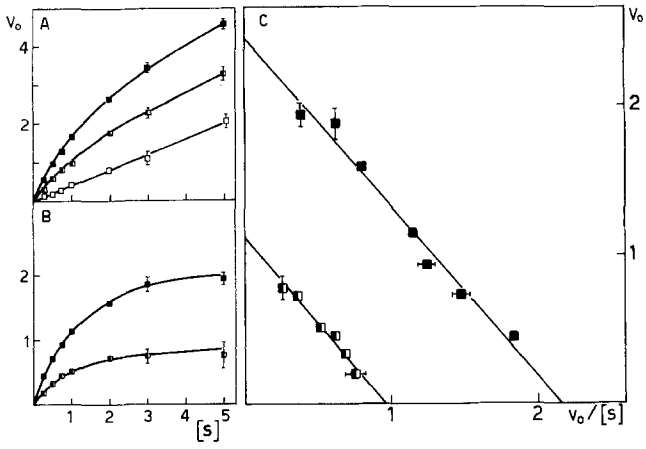

Fig. 6. Inhibition of D-alanine uptake by L-alanine. BBMV, resuspended in 100 mM mannitol, 10 mM HEPES-Tris, pH 7.4, were incubated in a medium of the following final composition: 100 mM mannitol, 10 mM HEPES-Tris, pH 7.4, 100 mM KSCN and 0.25–5 mM D-^{14}C-alanine, in the absence (■) or in the presence of 0.1 mM (◧) and 0.4 mM (□) L-alanine. Uptake after 15 sec incubation was measured. (*A*): Overall uptake *vs.* D-alanine concentration. (*B*): Uptake corrected for unsaturable component *vs.* D-alanine concentration. (*C*): Eadie-Hofstee plot of the corrected values. Kinetic parameters were obtained by linear regression analysis according to the least squares method and are reported in Table 3. v_o = nmol/15 sec/mg protein; [*S*] = mM D-alanine. Each point represents the mean ± SE of a typical experiment carried out in quadruplicate. When not given, SE bars were smaller than the symbol used

uptake was quite similar for D-and L- forms (Table 2); for both isomers the effectiveness of the tested anions corresponded fairly well to their putative permeabilities, thus confirming the role of the potential in the transport process examined.

The two transport systems behaved differently when the BBMV were prepared from frozen midgut. In this case, the overshoot for D-alanine was completely abolished, whereas that of L-alanine was only slightly reduced (data not shown).

To investigate the mutual inhibition of the two enantiomers, the uptake of D-alanine *versus* concentration was measured in the presence or in the absence of the L-form (Fig. 6). The overall uptake of D-alanine is reported in Fig. 6*A*: the kinetics are consistent with a Michaelis-Menten component plus a linear one. The nonsaturable component, calculated from the slope of the linear part of each curve, was similar in all cases and independent of the presence of L-alanine. The corrected values are

Table 3. Kinetic constants for L- and D-alanine uptake[a]

D-alanine uptake			L-alanine uptake		
	J_{max}	K_m		J_{max}	K_m
Control	2460 ± 94	1.14 ± 0.09	Control	5293 ± 158	0.38 ± 0.02
+ 0.1 mM L-alanine	1126 ± 68	1.17 ± 0.12	+ 0.5 mM D-alanine	5196 ± 123	0.51 ± 0.02
+ 0.4 mM L-alanine	—	—	+ 1.0 mM D-alanine	5113 ± 199	0.58 ± 0.05

[a] The constants were calculated from Figs. 6*C* and 7. J_{max} is expressed as pmol/15 sec/mg prot. ± SE; K_m is expressed as mM ± SE.

reported in Fig. 6*B*: 0.4 mM L-alanine completely abolished the carrier-mediated uptake of the D-form, whereas a concentration of 0.1 mM exerted a noncompetitive inhibition. This is more clearly shown by the Eadie-Hofstee plot reported in Fig. 6*C*, which allows calculation of the kinetic parameters J_{max} and K_m (Table 3). The calculated K_i for L-alanine was 84.4 μM. In contrast a competitive inhibition was exerted by D-alanine on L-alanine uptake, as is shown by Fig. 7, which shows the Eadie-Hofstee plot of uptake values corrected for the diffusional component, as above. The calculated kinetic parameters are reported in Table 3. The inhibition constant for D-alanine was calculated by a replot of the slopes in the Eadie-Hofstee plot *versus* inhibitor concentration (Fig. 7, inset): its value was 1.95 ± 0.36 mM.

The values of the kinetic parameters both for L- and D-alanine depend on potassium electrochemical potential. Figure 8 shows the kinetics of D- and L-alanine uptake in the presence of a potassium gradient (100 mM outside, 0 mM inside) or in the absence of the gradient, but in the presence of potassium (100 mM outside, 100 mM inside). Both for L- and D-alanine, the presence of an inwardly directed potassium gradient reduced the value of K_m and increased the value of J_{max} (Table 4). However, the effect of the potential difference was more pronounced on K_m, especially for L-alanine.

D-alanine is transported also by the intact midgut isolated *in vitro*, as shown in Table 5. The D-alanine influx (lumen to hemolymph) and efflux (hemolymph to lumen) were measured either in the presence or in the absence of L-alanine. As is evident from Table 5, there was a net flux of D-alanine, and this flux was substantially reduced in the presence of L-alanine. Nevertheless, D-alanine was not metabolized by the isolated midgut: extract of the tissue exposed to labeled D-alanine was separated by monodimensional thin-layer chromatography and autoradiographed. As is clearly evident in Fig. 9*B* (lanes *3–4*), there were no metabolic products of D-alanine present on the plate, but only one radioactive spot, corresponding to the position of authentic D-^{14}C-alanine. In contrast, in the same experimental conditions, L-^{14}C-alanine was extensively metabolized and showed almost six different radioactive spots (Fig. 9*A*, lanes *3–4*). However, it is noteworthy that neither D-alanine nor L-alanine were metabolized by BBMV (Fig. 9*A* and *B*, lanes 2). Furthermore, the search for possible enzymes that utilized D-alanine was unsuccessful. No activity of D-alanine racemase, D-alanine dehydrogenase, and D-alanine oxidase was detected in the crude midgut homogenate or in BBMV preparations. In contrast, the activity in the crude homogenate of a wide-spread enzyme that utilizes L-alanine, glutamic pyruvic transaminase, was 0.91 μmol/min/mg protein.

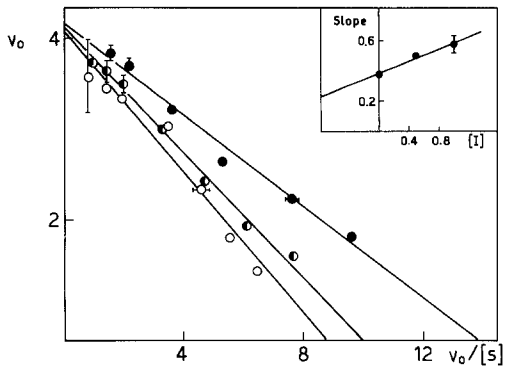

Fig. 7. Inhibition of L-alanine uptake by D-alanine. BBMV, resuspended in 100 mM mannitol, 10 mM HEPES-Tris, pH 7.4, were incubated in a medium of the following final composition: 100 mM mannitol, 10 mM HEPES-Tris, pH 7.4, 100 mM KSCN and 0.185-5.06 mM L-^{14}C-alanine, in the absence (●) or in the presence of 0.5 mM (◐) and 1 mM (○) D-alanine. Uptake after 15 sec incubation was measured and each value corrected for the nonsaturable component. The Eadie-Hofstee plot was constructed as indicated in Fig. 6. The obtained kinetic parameters are indicated in Table 3. v_o = nmol/15 sec/mg protein; [*S*] = mM L-alanine. In the inset, slope values are replotted as a function of D-alanine concentration. Each point in the Eadie-Hofstee plot represents the mean ± SE of a typical experiment carried out in quadruplicate. When not given, SE bars were smaller than the symbol used

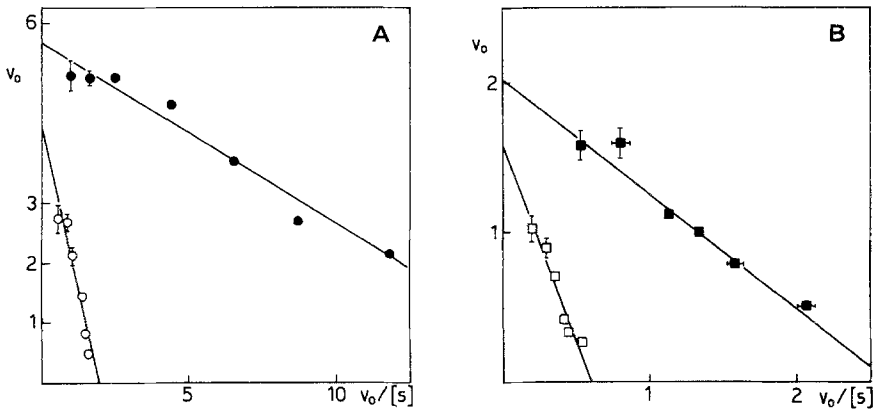

Fig. 8. Effect of a KSCN gradient on L-alanine and D-alanine uptake. (*A*): L-alanine uptake measured as reported in Fig. 7, in the presence of a KSCN gradient (100 mM outside, 0 mM inside) (●), or in the absence of a KSCN gradient but in the presence of KSCN (100 mM outside, 100 mM inside) (○). (*B*): D-alanine uptake measured as reported in Fig. 6, in the presence of KSCN gradient (100 mM outside, 0 mM inside) (■), or in the absence of a KSCN gradient but in the presence of KSCN (100 mM outside, 100 mM inside) (□). v_0 = nmol/15 sec/mg protein; [*S*] = mM L-alanine (*A*) or mM D-alanine (*B*). Each point represents the mean ± SE of a typical experiment carried out in quadruplicate. When not given, SE bars were smaller than the symbol used

Table 4. Kinetic constants for L- and D-alanine uptake in the absence and in the presence of a KSCN gradient[a]

	D-alanine uptake		L-alanine uptake	
	J_{max}	K_m	J_{max}	K_m
$K_{out} > K_{in}$	2072 ± 103	0.77 ± 0.07	5664 ± 167	0.30 ± 0.02
$K_{out} = K_{in}$	1587 ± 23	2.59 ± 0.33	4276 ± 423	2.20 ± 0.34

[a] The constants were calculated from Fig. 8. J_{max} is expressed as pmol/15 sec/mg prot. ± SE; K_m is expressed as mM ± SE.

Table 5. Unidirectional fluxes of D-alanine in isolated midgut of *Philosamia cynthia*[a]

	J_{l-h}	J_{h-l}	J_{net}
Control	54.0 ± 4.8 (4)	1.8 ± 0.1 (4)	52.2 ± 4.8
+ 5 mM L-alanine	37.8 ± 3.4 (4)	2.4 ± 0.3 (4)	35.4 ± 4.0

[a] D-^{14}C-alanine concentration was 5 mM. J_{l-h} = lumen to haemolymph flux; J_{h-l} = haemolymph to lumen flux. L-alanine was added in the lumen side. Fluxes are expressed as μmol/g dry wt/hr. Means ± SE, number of experiments in parenthesis.

Discussion

As demonstrated for L-phenylalanine and α-aminoisobutyric acid, in *P. cynthia* and for L-alanine in *Bombyx mori* (Giordana et al., 1982; Sacchi et al., 1984), the amino acid absorption takes place by means of a secondary active transport mechanism, due to the presence of a K^+-amino acid cotransport and an electrogenic K^+ pump. In this paper the characteristics of L- and D-alanine transport in BBMV from *P. cynthia* midgut have been studied. As shown by autoradiography (Fig. 9), the BBMV fulfill the requirements for a correct study of amino acid transport, since neither the L- nor the D- form are transformed during the experimental time. Both isomers are transported and accumulated inside the vesicles in the presence of an inwardly directed potassium gradient (Fig. 1). The uptake is quantitatively different, and the initial rate is 3.25-fold and the overshoot value 2.5-fold higher for L-alanine than for D-alanine. The concentrative uptake is abolished in the presence of the potassium ionophore valinomycin, which enhances potassium conductance across the membrane and thus dissipates the gradient. Therefore, L- and D-alanine are cotransported with potassium across the luminal

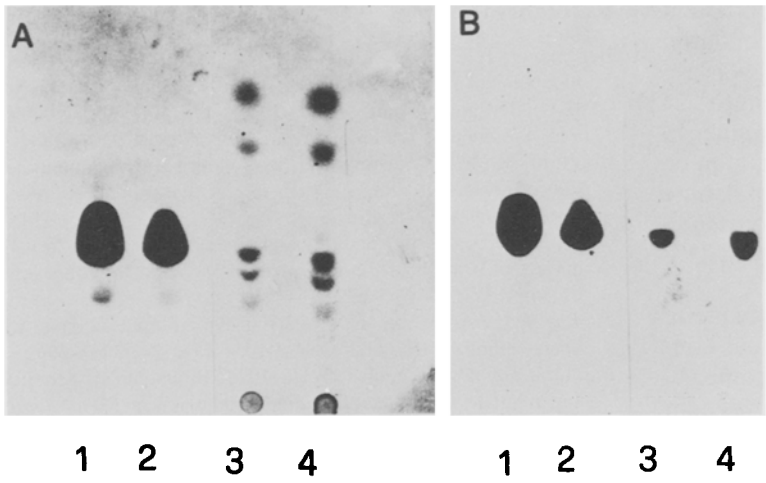

Fig. 9. Metabolism of L- and D-alanine by midgut and BBMV from midgut of *P. cynthia*. Tissue and vesicles were exposed to labeled amino acid, extracted, separated on thin-layer chromatography, and autoradiographed as indicated in Materials and Methods. (*A*): Autoradiography of samples incubated with L-^{14}C-alanine. (*B*): Autoradiography of samples incubated with D-^{14}C-alanine. *1*, Standard labeled amino acid. *2*, Vesicles exposed to labeled amino acid. *3*, Extract of the midgut exposed to labeled amino acid (0.75 μl deposition). *4*, Extract of the midgut exposed to labeled amino acid (1.5 μl deposition)

membrane of the enterocyte. The symport mechanism is electrogenic, since the presence of a transmembrane potential difference, generated by an outwardly directed proton flux in the presence of FCCP, increases both the initial rate and the overshoot value (Fig. 2).

Table 2 supplies further evidence of the involvement of the potential difference in the carrier-mediated translocation: the uptake of the two isomers is the fastest with the lipophilic anion, thiocyanate, and the slowest in the presence of the impermeable anion, gluconate. From Table 2 it can also be inferred that chloride permeability of BBMV is similar to that of sulphate and gluconate. Therefore, the accelerating effect observed in the presence of these anions with respect to the rate in the lack of any salt is probably due more to the presence of potassium than to the presence of an anion-generated potential difference.

Figure 3 shows that each isomer inhibits the uptake of the other form; the inhibiting effect of L-alanine on D-alanine uptake is larger than that of the latter on the former, since L-alanine overshoot is slightly reduced, whereas the uptake of D-alanine becomes merely equilibrative. Two possibilities can be taken into account to explain these results: the isomers (i) share the same transport agency with different affinity or (ii) are transported by two different carriers and exert a mutual inhibition and/or a reciprocal dissipation of potassium gradient.

To discriminate between these hypotheses, transstimulation experiments were performed (Figs. 4 and 5). Since a transstimulation effect takes place for each isomer only in the presence inside the vesicles of the same form, the two amino acids are taken up by two different transport systems. Fig. 4 shows that a slight effect on L-alanine uptake is also exerted by an outwardly directed D-alanine gradient: therefore D-alanine can be transported at least in part by the L-alanine carrier. In these experiments, any unspecific effect due to the electrical charge movement associated with the electrogenic carrier-mediated transport is prevented, thus short circuiting the vesicles by means of the potassium ionophore, valinomycin.

The same conclusion is suggested by the different pattern of specificity to cations shown by the two amino acids (Table 1). L-alanine exhibits the same cation specificity found for L-phenylalanine in *P. cynthia* and L-alanine in *B. mori* (Giordana et al., 1982), whereas D-alanine uptake is twofold higher in the presence of sodium than in the presence of potassium as cotransported cation.

A detailed investigation of the mutual inhibitory effect of the two isomers has produced evidence that D-alanine competitively inhibits L-alanine uptake (Fig. 7), in good agreement with transstimulation effect elicited by D-alanine on L-alanine uptake. This means that the two amino acids compete for the same binding site. In contrast, the inhibition of L-alanine on D-alanine uptake is noncompetitive (Fig. 6). This could be due either to a direct interaction with the D-alanine carrier or to a faster dissipation of the initial potassium gradient induced by L-alanine transport. The last possibility is ruled out, because an inhibition of D-alanine uptake was ob-

served also in the absence of a potassium gradient but in the presence of potassium. In this condition the initial rate of 1 mM D-alanine uptake was reduced from 931 ± 50 pmol/15 sec/mg protein to 524 ± 55 pmol/15 sec/mg protein by 1 mM L-alanine. In addition, it can be observed that 0.1 mM L-alanine determines a 54% inhibition of the J_{max} of D-alanine (Table 3), whereas in the absence of a potassium gradient the J_{max} of D-alanine is reduced only of 23% (Table 4).

Each transport system shows a different kinetic behavior when potassium is present at equal concentrations on both sides of the BBMV in comparison with the usual condition of 100 mM potassium gradient. Although a decrease of about 25% in J_{max} is observed for L- and D-alanine, the K_m value increases about sevenfold for L-alanine, but threefold for D-alanine (Table 4).

In conclusion, the data obtained on BBMV suggest that L- and D-alanine cross the mucosal barrier of the columnar cells via a secondary active transport operated by two different cotransport systems. The experiments carried out with the isolated midgut shows that the intestine actively absorbs both L-alanine (Sacchi & Giordana, 1980) and D-alanine (Table 5). The physiological significance of the L-alanine transport is quite evident, since L-alanine is extensively metabolized by the intestinal tissue, and all the required conditions exist *in vivo* for its active absorption, i.e., a potassium electrochemical gradient across the brush border membrane as high as −3320 cal/mol (Giordana et al., 1982) and an L-alanine concentration in the lumen content of 350 μM (*unpublished results*). In contrast, the physiological significance of a D-alanine transport mechanism is puzzling, mainly because D-alanine should be to our knowledge, practically lacking in the intestinal content, and it is not metabolized by the tissue. Nevertheless, not only is it transported *in vitro*, but the substitution of D-alanine for L-alanine in the diet has a toxic effect *in vivo* (Ito & Inokuchi, 1981). In addition, the D-alanine transport system exhibits characteristics somewhat different from the amino acid transports thus far described: it is dependent mainly on sodium as the cotransported cation, although sodium is almost totally absent in the lumen content of lepidopteran midgut (Giordana & Sacchi, 1978). Therefore, the D-alanine transport system has to be added to the number of structures in search of a function, perhaps the transport of some metabolite with a structure related to D-alanine.

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