THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 267, No. 22, Issue of August 5, pp. 15391-15397, 1992
Printed in U.S.A.

Kinetics of Leucine Transport in Brush Border Membrane Vesicles from Lepidopteran Larvae Midgut*

(Received for publication, December 20, 1991)

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The kinetics of K^+-leucine cotransport in the midgut of lepidopteran larvae was investigated using brush border membrane vesicles. Initial rate (3 s) of leucine uptake was determined under experimental conditions similar to those occurring *in vivo*, *i.e.* in the presence of $\Delta\psi >> 0$ (inside negative) and a ΔpH of 1.4 units ($7.4_{in}/8.8_{out}$). Leucine and K^+ bind to the carrier according to a sequential mechanism, and the binding of one substrate changed the dissociation constant for the other substrate by a factor of 0.15. Both *trans*-K^+ and *trans*-leucine were mixed-type inhibitors of leucine uptake. Moreover, a portion of total leucine uptake was K^+ independent, and it was competitively inhibited by *trans*-leucine. We interpret the *trans* inhibitory effects to mean that the partially loaded K^+ only form is virtually unable to translocate across the membrane, whereas the binary complex carrier, leucine, can isomerize from the *trans* to the *cis* side of the membrane. However, the K^+-independent leucine uptake occurs with a $K_{eq} > 1$, *i.e.* the efflux route through the partially loaded leucine only form is slower than the rate of isomerization of the unloaded carrier from *trans* to *cis* side. Taken together, these results suggest a model in which transport occurs by an iso-random Bi Bi system. Since K^+ does not act as a pure competitive activator, this model is different from that proposed for most of the Na^+-linked solutes transport agencies and may be related to the broadening of the cation specificity of the amino acid transporters in lepidopteran larvae.

Over the past 30 years it has became evident that most solutes are transported into the cells by a coupling to the downhill movement of specific ions: Na^+ in animal cells and H^+ in prokaryotes and walled eukaryotes. As far as Na^+-dependent systems are concerned, several kinetic models describing the events associated with the carrier-mediated transport of organic substrates have been proposed for vertebrates (Schultz and Curran, 1970; Heinz *et al.*, 1972; Alvarado and Mahmood, 1974; Geck and Heinz, 1976; Sepulveda and Robinson, 1978; Hopfer and Groseclose, 1980; Turner, 1981). Kinetic experiments performed in mammals show that, in many instances, the Na^+-dependent carriers display an ordered kinetic mechanism where Na^+ binds first to increase the affinity of the carrier for the organic cosubstrate (Kessler and Semenza, 1983; Wright *et al.*, 1983; Stevens and Wright, 1987). Alternatively, a random model for the transport of neutral amino acids in small intestine was proposed (Alvarado and Mahmood, 1974; Sepulveda and Robinson, 1978). Although in general, Na^+ acts as a pure competitive activator, in other vertebrates this effect of Na^+ on the amino acid cotransport mechanism was not confirmed, and a different order of binding was proposed for the Na^+-proline cotransporter in eel intestine (Maffia *et al.*, 1990). By contrast for the prokaryotic H^+ organic solutes cotransporter, a model based on a random order of binding of substrates has been proposed (Sanders, 1986).

In addition to these two classes of cotransport systems, whose characterization is constantly increasing, a third group of transport agencies should be considered, those which couple the intracellularly directed K^+ and amino acid fluxes in the midgut of lepidopteran larvae (Hanozet *et al.*, 1980). On the basis of experimental evidence obtained both from membrane vesicles studies and from the whole intestine isolated *in vitro*, a model for the K^+-dependent secondary active transepithelial transport of amino acids has been proposed (Giordana *et al.*, 1982). The driving force for this process is supplied *in vivo* by the K^+ electrochemical gradient maintained by a luminally directed active potassium transport located at the luminal border of goblet cells (Dow *et al.*, 1984; Wieczorek *et al.*, 1989). However, studies with membrane vesicles have shown that this cotransport mechanism is not strictly K^+ dependent, since sodium, and in some cases lithium, can activate the transport (Hanozet *et al.*, 1984; Giordana *et al.*, 1985). From a kinetic point of view, as external K^+ increases there is a hyperbolic or a sigmoid activation of amino acid uptake, depending on the amino acid considered. The transport is improved by the presence of both an inside-negative $\Delta\psi$[1] and a pH gradient (alkaline outside) similar to those occurring *in vivo* (Sacchi *et al.*, 1990). Although many transport systems with different amino acid and cation specificities have been identified (Giordana *et al.*, 1989), a kinetic model for the K^+ amino acid cotransport in the enterocytes from lepidopteran larvae has not yet been defined. In this study, we focus on a well characterized carrier system, the brush border neutral amino acid transporter, to develop a model for K^+-leucine cotransport. The results obtained indicate that the process is described by an iso-random Bi Bi reaction kinetics.

EXPERIMENTAL PROCEDURES

Brush Border Membrane Preparation and Transport Experiments—BBMV were prepared from frozen midguts of fifth instar larvae of *Philosamia cynthia* (Lepidoptera, Saturnidae) as previously described (Sacchi *et al.*, 1990). Vesicles were resuspended in 160 mM mannitol, 90 mM HEPES, 45 mM Tris, pH 7.4, and variable amounts

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[1] The abbreviations used are: $\Delta\psi$, transmembrane electrical potential difference; BBMV, brush border membrane vesicles; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

of K_2SO_4 or leucine as specified in the experiments, at a protein concentration of 2–3 mg/ml, determined according to Bradford (1976) with a Bio-Rad kit and bovine serum albumin as standard. A linear relationship between leucine uptake and BBMV protein concentration was obtained in the range between 0.25–8.00 mg of protein/ml (data not shown).

Transport of [^{3}H]leucine was performed in quadruplicate at 24 °C by a rapid filtration technique using an automated apparatus for the determination of initial uptake rates as described by Sacchi *et al.* (1990). The uptake was measured at 3 s of incubation time by mixing 10 μl of BBMV with 40 μl of radioactive mixture containing 160 mM mannitol, 60 mM Tris, pH 10.1, 40 μCi/ml [^{3}H]leucine, 90 μM FCCP, 0–48 mM K_2SO_4, and 0–5 mM leucine as specified in the experiments. Final pH of the extravesicular medium was 8.8. The osmolarity of uptake buffer was always adjusted to that of the intravesicular buffer using D-mannitol.

The initial rates of leucine uptake were determined under zero-*trans* conditions, *i.e.* when the initial *trans*-leucine and K^+ concentrations are zero, and under non-zero-*trans* conditions, *i.e.* when the intravesicular compartment was loaded with leucine or K^+ by resuspending the pellet of the second centrifugation step in a medium containing the leucine and K^+ concentrations as reported in the experiments. Preliminary experiments have shown that both leucine and K^+ equilibrate with the vesicles under these conditions. The intravesicular leucine concentration was assayed with ninhydrin as described elsewhere (Moore and Stein, 1948), whereas the presence of intravesicular K^+ was evaluated by recording the fluorescence quenching of the voltage-sensitive cyanine dye 3,3′-diethylthiocarbocyanine iodide as described by Cassano *et al.* (1988).

Calculations—Experimental data are given ±S.E. of triplicate determinations and were analyzed by computer and plotted on a digital plotter using a multiparameter, iterative, nonlinear regression program based on the Marquardt-Levenberg algorithm (SigmaPlot, Jandel, CA). The calculated constants are given ±S.D.

Materials—L-[4,5-^{3}H]Leucine was purchased from the Radiochemical Centre (Amersham International, Amersham, United Kingdom). Valinomycin was obtained from Boehringer (Mannheim, Federal Republic of Germany (F. R. G.)), and FCCP was from Sigma. All other reagents were analytical grade products from Merck (Darmstadt, F. R. G.).

RESULTS

cis-K^+ and cis-Leucine Effects—Leucine uptakes under both zero-*trans* and non-zero-*trans* conditions in the presence of $\Delta\psi$ and of a pH gradient were linear from 0 to 9 s. One example is shown in Fig. 1 for 0.5 and 5 mM leucine in the presence of extravesicular 50 mM K_2SO_4. Thus, the estimation of initial rates from 3-s uptakes provided the necessary accuracy to minimize interference from dissipation of gradients and volume changes.

The influx of leucine through the neutral amino acid transporter was measured under zero-*trans* conditions at four different *cis*-K^+ concentrations (10, 20, 40, and 150 mM) and at *cis*-leucine concentrations ranging from 0.05 to 5 mM (Fig. 2). In all conditions, these data fitted with a simple Michaelis-Menten equation, as confirmed also by the linear behavior of the Eadie-Hofstee plot reported in *panel B*. A nonsaturable component was not observed in our experimental conditions. The parameters, calculated by nonlinear regression analysis program, are reported in Table I. Note that changing the external K^+ concentration from 10 to 150 mM produced a 2-fold increase in V_{max} and a 6-fold decrease in K_m for leucine.

The K^+ dependence of leucine uptake was explored by measuring the K^+ activation curves at fixed *cis*-leucine concentrations (0.1, 0.14, 0.2, 0.5, 0.8, 1.0, and 5.0 mM) and at *cis*-K^+ concentrations ranging from 0 to 100 mM. Some of these curves were plotted in Fig. 3. As $[K^+]_{cis}$ increased, there was an hyperbolic activation of leucine uptake. Moreover, in the absence of K^+ a small but significant amount of leucine crosses the brush border membrane, giving a positive intercept

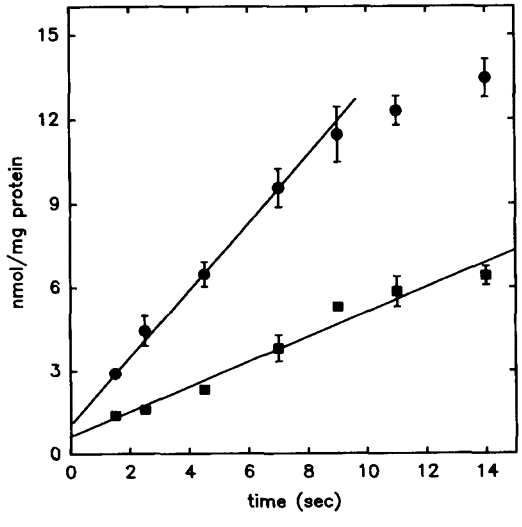

FIG. 1. **Time course of leucine uptake.** Total leucine uptakes were determined in quadruplicate under zero *trans* conditions as described under "Experimental Procedures," with 0.5 mM (*squares*) or 5 mM (*circles*) *cis*-leucine in the presence of 75 mM K_2SO_4. When not given *error bars* are smaller than the symbols used.

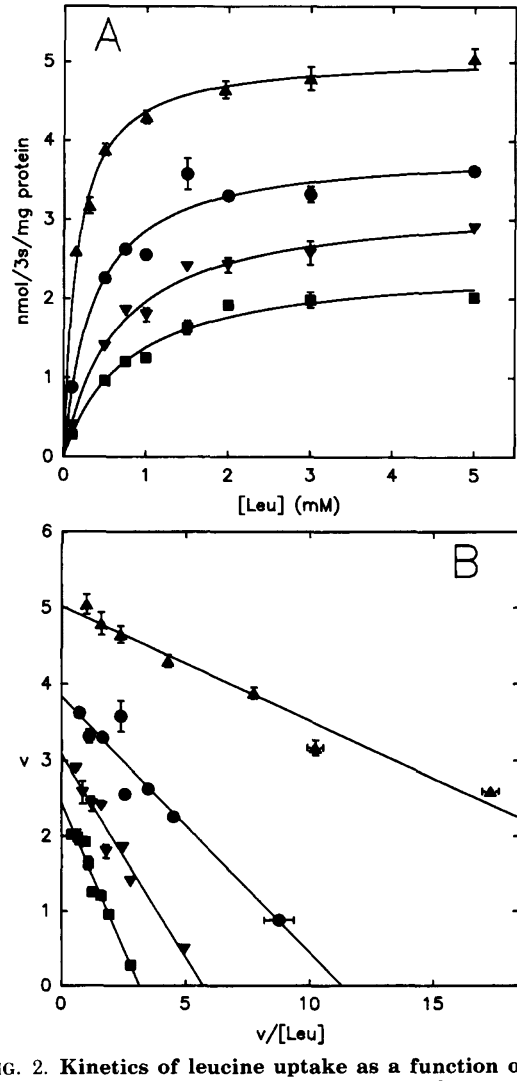

FIG. 2. **Kinetics of leucine uptake as a function of *cis* K^+.** Experiments were performed under zero *trans* conditions at 5 (■), 10 (▼), 20 (●), and 75 (▲) mM *cis*-K_2SO_4. *Panel A*, overall uptake *versus* leucine concentration; *panel B*: Eadie-Hofstee plot of the data; v = nmol/3 s/mg protein. The kinetic parameters are given in Table I. When not given *error bars* are smaller than the symbols used.

TABLE I

Zero-trans kinetics of leucine transport

Summary of the effects of *cis*-K^+ and *cis*-leucine on the kinetics of leucine uptake. See Figs. 2 and 3 for experimental details.

[Leu]	[K^+]	K_m	V_{max}
	mM		*pmol/3 s/mg protein*
0.1	0–100	108.0 ± 8.0	1572 ± 70
0.14	0–100	57.7 ± 6.1	2163 ± 100
0.2	0–100	35.0 ± 5.3	2494 ± 169
0.8	0–100	27.2 ± 0.9	3420 ± 144
1.0	0–100	15.7 ± 6.2	3620 ± 121
5.0	0–100	8.3 ± 1.5	4073 ± 212
0.05–5	10	0.78 ± 0.11	2452 ± 124
0.05–5	20	0.65 ± 0.05	3250 ± 130
0.05–5	40	0.36 ± 0.05	3887 ± 202
0.05–5	150	0.13 ± 0.02	4949 ± 331

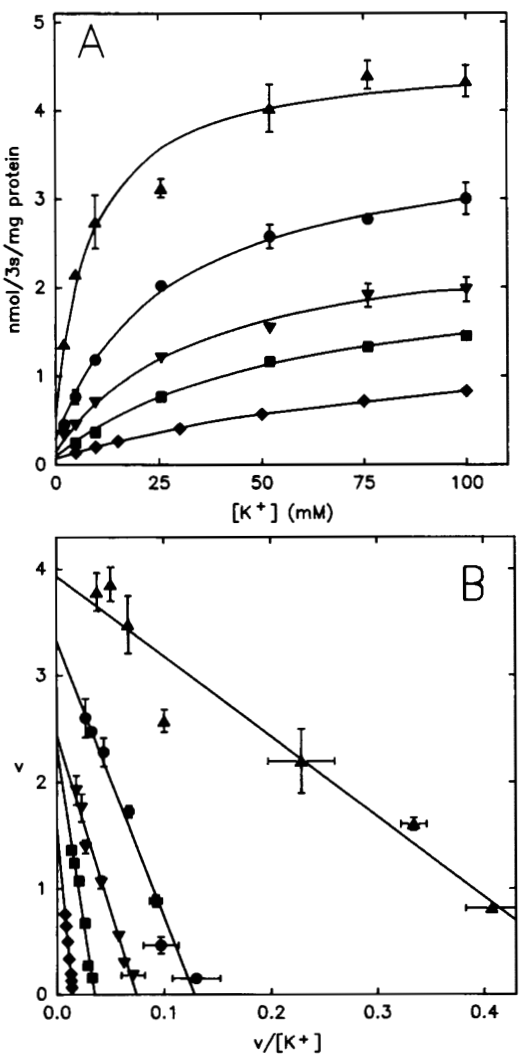

FIG. 3. **K^+ activation of leucine uptake.** Experiments were performed under zero *trans* conditions at 0.1 (◆), 0.14 (■), 0.2 (▼), 0.8 (●), 5.0 (▲) mM *cis* leucine. *Panel A*, overall uptake *versus* potassium concentration after subtraction of the K^+-independent leucine uptake; *panel B*, Eadie-Hofstee plot of the data; v = nmol/3 s/mg protein. The kinetic parameters are given in Table I. When not given *error bars* are smaller than the symbols used.

on the vertical axis of the activation curves (Fig. 3, *panel A*). This K^+-independent leucine transport accounted for 10–15% of the V_{max} values calculated from each curve. The K^+-dependent leucine uptake, with the calculated kinetic parameters reported in Table I, gives a straight line on the Eadie-Hofstee plot (Fig. 3, *panel B*). Increasing the $[Leu]_{cis}$ from 0.1 to 5.0 mM produced a 12-fold decrease of the apparent K_m for K^+ and a 2.5-fold increase of V_{max}.

Assuming that the rate-limiting step is the translocation of the ternary complex, the estimates reported in Table I can be used to calculate dissociation constants for K^+ and for leucine from the carrier (K_a and K_b, respectively) and from the binary complexes carrier-leucine and carrier-K^+ (αK_a and αK_b, respectively) according to the following equations (Segel, 1975)

$$K_a' = \alpha K_a(1 + K_b/[Leu])/(1 + \alpha K_b/[Leu]) \quad (1)$$

and

$$K_b' = \alpha K_b(1 + K_a/[K^+])/(1 + \alpha K_a/[K^+]) \quad (2)$$

where K_a' and K_b' are the apparent dissociation constants for K^+ and leucine and α is the factor by which the binding of one substrate changes the dissociation constant for the other substrate. Solving Equations 1 and 2, the estimates of the parameters were K_a 73.8 ± 8.1 mM, K_b 0.73 ± 0.12 mM and α 0.15 ± 0.02. By multiplying the α-factor value by K_a and K_b, Michaelis constants for the respective ligand in the presence of a saturating concentration of the other ligand were obtained. The K_m values for K^+ and leucine were 11.1 ± 1.0 and 0.11 ± 0.01 mM, respectively. The calculated parameters can be also used to estimate the V_{max} of the system, which can be obtained from the following equation (Segel, 1975)

$$V_{max}' = V_{max}(1 + \alpha K_b/[Leu]) \quad (3)$$

where V_{max}' is the maximal velocity measured at fixed *cis*-leucine concentrations. The estimate was V_{max} 5760 ± 350 pmol/3 s/mg protein. This value represented the K^+-dependent component of leucine uptake. The estimate of the V_{max} of the K^+-independent component may be obtained from the data represented in Fig. 3*A*. As a result, in these experimental conditions a value of 445 ± 13 pmol/3 s/mg protein was calculated.

trans-K^+ Inhibition—The results obtained from zero-*trans* kinetics of leucine transport are consistent with two kinetic mechanisms: a rapid equilibrium iso-random Bi Bi system and a steady-state iso-ordered Bi Bi mechanism. To distinguish between these two possibilities *trans*-leucine and *trans*-K^+ effects on leucine uptake were investigated in detail. The nature of *trans*-K^+ inhibition was analyzed by measuring leucine uptake kinetics at $[K^+]_{trans}$ = 0, 25, or 50 mM. An inhibitory effect by *trans*-K^+ was always observed both at saturating (150 mM) and non-saturating (20 mM) *cis*-K^+ concentration. This effect was accounted for by a decrease of V_{max} and an increase in K_m for leucine (Fig. 4, Table II). The same pattern of inhibition was observed when *trans*-K^+ effects were measured at fixed non-saturating (0.2 mM) and saturating (5 mM) *cis*-leucine. As a matter of fact $[K^+]_{trans}$ = 50 mM significantly affected the K^+ activation curve of leucine uptake via a 2-fold increase of the apparent K_m for K^+ and a 20% decrease of the V_{max} value (Table II). Taken together, these data indicated that *trans*-K^+ behaved as a mixed-type inhibitor of the K^+-leucine cotransporter.

trans-Leucine Effects—In a second set of experiments *trans* effects of leucine on leucine uptake was investigated. As shown in Fig. 5, 0.5 and 1.0 mM *trans*-leucine concentrations significantly inhibited the K^+-activated leucine uptake. The presence of leucine at the *trans* side reduced the affinity for K^+ at the *cis* side and decreased the V_{max} of the system. A saturating *cis*-leucine concentration did not abolish the inhibitory effect of *trans*-leucine (Fig. 5*B*). The kinetic parameters calculated in these experimental conditions and those ob-

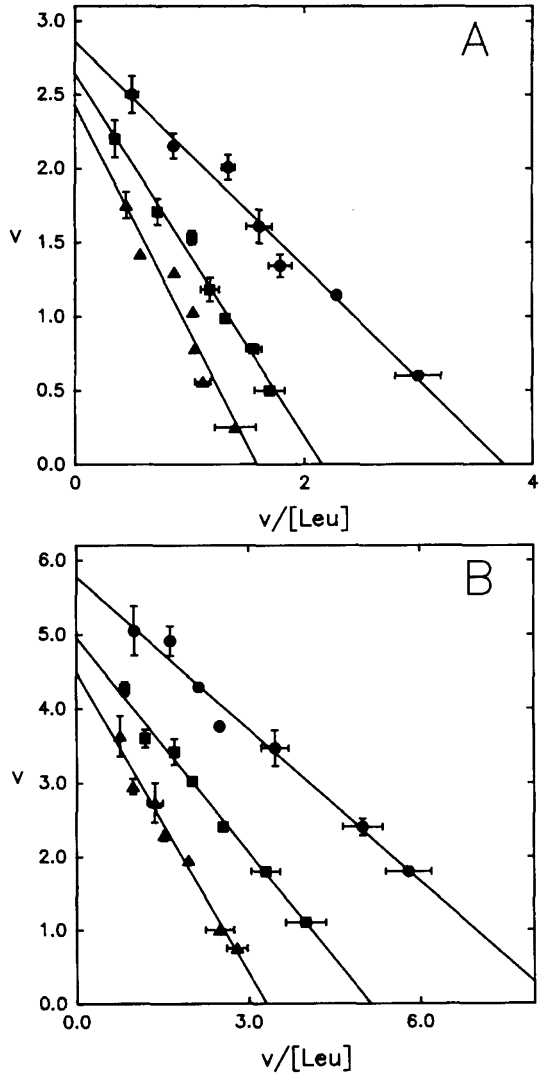

FIG. 4. ***trans*-K⁺ inhibition of leucine uptake kinetics.** Experiments were performed under non-zero *trans* conditions. *Cis*-leucine kinetics were measured at fixed $[K^+]_{cis}$ = 20 mM (*panel A*) or 150 mM (*panel B*) and at $[K^+]_{trans}$ = 0 (●), 25 (■), or 50 mM (▲). The kinetic parameters are reported in Table II. v = nmol/3 s/mg protein. When not given *error bars* are smaller than the symbols used.

tained from leucine uptake kinetics at fixed $[K^+]_{cis}$ = 0, 25, or 150 mM, are summarized in Table III. In all experimental conditions, a mixed-type inhibition by *trans*-leucine was observed, since the presence of leucine within the vesicles caused a decrease both in the affinity for substrates at the *cis* side of the carrier and in the maximal velocity of the cotransporter. *trans*-leucine also affected the initial rate of leucine uptake in the absence of K^+ (Fig. 6), indicating that isomerization rate of the leucine only form was lower than that of the unloaded carrier.

Model for K⁺-Leucine Cotransport—Table IV reports the kinetic model for leucine-K^+ translocation inferrable for the product inhibition patterns using Cleland rules to predict the effect of K^+ and leucine on the initial velocity of leucine uptake. Among the bireactant mechanisms considered, our data are compatible with a system in which K^+ and leucine bind randomly to the carrier and both the fully loaded complex and the leucine only form are able to translocate across the membrane. Fig. 7 shows the kinetic model for the K^+-coupled cotransport of leucine across intestinal brush border membranes from lepidopteran larvae.

TABLE II

Summary of trans-K⁺ effects on leucine uptake

Leucine uptakes were measured as described under "Experimental Procedures." Kinetic parameters were obtained after subtraction of the K^+-independent component of leucine uptake and were calculated using a nonlinear regression analysis program.

$[Leu]_{cis}$	$[K^+]_{cis}$	$[K^+]_{trans}$	K_m		V_{max}
			K^+	Leu	
		mM			*pmol/3 s/mg*
0.2	0–100	0	44 ± 7		1735 ± 26
0.2	0–100	25	78 ± 13		1545 ± 24
0.2	0–100	50	112 ± 11		1367 ± 37
5.0	0–100	0	5.5 ± 0.8		4211 ± 162
5.0	0–100	50	9.9 ± 0.9		3500 ± 190
0.05–5	150	0		0.13 ± 0.02	4949 ± 331
0.05–5	150	25		0.19 ± 0.02	3819 ± 221
0.05–5	150	50		0.37 ± 0.07	3584 ± 274
0.05–5	20	0		0.48 ± 0.09	3417 ± 256
0.05–5	20	25		0.87 ± 0.19	2958 ± 102
0.05–5	20	50		1.07 ± 0.04	2411 ± 164

DISCUSSION

In this study we have measured the kinetics of K^+-leucine cotransport across brush border membranes from the midgut of lepidopteran larvae in experimental conditions which resemble those occurring *in vivo*. These conditions require the presence of both a ΔpH ($7.4_{in}/8.8_{out}$) and a constant $\Delta\psi$ (≃94 mV, inside negative) across the membrane. Since our membrane preparations have a low intrinsic potassium permeability,[2] the latter condition may be obtained in the presence of the protonophore FCCP when sulfate is the accompanying anion for potassium. The overshoot phenomenon of leucine uptake in the presence of a K_2SO_4 gradient is not different from that obtained in the presence of a chemical potassium gradient in voltage-clamped conditions (Sacchi *et al.*, 1990).

Leucine is transported by a system which is responsible for the transfer of most neutral amino acids (Giordana *et al.*, 1989). This system is strongly dependent upon K^+ concentrations on each side of the membrane and $\Delta\psi$. Previously, experiments have shown that the transporter is greatly stimulated by the presence of an outwardly directed (alkaline outside) pH gradient (Sacchi *et al.*, 1990). The membrane potential affects both K_m and V_{max} for leucine transport, whereas the pH gradient influences only the V_{max}. By contrast, the presence of *trans*-K^+ has an inhibitory effect on leucine uptake, decreasing both the affinity and the V_{max} of the carrier. On the basis of these observations and the data presented in this paper, we now propose a tentative kinetic model for leucine transport in BBMV from lepidopteran larvae midgut. The model (Fig. 7) shows the transporter depicted as a carrier that combines with K^+ and leucine without an obligate order of binding to form a ternary complex. This complex then isomerizes to release the substrates at the *trans* side of the membrane. Alternatively a binary complex, the leucine only form, can translocate to unload leucine within the vesicles. Afterward the unloaded carrier returns to the *cis* side to complete the cycle. Although the translocator is represented as a mobile carrier, a gated channel mechanism is not eliminated by this formalism. All the experimental evidences are compatible with a coupling ratio of 1 K^+/1 leucine. In the terminology of enzyme kinetics, the system can be described as an iso-random Bi Bi reaction. The

[2] P. Parenti, unpublished observations with cationic dye experiments.

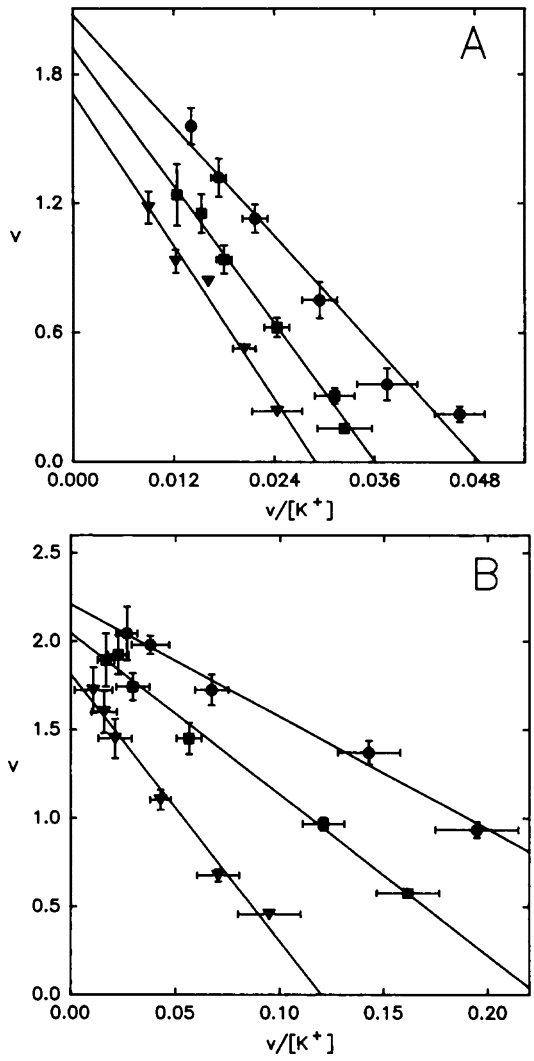

FIG. 5. ***trans*-Leucine inhibition of leucine uptake kinetics.** Experiments were performed under non-zero *trans* conditions. *Cis*-leucine kinetics were measured at fixed $[Leu]_{cis}$ = 0.2 mM (*panel A*) or 5 mM (*panel B*) and at $[Leu]_{trans}$ = 0 (●), 0.5 (■), or 1 mM (▲). The kinetic parameters are reported in Table III. v = nmol/3 s/mg protein. When not given *error bars* are smaller than the symbols used.

details of the model will be now discussed in relation to the experimental findings reported in this paper.

The kinetics of K^+-leucine cotransport was first investigated by studying the relationship between the apparent K_m for leucine and *cis*-K^+ and between the apparent K_m for K^+ and *cis*-leucine (zero *trans* conditions). The experimental observations that changes in $[K^+]_{cis}$ (Fig. 2) and changes in $[Leu]_{cis}$ (Fig. 3) affect both K_m and V_{max} suggest that, in lepidopteran midgut, a different mechanism from that described for some amino acid cotransporters in vertebrates (Stevens and Wright, 1987; Maffia *et al.*, 1990) is operative. In fact, the data reported in Figs. 2 and 3 excluded the presence of a rapid equilibrium ordered mechanism, since in this case the V_{max} should depend on *cis*-leucine concentration if K^+ binds first or should depend on *cis*-K^+ if leucine binds first. Moreover, the K^+ activation curves (Fig. 3) are characterized by a positive intercept on the y axis, indicating that leucine is partially transported across the membrane via a potassium-independent system. Some indirect evidences strongly suggested that this non-potassium-mediated transport may be accounted for by the translocation of the leucine only form (Sacchi *et al.*, 1990). Therefore, under zero *trans*

TABLE III

Summary of trans-leucine effects on leucine uptake

Leucine uptakes were measured as described under "Experimental Procedures." Kinetic parameters were calculated using a nonlinear regression analysis program.

$[K^+]_{cis}$	$[Leu]_{cis}$	$[Leu]_{trans}$	K_m		V_{max}
			K^+	Leu	
		mM			*pmol/3 s/mg*
0–100	0.1	0	97 ± 5		2163 ± 100
0–100	0.1	0.25	136 ± 7		1632 ± 132
0–100	0.1	0.50	157 ± 17		1212 ± 202
0–100	0.2	0	44 ± 1		2300 ± 58
0–100	0.2	0.5	53 ± 2		2045 ± 58
0–100	0.2	1.0	79 ± 6		1706 ± 110
0–100	5.0	0	7.7 ± 1.5		4322 ± 107
0–100	5.0	0.5	19.3 ± 3.6		3970 ± 155
0–100	5.0	1.0	36.5 ± 3.3		3676 ± 101
150	0.05–5	0		0.12 ± 0.01	4443 ± 63
150	0.05–5	0.5		0.26 ± 0.03	4030 ± 74
150	0.05–5	1.0		0.56 ± 0.05	3552 ± 101
25	0.05–5	0		0.59 ± 0.07	3162 ± 175
25	0.05–5	0.5		1.08 ± 0.05	2711 ± 73
25	0.05–5	1.0		1.55 ± 0.08	2230 ± 74
0	0.05–5	0		0.91 ± 0.08	284 ± 7
0	0.05–5	0.5		1.41 ± 0.12	280 ± 29
0	0.05–5	1.0		1.66 ± 0.10	260 ± 13

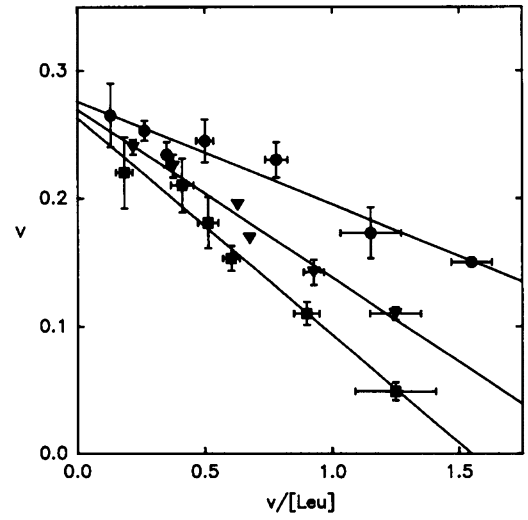

FIG. 6. ***trans*-Leucine inhibition of the K^+-independent leucine uptake kinetics.** Experiments were performed under non-zero *trans* conditions. *Cis*-leucine kinetics were measured in the absence of K^+ and at $[Leu]_{trans}$ = 0 (●), 0.5 (▼) or 1 mM (■). The kinetic parameters are reported in Table III. v = nmol/3 s/mg protein. When not given *error bars* are smaller than the symbols used.

conditions and using the usual assumptions associated with the equilibrium carrier model (Turner, 1981), the unidirectional flux of the leucine inside the vesicles is given by

$$J_{Leu} = k[CLeu] + h[CK^+Leu] \quad (4)$$

where k and h are the rate constants for the inward translocation of the partially loaded (CLeu) and the loaded (CK^+Leu) carrier species. Equation 4 can be solved for a random model in term of maximal velocity of transport and Michaelis constants for the amino acid and the activator according to the following equation

$$J_{Leu} = \frac{(V_0 K_{\infty(Leu)} + V_\infty[K^+])[Leu]}{K_{\infty(K)}(K_{0(Leu)} + [Leu]) + [K^+](K_{\infty(Leu)} + [Leu])} \quad (5)$$

where V_0 and V_∞ are the maximum fluxes of leucine into the vesicle when the external $[K^+]$ is zero or tend to infinite, respectively; $K_{0(Leu)}$ and $K_{\infty(Leu)}$ are the apparent Michaelis constants for leucine when the external $[K^+]$ is zero or tend to infinite, respectively; $K_{\infty(K)}$ is the apparent Michaelis constant for K^+ when the external [Leu] tend to infinite; $[K^+]$ and [Leu] are the extravesicular potassium and leucine concentrations. The data reported in Figs. 2 and 3 fitted with Equation 5 and the best fitting gives the following value for the parameters: $K_{\infty(K)}$ 15.4 ± 1.0 mM, $K_{0(Leu)}$ 2.2 ± 0.2 mM, $K_{\infty(Leu)}$ 0.22 ± 0.01 mM, V_0 514 ± 95 pmol/3 s/mg protein; V_∞ 5204 ± 90 pmol/3 s/mg protein. These data are in a rather good agreement with initial estimate of the parameters obtained by solving Equations 1 and 2 and indicated that "slippage" contributed significantly to leucine transport across the luminal membrane of the enterocytes only when *cis*-leucine concentration is higher than 1 mM. As a matter of fact the maximal velocity of leucine uptake in the absence of potassium is 10-fold lower, and the cation increased the affinity of the cotransporter for leucine by a factor of 10. Therefore, since intraluminal leucine concentration in *P. cynthia* larvae is 0.58 ± 0.13 mM (Parenti *et al.*, 1984) more than 90% of the amino acid is translocated via the ternary complex.

TABLE IV

Possible product inhibition patterns for the K⁺-leucine cotransport in BBMV from the midgut of P. cinthya larvae

Product inhibition patterns are developed using the general rules formulated by Cleland (1963) or adapted from Segel (1975). All the systems considered are iso Bi Bi mechanisms. In the Rapid Equilibrium systems, model B refers to a model in which the isomerization step is included in the rapid equilibrium segment, whereas in model A this is not. The inhibition profile is obtained evaluating the effects on slope and v axis intercept on a Eadie-Hofstee plot. C (competitive) = only slope changes; UC (uncompetitive) = only v axis intercept changes; MT (mixed-type) = both slope and v axis intercept change. A horizontal line means no inhibition. US and S refer to unsaturating and saturating conditions with the fixed substrate, respectively.

Mechanism	Product inhibitor	Varied substrate			
		K^+		Leucine	
		US	S	US	S
Steady-state ordered					
K^+-leucine	Leucine	MT	UC	MT	MT
	K^+	MT	MT	MT	UC
Random	Leucine	MT	MT	MT	MT
	K^+	MT	MT	MT	MT
Rapid equilibrium ordered					
K^+-leucine					
Model A	Leucine	—	—	—	—
	K^+	MT	MT	MT	MT
Model B	Leucine	—	—	—	—
	K^+	C	—	C	—
Random					
Model A	Leucine	MT	MT	MT	MT
	K^+	MT	MT	MT	MT
Model B	Leucine	C	—	C	—
	K^+	C	—	C	—

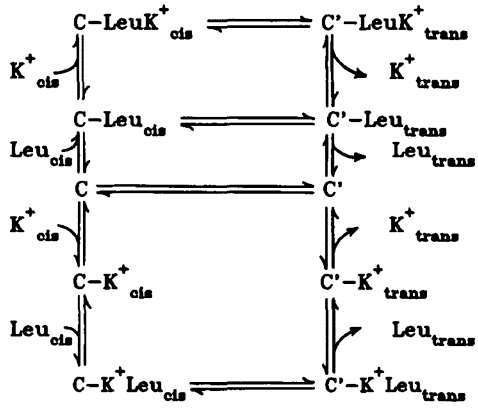

FIG. 7. **Model for the K⁺-dependent transport of leucine into BBMV from the midgut of *P. cynthia* larvae.** *C* represents the neutral amino acid transporter. The fully loaded complex and the partially loaded Leu only form isomerize to release leucine to the *trans* side.

The results obtained with *trans* inhibition experiments confirmed that the kinetic model describing the K^+-leucine cotransport is a random mechanism in which three carrier species, the unloaded, the fully loaded, and the leucine only forms, can undergo isomerization. Even though there is no information about the mobility of the K^+ only form, *trans* inhibition experiments seem to indicate that this form is virtually immobile. This is reasonable in view of the consideration that a significant mobility of the K^+-carrier complex would uncouple the cotransport process.

The pattern of product inhibition summarized in Tables II and III, revealed a mixed-type inhibition both at saturating and at non-saturating *cis* concentrations of substrates. This is the profile expected for an iso-random Bi Bi mechanism (Table IV). The presence of slippage does not modify the pattern of product inhibition, even though, in the formalism introduced by Cleland (1963), the translocation of binary complexes introduces new possible patterns between the carrier form that bind the varied substrate and that binding the product. According to our model (Fig. 7), *trans*-K^+ inhibits leucine uptake by shifting C' to C'K^+ and C'Leu to C'LeuK^+. In doing so, the carrier is locked to the *trans* face of the membrane to reduce the number of carriers available on the *cis* face and, in addition, the dissociation of leucine from C'Leu. *Trans*-K^+ inhibition cannot be significantly relieved by *trans*-leucine, since the amino acid *trans* inhibits the uptake of *cis*-leucine via the binary complex. The inhibitory effect of *trans*-leucine in the absence of *trans*-K^+ may be explained by supposing that the route (Leu_{trans} + C' → C'Leu → CLeu → Leu_{cis} + C) is slower that the rate of C regeneration via the influx route (C' → C). Therefore, binding of leucine at the *trans* side of the membrane decrease the number of carriers available for transport at the *cis* side. Since a *trans* stimulatory effect of leucine on K^+-independent leucine uptake has been previously described in different experimental conditions (Sacchi *et al.*, 1990), these results seem to indicate that pH conditions may have an influence not only on kinetic parameters but also on relative mobilities of the carrier forms.

Our experimental data cannot discriminate between a rapid equilibrium and a steady state random bireactant system. Indeed the two models are indistinguishable from the product inhibition pattern (Table IV), and initial velocity patterns shown in Figs. 2*B* and 3*B* are in agreement with a rapid equilibrium kinetics, although they do not rule out the possibility of the mechanism being steady state (see Gulbinsky and Cleland, 1968). Actually, the discrepancy observed in the calculation of the limiting Michaelis-Menten constants using Equations 1 and 2 on the one hand and Equation 5 on the other may suggest that free carrier, binary complexes and substrates are not at equilibrium. Clearly, in view of the complexity of the random order steady state kinetics, and considering also that routes to ternary complex could be not equally favored, more definite tests are needed to distinguish

which pathway is operative in our brush border membrane preparation.

The results reported in the present paper indicate that absorption of leucine, and possibly of the other neutral amino acid, across the midgut of lepidopteran larvae, is sensitive to the luminal and intracellular concentration of K^+ and leucine. The experimental protocol adopted combines quasi physiological conditions of *cis* and *trans* pH and of $\Delta\psi$. As a matter of fact, in view of the ionic and electrical characteristics of the tissue, the pH gradient and $\Delta\psi$ have a considerable influence on the kinetics of amino acid uptake (Giordana *et al.*, 1985, 1989; Sacchi *et al.*, 1990). Compared to the model depicted for solutes cotransport in vertebrate, our results are consistent with a mixed-type model instead of an affinity type model, which appears to be the mechanism shared by the different Na^+-cotransport systems. Although further studies are requested to clarify if the model proposed for K^+-leucine cotransport fit also to other K^+-dependent amino acid transport agencies, the evolution of a mixed-type model may be a consequence of the most evident feature of the lepidopteran amino acid cotransporter, *i.e.*, the broadening of the cation specificity of the carriers. An improved characterization of the K^+-linked systems may be relevant in understanding the biochemical events associated with the translocation of substances across biological membranes.

Acknowledgments—We thank Drs. Barbara Giordana and Franca Sacchi for valuable comments on the manuscript.

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