Archives of Insect Biochemistry and Physiology 63:110–122 (2006)

Leucine Transport in Brush Border Membrane Vesicles From Freshwater Insect Larvae

Matilde Forcella, Elisa Berra, Roberto Giacchini, and Paolo Parenti*

Leucine transport across brush border membrane vesicles prepared from four insect species common to European freshwater streams has been characterized. The species studied were: *Ephemera danica* (Ephemeroptera: Ephemeridae), *Isoperla grammatica* (Plecoptera: Perlodidae), *Hydropsyche pellucidula* (Trichoptera: Hydropsychidae), and *Hybomitra bimaculata* (Diptera: Tabanidae). The transport differed among the studied taxa for several features, including pH and sodium dependence, substrate affinity and specificity, and efficiency. In *H. pellucidula* and *E. danica*, leucine uptake was higher at pH 7.4 than at more alkaline or acidic pH values, whereas in *I. grammatica* and *H. bimaculata*, the uptake was rather constant when pH varied from 5.0 to 7.4, then strongly decreased at pH 8.8. All but *E. danica* displayed a transient intravescicular leucine accumulation in the presence of sodium, suggesting the existence of a cation-leucine symport mechanism. The sodium dependence ranged according to the following order: *H. pellucidula* > *I. grammatica* > *H. bimaculata* > *E. danica*. Moreover, in *H. pellucidula* and *I. grammatica*, the sodium-dependence was stronger at pH 8.8 than at pH 7.4. In *E. danica*, leucine uptake was sodium-independent at all pH values. The highest value of V_{max} (45.3 pmol·s^{-1}·mg proteins^{-1}) was in *E. danica*, which, however, displayed the lowest affinity (K_m137 μM) when compared to the kinetic parameters of other taxa. The V_{max} and K_m values were: 40 and 52.5, 32.1 and 12.5, and 4.5 and 230 for *H. bimaculata*, *H. pellucidula*, and *I. grammatica*, respectively. The obtained results are discussed within our current knowledge of amino acid transport systems in insects. Arch. Insect Biochem. Physiol. 63:110–122, 2006.

KEYWORDS: leucine transport; kinetics; brush border membranes; freshwater insects

INTRODUCTION

Specific membrane transport proteins are required by insects in order to acquire essential amino acids. Usually, the first step of amino acid absorption in insects is represented by transport from the gut lumen into the cells of the midgut and the hindgut (Wright and Ahearn, 1997). However, uptake of amino acids through epidermis has been described in some species (Tomlin et al., 1993; Giordana et al., 2003). In all instances, amino acid transport is apparently mediated by specific membrane proteins (transporters), which operate as secondary active transport systems (symporters) or mediate a facilitated diffusion (uniporters) (Wolfersberger, 2000). Three kinds of amino acid transporters have been detected in insects: (1) the K^+-dependent system, which has been characterized extensively in several phytophagous lepidopteran larvae (Hanozet et al., 1980; Parenti et al., 1992, 2000; Hennigan et al., 1993; Reuveni and Dunn, 1994; Parthasarathy et al., 1994; Gior-

Department of Environmental Sciences, University of Milano-Bicocca, Milano, Italy

Contract grant sponsor: University of Milano-Bicocca (Progetto F.A.R.).

Abbreviations used: CAPS = 3-cyclohexylamino-1-propansulfonic acid; HEPES = 4-2-hydroxyethyl)-1-piperazineethansulfonic acid; MES = 4-morpholineethansulfonic acid; TMA = tetramethylammonium.

*Correspondence to: Prof. Paolo Parenti, Dipartimento di Scienze dell'Ambiente e del Territorio, Università di Milano-Bicocca, Piazza della Scienza, 1, 20126 Milano, Italy. E-mail: paolo.parenti@unimib.it

Received 20 December 2005; Accepted 17 June 2006

DOI: 10.1002/arch.20147
Published online in Wiley InterScience (www.interscience.wiley.com)

dana and Parenti, 1994; Giordana et al., 2002; Castagna et al. 2002) and in the sucker bug *Dysdicercus peruvianus* (Hemiptera) (Silva and Terra, 1994); (2) the Na^+-dependent system, which is present in the midge *Chironomus riparius* (Parenti et al., 2001), in *Locusta migratoria* (P. Parenti, unpublished data), in the cockroach *Blabera gigantea* (Parenti et al., 1986), and in the colorado beetle *Leptinotarsa decemlineata* (Hong et al., 1995); and (3) the cation-independent system, which has been detected again in the coleopteran *L. decemlineata* (Reuveni et al., 1993; Neal et al., 1996), and in the honeybee *Apis mellifera* (Haszonits and Crailsheim, 1990). With the exception of *C. riparius*, all taxa cited above represent terrestrial species.

Freshwater insects are integral and important food web components of most stream ecosystems throughout the world; they exploit food resources with widely different nutritive characteristics, and they play a significant role in nutrient cycling in streams and lakes (Resh and Rosenberg, 1984). However, the physiology of digestion in aquatic insects has received comparably little attention, usually focused on digestive enzymes (Martin et al., 1980; Kesler, 1982; Grant and Minshall, 1984). With the exception of midge larvae, to our knowledge there is no information about molecular physiology of membrane transport in freshwater insects midgut. The aim of this work is to address this gap.

MATERIALS AND METHODS

Animals

Caddisfly larvae belonging to *Hydropsyche pellucidula* (Curtis, 1834) (Trichoptera: Hydropsychidae), stonefly nymphs of the species *Isoperla grammatica* (Poda, 1761) (Plecoptera: Perlodidae), mayfly nymphs of the species *Ephemera danica* (Müller, 1764) (Ephemeroptera: Ephemeridae), and horse fly larvae of the species *Hybomitra bimaculata* (Macquart, 1826) (Diptera: Tabanidae) were used. They were collected in the wild from sites of the Ticino and Taro rivers, northern Italy, and maintained in large vessels in a 2-cm water layer at 4°C and reared with TetraMin® purchased from a local aquarium fish dealer. Collection was made in spring to autumn and, as several individuals were used for each experiment, attention was paid to gather specimens of the same size. As a control, second instar larvae of the terrestrial lepidopteran *Philosamia cynthia* collected in several localities south of Milano were also used in preliminary experiments.

Chemicals

L-[4,5-^{3}H]-leucine 152 Ci/mmol was purchased from Amersham International plc, U.K. Amino acids and derivatives and all other reagents of analytical grade were from Sigma-Aldrich Italia.

Membrane Preparation

Membrane vesicles were prepared from either fresh larvae or nymphs using the protocol described by Wolfersberger et al. (1987), with minor modifications. Briefly, whole larvae or nymphs (roughly 3 g in each experiment) were suspended with 9 volumes of a hypotonic Tris buffer (Tris-HCl 17 mM, pH 7.4, mannitol 100 mM, EGTA 5 mM) and homogenized at 4°C in a Waring-mixer, set at 11,400 rpm, for two 2-min periods, separated by a 4-min cooling interval. The suspension was then filtered over two layers of surgical gauze and represented the homogenate, from which 1-ml aliquots were withdrawn for enzyme assays. An equal volume of 24 mM $MgCl_2$ solution was added to the homogenate. The resulting mixture was thoroughly stirred for 15 min in an ice bath and then centrifuged at 2,500*g* for 15 min at 4°C. The resulting pellet was discarded and the supernatant centrifuged at 48,000*g* for 20 min at 4°C. The pellet was resuspended in 0.5 homogenate volume of ice cold Tris buffer and homogenized in a glass and Teflon Potter-Elvehjem with two cycles of 6 strokes at 2,000 rpm, separated by 1 min in ice. An equal volume of 24 mM $MgCl_2$ was added, the mixture stirred for 15 min in an ice bath, and centrifuged at low speed and then at high speed as above. The final pellet was resuspended in 1 vol-

ume homogenate buffer, homogenized in Potter-Helvehjem as before, and centrifuged again at 48,000*g* for 20 min at 4°C. The obtained pellet was resuspended in HEPES-Tris 10 mM, pH 7.4, mannitol 100 mM at 2–3 mg/ml of protein as determined according to Bradford (1976), using bovine serum albumin as standard. The suspension represented the membrane preparation used for transport experiments. The recovery was about 0.6 ± 0.1 mg membrane proteins/g wet fresh larvae. The purity of the membrane preparation was assessed routinely by measuring the leucine aminopeptidase activity according to Tuppy et al. (1962). Cytochrome c oxidase was assayed according to Smith (1955). Enzyme assays were performed at 25°C in a Cary3 Spectrophotometer, recorded on a personal computer, and analysed by the Cary Win UV application software for Windows2000.

Amino Acid Transport Measurements

Tracer experiments were performed by incubating one volume of vesicles and four volumes of a radioactive cocktail buffered at different final pH values, containing 30 µCi/ml L-[^{3}H]leucine and the substances specified in the figure legends. Uptakes were carried out according to the rapid filtration technique as described elsewhere (Giordana et al., 1982).

Calculations

Experimental data on uptake measurements are usually given as nmol/15 s/mg proteins, except for time course experiments in which the uptake was followed over 60 min. Kinetics were analysed by computer and plotted using a multiparameter, iterative, non-linear regression program based on the Marquardt-Levenberg algorithm (Sigma Plot 6.0, Jandel, CA). The calculated constants are given as ± S.D.

pH Measurements In Vivo

Estimation of the lumen pH was performed as described by Zhuang et al. (1999). Briefly, single larvae were dissected alive on a microscopic slide, the intestine was isolated and examined under a Leica stereomicroscope adding one of the following indicators (in powder to not dilute body fluids): phenolphthalein, thymol blue, and cresol red. Segments were compared visually with standards prepared in steps of approximately 0.5 pH units.

RESULTS

The activity of leucine aminopeptidase, a typical brush border marker enzyme, as measured in membrane preparations obtained from *E. danica*, *I. grammatica*, *H. pellucidula*, and *H. bimaculata* is reported in Table 1. In all preparations, the enrichment factor was significantly higher than 1, indicating that the obtained subcellular fractions were mainly constituted by brush border membranes. An assay of cytochrome c oxidase in the same preparations revealed a significantly lower specific activity compared to that measured in crude extracts. These data agree with low levels of cross-contamination by other kinds of cell membranes.

The first step for the characterization of amino acid transport was the evaluation of the pH dependence of the initial rate of leucine uptake. Using a standard incubation medium containing 100 mM NaCl, the uptake of 0.01 mM L-leucine was measured at pH values ranging from 5.0 through 8.8. Results are plotted in Figure 1. As shown, the uptake rate was high at slightly alkaline pH (pH 7.4), for *E. danica* (33.0 pmol · 15s^{-1} · mg protein^{-1}) and *H. pellucidula* (325 pmol · 15s^{-1} · mg protein^{-1}), whereas the optimum uptake rate for *H. bimaculata* (109 pmol · 15s^{-1} · mg protein^{-1}) and *I. grammatica* (3.9 pmol · 15s^{-1} · mg protein^{-1}) was observed at pH 5.0. In vivo measurements of lumen pH in single larvae emphasised, in most instances, an alkaline pH in the midgut, more clearly observable in the anterior midgut of *E. danica* and *H. pellucidula* with values in the range 7.5–8.5. When cresol red + thymol blue came in contact with the inner content of the anterior midgut, the yellow colour suddenly changed to a deep violet denoting a pH above 8.3. The phenomenon, more marked in the anterior part of the midgut, was also observable in the

TABLE 1. Enzyme Activity in Membrane Preparations From Freshwater Insect Larvae*

		Enzyme activity					
		Homogenate (H)		Membrane vesicles (V)		Purification	Yield
Species	Enzyme	Specific	Total	Specific	Total	(V/H)	(%)
E. danica	*LAP*	122 ± 12	9826	762 ± 59	409	6.2	4.1
	Cyt c OX	22.5 ± 1.5	1771	1.1 ± 0.1	0.59	0.05	—
I. grammatica	*LAP*	18 ± 4	2860	147 ± 12	65	8.8	2.3
	Cyt c OX	4.5 ± 0.8	715	0.25 ± 0.01	0.11	0.06	—
H. pellucidula	*LAP*	5 ± 2	299	151 ± 9	21	30.2	6.9
	Cyt c OX	1.2 ± 0.2	71.8	0.08 ± 0.01	0.011	0.07	—
H. bimaculata	*LAP*	42 ± 5	7333	1055 ± 22	643	25.4	8.8
	Cyt c OX	12.8 ± 1.0	2234	0.75 ± 0.02	0.46	0.06	—

*The specific activities of leucine aminopeptidase (LAP) and cytochrome c oxidase (Cyt cOX) were determined as described in Materials and Methods and are expressed as nmol/min/mg protein. Total activities are calculated on the basis of the whole volume of homogenate (18–20 ml) and vesicle suspension (0.8–1.0 ml). The yield was calculated from the membrane marker enzyme leucine aminopeptidase. Numbers are mean ± S.E. of triplicates of a typical membrane preparation.

middle part. The colour change was not evident in *I. grammatica* and *H. bimaculata*.

The extent of sodium dependence of leucine uptake is illustrated in Figure 2. Aliquots of membrane vesicles were incubated in the presence of either 100 mM Na^+ or 100 mM K^+, accompanied by anions known to have different mobility across plasma membranes ($SCN^- > Cl^- > SO_4^{2-}$). This generated a series of transmembrane electrochemical gradient of different magnitude. Control uptake was performed in the presence of 100 mM mannitol or 100 mM tetramethylammonium sulphate. As shown in Figure 2, in all instances, the uptake rate was lower in the presence of potassium than in the presence of sodium. However, the sodium dependence ranged from high to very low, according to the following order: *H. pellucidula* > *I. grammatica* >> *H. bimaculata* > *E. danica*. Actually, the leucine transport into BBMV from *E. danica* should be regarded as cation independent. In this species, the value of leucine uptake in the absence of added salt was almost identical to that measured in the presence of sodium. The lower uptake rate obtained when sodium was substituted with potassium was interpreted as an inhibitory effect of potassium. Such an effect was not observed in the other three populations of membrane vesicles. All preparations shared a higher uptake rate in the presence of NaSCN. *Isoperla grammatica* and *H. pellucidula* were the species in which this effect was more pronounced compared to *H. bimaculata* and *E. danica* (compare NaSCN vs. Na_2SO_4).

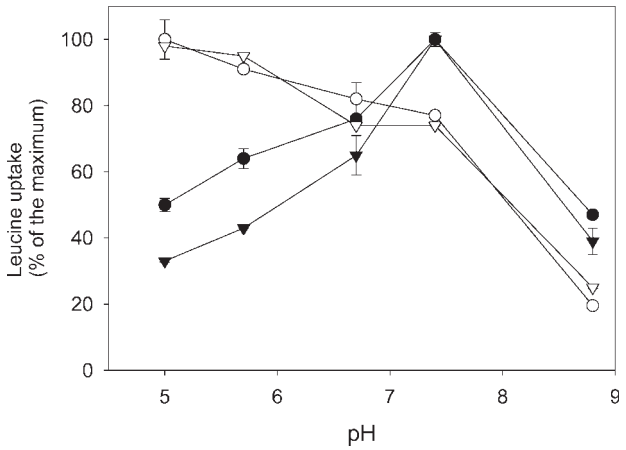

Fig. 1. Effect of external pH on initial rate of leucine uptake into BBMV from freshwater larvae. Membrane vesicles from *E. danica* (solid circles), *I. grammatica* (open circles), *H. pellucidula* (solid triangles), and *H. bimaculata* (open triangles) were prepared at pH 7.4 and diluted in buffers at the indicated pH values adjusted using concentrated Hepes-Tris solution for neutral and alkaline pH and Mes-Tris for acidic pH. Buffer final concentration was 20 mM. Uptake solution contained 0.01 mM ^{3}[H]-L-leucine and 100 mM NaCl. Data represent means ± S.E. of a typical experiment made in triplicate.

As shown above, the uptake rate was either decreased or increased when medium pH was shifted from 7.4 to 8.8. This stimulated our interest in investigating whether or not alkaline pH affects the sodium-dependent component of the total uptake. The reduction of total leucine uptake ranged from 31% (*H. bimaculata*) to 63% (*E. danica*) (see leg-

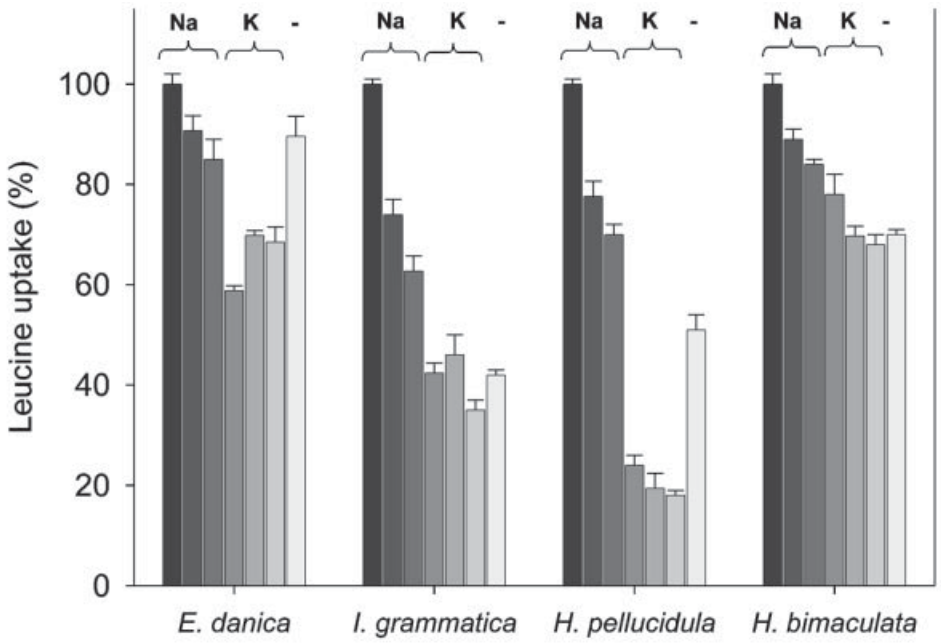

Fig. 2. Ion dependence of leucine uptake into BBMV from freshwater larvae. Membrane vesicles from *E. danica, I. grammatica, H. pellucidula,* and *H. bimaculata* were prepared at pH 7.4 and diluted in 10 mM Hepes-Tris, pH 7.4, containing 100 mM (as cation) of the following salts (from dark gray to white): NaSCN, NaCl, Na_2SO_4, KSCN, KCl, K_2SO_4, TMA_2SO_4. Uptake was measured at 0.01 mM ^{3}[H]-L-leucine and expressed as percent of maximal uptake rate observed in the presence of NaSCN. The maximal uptake rates were (nmol $\cdot$ 15s^{-1} $\cdot$ mg proteins^{-1}): 26.4 ± 1.7, 2.7 ± 0.3, 267 ± 16, and 115 ± 17 for *E. danica, I. grammatica, H. pellucidula,* and *H. bimaculata,* respectively.

ends for Figures 2 and 3 for uptake values) with differences among species (Fig. 3). In *H. pellucidula* and *I. grammatica*, the sodium-dependence was stronger at pH 8.8 than at pH 7.4. Specifically, in *I. grammatica* the sodium-independent component was drastically reduced from 42 to 10%, whereas in *H. pellucidula* it remained almost unchanged, slightly decreasing from 51 to 45%. In contrast, no changes were measured in *E. danica*, which remains sodium-independent at all pH values, including alkaline and acidic pH values (this latter not shown). The opposite effect was observed in *H. bimaculata*, which switched toward a cation-independent system.

To confirm the presence of ion-driven leucine uptake, time course experiments were established for each species. A membrane preparation obtained from whole second instar larvae of the lepidopteran species *Philosamia cynthia* was added as control. As illustrated in Figure 4, all but *E. danica* displayed a transient intravesicular leucine accumulation in the presence of either sodium or potassium according to species, suggesting the existence in these species of a cation-leucine symport mechanism. Moreover, the result obtained with *P. cynthia* larvae, which were superimposable with those reported in the literature with the midgut as starting material, confirmed that subcellular preparations obtained from whole animals using the Mg-precipitation method were suitable for transport experiments.

Next, the kinetics of L-leucine uptake as a function of leucine concentration was studied. The mayfly *E. danica* and the caddisfly *H. pellucidula* displayed complex saturation kinetics in which two components could be separated, a high-affinity component and a low-affinity component apparently indistinguishable from simple diffusion (Fig. 5A,C). Deviation from simple Henri-Michaelis-Menten kinetics can be also emphasized by plotting an initial rate according to Eadie-Hofstee linearization (Fig. 5A,C, inset). An upward curvature in the linear plot is diagnostic for the pres-

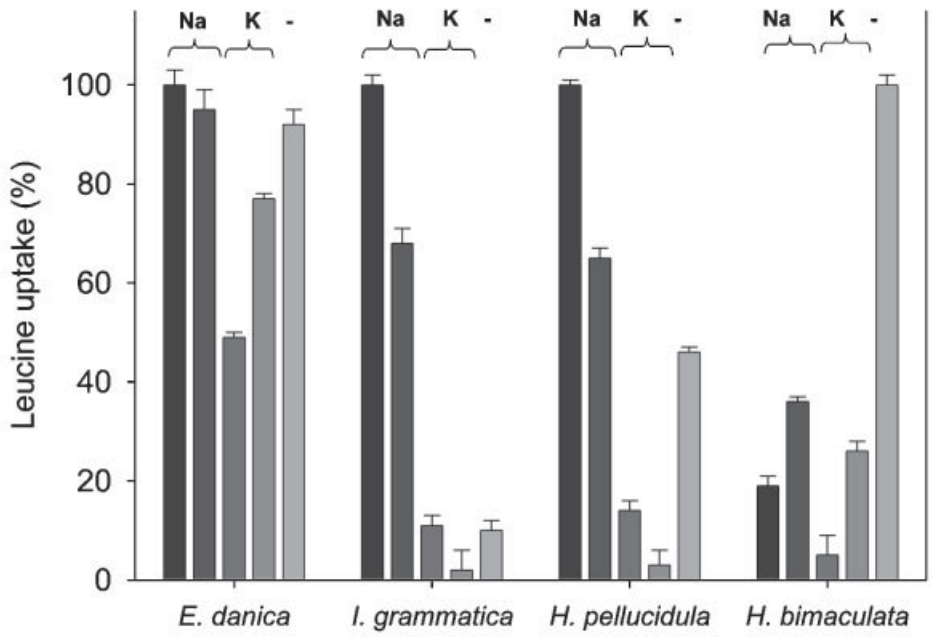

Fig. 3. Sodium-dependent leucine uptake at alkaline pH. Membrane vesicles from *E. danica, I. grammatica, H. pellucidula,* and *H. bimaculata* were prepared at pH 7.4 and diluted in 50 mM Tris, pH 8.8 (final pH), containing 0.01 mM ^{3}[H]-L-leucine and 100 mM (as cation) of the following salts (from dark gray to white): NaSCN, NaCl, KSCN, KCl, TMA_2SO_4. Uptake was expressed as percent of maximal uptake rate, which was observed in the presence of NaSCN except in *H. bimaculata,* which was measured without salt added. The maximal uptake rates were (nmol/15s/mg proteins): 8.8 ± 0.2, 1.8 ± 0.1, 167 ± 21, and 78 ± 10 for *E. danica, I. grammatica, H. pellucidula,* and *H. bimaculata,* respectively.

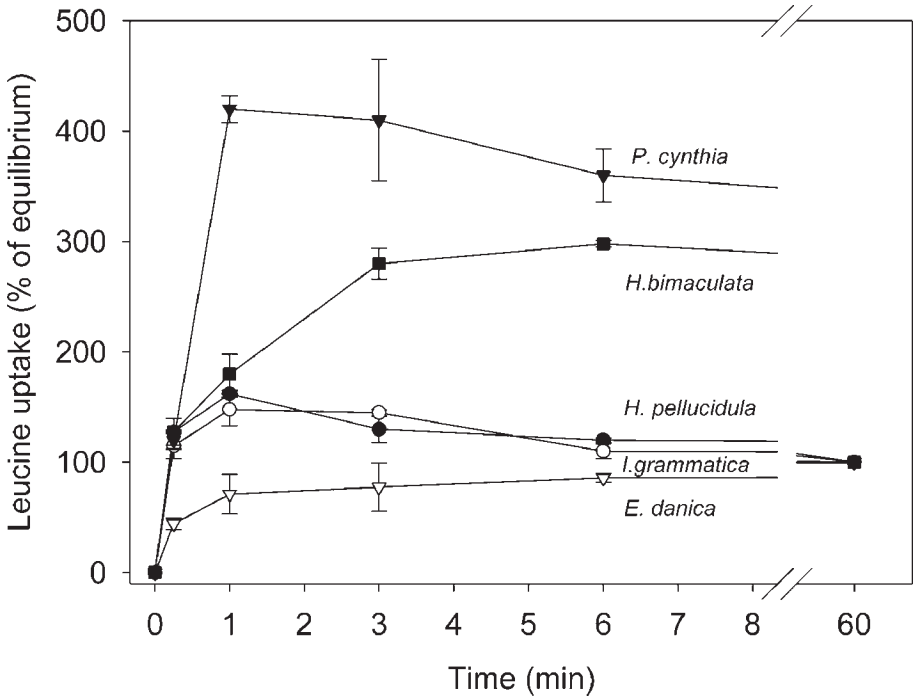

Fig. 4. Time course of leucine uptake in insect larvae. Membrane vesicles from the indicated species were prepared in 10 mM Hepes-Tris, pH 7.4, mannitol 100 mM, and diluted 1:4 in the same buffer containing 100 mM NaCl and 0.01 mM ^{3}[H]-L-leucine, except that for *P. cynthia* in which NaCl was substituted with KCl. At the selected time, 20 µl of the mixture was withdrawn and diluted in ice-cold 150 mM NaCl, 1 mM Hepes-Tris, pH 7.4, and filtered as reported in Materials and Methods. Uptake is expressed as percent of the equilibrium value obtained for each curve.

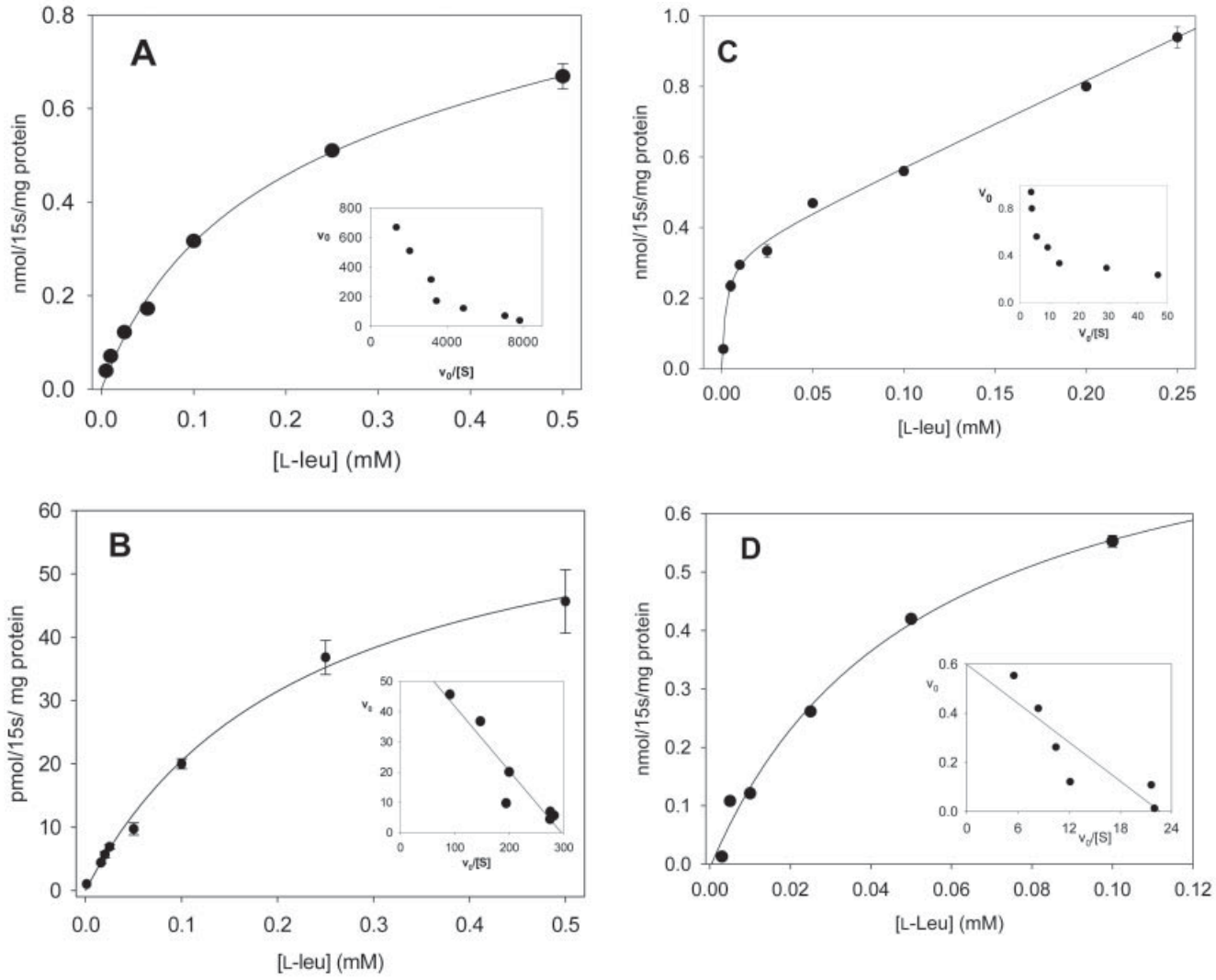

Fig. 5. Initial rate of leucine uptake as a function of leucine concentration. Membrane vesicles were incubated in the presence of the indicated leucine concentrations and the initial rate was measured at pH 7.4 and in the presence of 100 NaCl. Measurements were performed in quadruplicate. The insect represents an Eadie-Hofstee plot of the original data in the main panel. **A:** *E. danica*; **B:** *I. grammatica*; **C:** *H. pellucidula*; **D:** *H. bimaculata*.

ence of two systems, which are involved in the same reaction with different affinity and maximal velocity. The curvature was not evident in *I. grammatica* and *H. bimaculata* (Fig. 5B, D, inset). Using a non-linear iterative algorithm as specified in Materials and Methods, experimental data obtained for *E. danica* and *H. pellucidula* were fitted to the following equation:

$$v_0 = V_{max} \cdot [Leu]/(K_{leu} + [Leu]) + C \cdot [Leu] \quad (1)$$

where v_0 is the initial rate of leucine uptake; V_{max}, the maximal initial rate; K_{leu} is the Michaelis-Menten constant for leucine; and C is the diffusional component in min^{-1}. The uptake rate in *I. grammatica* and *H. bimaculata* followed a simple Henri-Michaelis-Menten equation and data were fitted to the following equation:

$$v_0 = V_{max} \cdot [Leu]/(K_{leu} + [Leu]) \quad (2)$$

Kinetic parameters are reported in Table 2. The highest value of V_{max} was obtained in an *E. danica* membrane preparation, which, however, displayed the lowest affinity (high K_m) compared to the other taxa. The V_{max}/K_m, which is a measure of the catalytic

TABLE 2. Kinetic Parameters of Leucine Uptake Into BBMV From Freshwater Insect Larvae*

Species	V_{max}	K_m	C	V_{max}/K_m
E. danica	45.3 ± 2.8	137 ± 5	4.6 ± 0.8	0.33
I. grammatica	4.5 ± 0.4	230 ± 15	—	0.02
H. pellucidula	32.1 ± 1.0	12.5 ± 0.7	24.1 ± 0.9	2.58
H. bimaculata	40.0 ± 0.4	52.5 ± 0.8	—	0.76

*Parameters were calculated by weighted non-linear regression analysis using the equations reported in the text and the experimental data shown in Figure 3. Maximal velocity (V_{max}) is expressed in pmol·s^{-1}·mg protein^{-1}; affinity constant (K_m) in μmol·L^{-1}; diffusional component (C) in s^{-1}; catalytic efficiency (V_{max}/K_m) normalized for protein concentration is in s^{-1}.

efficiency, is highest in *H. pellucidula* due to high V_{max} and very low K_m. The ratio decreased in the horse fly larva *H. bimaculata* and then again in *E. danica* due to an increased K_m value. *Isoperla grammatica*, having the highest K_m value and a relatively low V_{max} , displayed the lowest catalytic efficiency.

The last set of experiments was to study the substrate specificity. Vesicles were incubated in the presence of a leucine concentration close to the K_m value, as determined above for each species, and a 20-fold excess of a number of amino acids and analogues. The initial rate was measured and results were reported as a percent of control without inhibitor (Table 3). In all species, leucine uptake was strongly inhibited by hydrophobic amino acids and barely affected by glycine (except *E. danica*), basic and acidic amino acids. Differences, however, were observed in the presence of proline, oxoproline, β-alanine, the analogues BCH and acivicin, and the leucine esters. In particular, the transport system in *E. danica* was characterized by the broadest specificity; the system of *I. grammatica* was strongly inhibited by proline, but was not inhibited by the bulky substrate BCH, which had a slight stimulatory effect; in *H. pellucidula*, an apparent stimulation of uptake rate was observed in the presence of leucine esters, proline, and β-alanine; all but *H. pellucidula* are strongly inhibited by acivicin, an histidine analogue. Data obtained under the same experimental condition from membrane vesicles of the freshwater midge *C. riparius* taken from Parenti et al. (2001) are included for comparison.

TABLE 3. Inhibition of Leucine Uptake Into BBMV From Freshwater Insect Larvae*

	Residual activity				
Inhibitor	*Ephemera danica*	*Hydropsyche pellucidula*	*Isoperla grammatica*	*Hybomitra bimaculata*	*Chironomus riparius*[a]
L-leucine	18.9	15.5	26.0	24.5	23
L-alanine	36.0	25.3	27.2	32.0	30
L-phenylalanine	26.7	23.0	27.5	22.0	71
L-methionine	19.7	39.3	25.5	25.5	22
Glycine	47.2	73.0	74.8	69.0	122
L-lysine	87.1	98.0	121.5	112.0	93
L-glutamate	82.5	104.8	87.0	97.0	n.d.
L-proline	61.1	143.5	35.5	95.5	118
Oxoproline	64.0	123.0	59.0	88.3	n.d.
Beta-alanine	79.2	137.6	106.0	82.0	117
BCH	54.2	51.5	143.5	61.0	63
Acivicin	34.3	78.4	38.0	27.5	85
L-leuOMe	66.9	117.5	90.0	95.5	96
L-leuOEt	65.5	137.5	68.5	98.2	n.d.

*Leucine uptake was measured at ph 7.4 in the presence of a 20-fold excess of the indicated inhibitor after 15 sec of incubation and at the K_m value as determined from leucine kinetics and reported in Table 2. Results are expressed as percent of a control in the presence of mannitol.

Acivicin, a-amino-3-chloro-4,5-dihydro-5-isoxazolacetic acid; BCH, 2-amino-2-norbornanecarboxylic acid; leuOMe, leucine methyl ester; leuOEt, leucine ethyl ester; n.d., no data.

[a]Data from Parenti et al. (2001).

DISCUSSION

For two decades, brush border membranes prepared from isolated midgut of relatively large lepidopteran larvae have proved invaluable tools for several in vitro studies, including transport studies and receptor binding studies (Wolfersberger, 2000). Small insects have been largely neglected and protocols from whole animals have been developed for binding studies (Abdul-Rauf and Ellar, 1999). Recently, we have successfully employed a procedure based on Mg^{2+} precipitation for the isolation of brush border membranes from whole larvae of the midge *C. riparius*. The preparation was constituted mostly of closed vesicles of midgut origin and has been demonstrated suitable for transport experiments (Parenti et al., 2001). In this report, the same procedure has been applied to four freshwater insect larvae, chosen as representative of the most ecologically diverse groups of benthic insects (Resh and Rosenberg, 1984). To further support the trustworthiness of our membrane preparations as a tool for solute transport experiments, the protocol was applied to larvae of similar size (second instar) of a phytophagous lepidopteran species

largely used in the last decade to characterize midgut amino acid transport (Parenti et al., 1992; Sacchi et al., 1994). As shown in Figure 4, *P. cynthia* membrane preparation displayed a large, potassium-driven overshoot (i.e., transient accumulation) of the labelled amino acid, whose magnitude was close to that usually obtained from typical brush border membranes purified from isolated midgut.

All freshwater species included in this study spend the larval stage of their life cycle strictly associated with sediment of rivers and lakes, where they occupy different positions along the trophic chain. Caddisfly larvae (Hydropsychidae), here represented by the species *H. pellucidula*, are primary consumers and one of the most common filter-feeding insect group in streams. Mayfly nymphs (Ephemeridae), here represented by *E. danica*, and horse fly larvae, *H. bimaculata* (Tabanidae), are deposit collectors, which is another category of primary consumers, and occasionally feed on small invertebrates. Stonefly nymphs of the species *I. grammatica* (Perlodidae) are predaceous. The gut of many primary consumers is well adapted to hydrolyse cellulose and other carbohydrates for the presence of several cellulases and glycosidases (Lambert and Moore, 1984), whereas the digestion of protein is promoted by a battery of endo- and exopeptidases (Terra and Ferreira, 1994). In insects, net absorption of amino acid through the brush border is highly sensitive to lumen pH and, in most instances, it is largely stimulated by alkaline to strongly alkaline pH (Giordana et al., 1998; Parenti et al., 2001). In *H. pellucidula* and *E. danica* leucine, uptake is higher at pH 7.4 than at more alkaline or acidic pH values, whereas in *I. grammatica* and *H. bimaculata*, the uptake is rather constant when pH was varied from 5.0 to 7.4, with slightly higher activity at pH 5.0 and a strong decrease at alkaline values. This result was in agreement with our pH measurements in vivo. Data indicate considerable variations among freshwater larvae and these could be related to different protein content of the diet: when protein are the primary food material the lumen, pH tends to be slightly acidic as in the cockroach *Periplaneta americana* (O'Riordan, 1969).

Kinetic parameters of leucine uptake into brush border membrane vesicles indicate a different transport efficiency among the studied taxa. Assimilation efficiency of nutrients in primary consumers has been studied in several species and has been found highly variable (Lamberti and Moore, 1984). In particular, in filter-feeding caddisflies it is low (Bencke and Wallace, 1980). Data reported in Table 2 seem to disagree with such a feature, as *H. pellucidula* showed the highest uptake rate compared to *E. danica, H. bimaculata* (Table 2), and *C. riparius* (Parenti et al., 2001). However, extrapolation to an in vivo situation is complicated since assimilation efficiency depends on type of food, total amount of food eaten per day (which may exceed the body weigh), and food retention time, which may vary from less than 10 min up to several hours (Lamberti and Moore, 1984). On the other hand, an in vitro uptake assay with leucine as a tracer allows us comparisons with transport systems described in the literature. For example, kinetic parameters summarized in Table 2 show that *H. pellucidula* shares with the closely related order of Lepidoptera the high efficiency typical of cation-dependent symport mechanisms. Based on kinetic parameters previously reported for *P. cynthia* larvae (Parenti et al., 1992), the calculated catalytic efficiency V_{max}/K_m in *H. pellucidula* is only 6.7-fold lower than that measured under similar experimental conditions in Lepidoptera. Close values of catalytic efficiency were found for the dipterans *H. bimaculata* (0.76 s^{-1}) and *C. riparius* (0.62 s^{-1}), this latter calculated from Parenti et al. (2001). Comparison between sodium-independent systems, as resulted from experiments shown here on the mayfly *E. danica* and those reported in the potato beetle *Leptinotarsa decemlineata* (Reuveni et al. 1993), revealed strong differences: a value of 0.33 s^{-1} is found here for the former and 0.02 s^{-1} can be calculated for the latter. In transport study, catalytic efficiency is affected not only by the intrinsic structural properties of the transport protein, but also by the general permeability of the membrane. This means that leaky vesicles uncouple cation-driven symport mechanisms and, as a consequence, reduce uptake rate. That the observed differences in catalytic efficiencies here reported could be due

to an artefact introduced during membrane preparation is apparently ruled out by results obtained with *P. cynthia* larvae (Fig. 4).

In another set of experiments we observed that leucine uptake, with the exception of *E. danica*, was affected by the magnitude of the transmembrane electrical potential and by sodium ions. Uptake was higher when sodium is accompanied by the anion thiocyanate, compared to chloride and sulphate. Thiocyanate has a higher permeability than chloride and sulphate, thus raising membrane potential. In *I. grammatica* and *H. pellucidula* the stimulatory effect of sodium was enhanced at pH 8.8 of the incubation medium. This can be due to a reduced competition of sodium cation with protons. Uptake rates at alkaline pH, however, were reduced to 35–45% of the rate measured at pH 7.4. In this feature, these sodium-dependent systems seem to be different from other cation-dependent transport systems described in insect larvae, such as the sodium-dependent leucine transport of *C. riparius* (Parenti et al, 2001) and the potassium-dependent lepidopteran system (Giordana et al., 1998), both of them strongly activated by an alkaline pH in the medium.

Inhibition experiments in the presence of excess of amino acids or analogues revealed the existence in all but *E. danica* of a transport system compatible with the sodium-dependent so-called B° system. The inhibition pattern found in the mayfly *E. danica* appeared closest to the sodium-independent system-L, functionally characterized in mammalian plasma membranes (Palacin et al., 1998). The first group could be further divided in two on the basis of the effect of the bulky analogue BCH, which was not accepted in *I. grammatica*, whereas it was in *H. pellucidula* and *H. bimaculata*. Therefore, the system in these two latter species appears again much closer to that known for lepidopteran and dipteran larvae. The idea that the system in *I. grammatica* could be a variant of the typical B° system is supported by the inhibition of proline and oxoproline, a feature of the so-called mammalian IMINO carrier. BCH inhibited leucine uptake in *E. danica* as expected for a typical system-L. The lacking of inhibition by cationic and anionic amino acids supports the presence of a B° transport system.

Finally, in order to give a comprehensive picture of the state of the art of intestinal amino acid

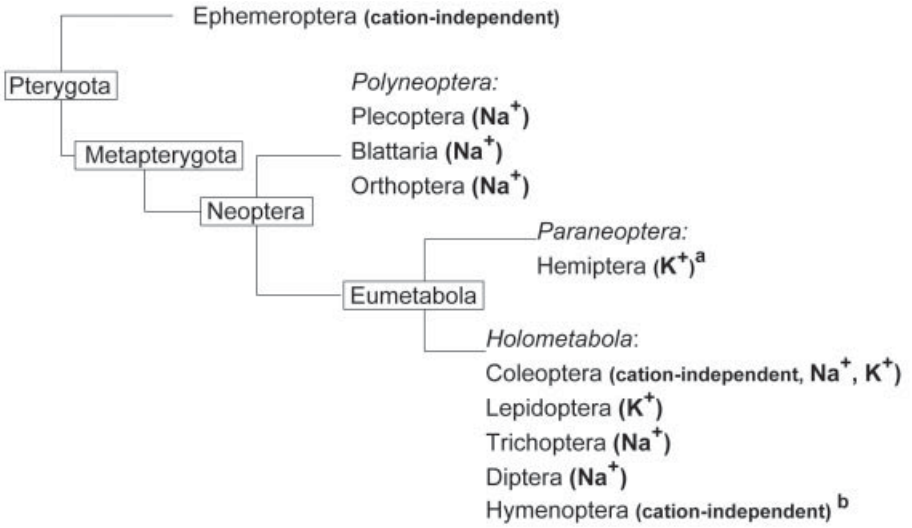

Fig. 6. Summary of our present knowledge of amino acid transport in insects. The cladogram reports taxa for which data on intestinal amino acid transport are available. The majority of the results came from studies on brush border membrane vesicles. Data on Hemiptera (**a**) and Hymenoptera (**b**) have been obtained in isolated midgut and isolated enterocytes, respectively. Appropriate references are listed in the Introduction but see Discussion for further details. The phylogenetic tree is based on Wheeler et al. (2001).

transport in insects, we have put all data available in a cladogram of interrelationship among extant insect taxa as recently proposed by Wheeler et al. (2001). Albeit incomplete, the scheme illustrated in Figure 6 shows that most taxa have developed secondary active Na^+-dependent transport mechanisms. This is compatible with high-Na^+ diets and the presence of Na^+, K^+-ATPase in the midgut cell basal membrane. Lepidoptera larvae have evolved a potassium-dependent transport system, consistent with the K^+-rich plant diet. They lack a typical Na^+, K^+-ATPase and they express another important ion pump, the H^+, K^+-ATPase, which is primarily responsible for maintenance of the ion gradient needed for net absorption of essential and nonessential amino acids (Giordana et al., 1998). This system is regarded as a variant of the sodium-dependent symport, as supported by its ability to use sodium in vitro, though with less efficiency (Giordana et al, 1982; Sacchi et al., 1994). In Hemiptera, the role of cations in solute transport has been studied in the cotton seed sucker bug *D. peruvianus* using isolated midgut (Silva and Terra, 1994): leucine uptake is strongly inhibited by sodium and is affected by the presence of potassium. In Coleoptera, there are both cation-dependent and cation-independent transport systems. In particular, the uptake of leucine (Reuveni et al., 1993) and histidine (Neal et al., 1996) is cation-independent, whereas tyrosine uptake is stimulated by sodium and, to a lesser extent, by potassium (Hong et al., 1995). Cation-independent amino acid transports, also called uniporters, are present in several lineages of the Eumetabola, including Coleoptera, Hymenoptera, and Lepidoptera (Leonardi et al., 1998) and it is demonstrated here for the first time also in basal Pterygota, the Ephemeroptera.

Assuming that the cation-independent system is plesiomorphic, it remains unclear which was the evolutionary advantage to revert from sodium dependence to cation independence in Eumetabola. The utilization by the colorado beetle *L. decemlineata* of both ion-dependent and ion-independent pathways has been claimed comparable to mammals that use both Na^+-stimulated and Na^+-independent amino acid symporters (Neal et al., 1996). Probably a generalization is presently still too speculative and many more species should be investigated. A reason, however, could be found in the larger energy cost of secondary active mechanisms compared to uniporters. Actually, the uniporter may guarantee efficient absorption of solutes if some sort of biochemical trapping mechanism exists (e.g., transamination for amino acids or phosphorylation for glucose). Elucidation of these aspects is currently in progress in our laboratory.

LITERATURE CITED

Abduf-Rauf M, Ellar DJ. 1999. Isolation and characterization of brush border membrane vesicles from whole *Aedes aegypti* larvae. J Invert Pathol 73:45–51.

Bencke AC, Wallace JB. 1980. Trophic basic of production among net-spinning caddisflies in a southern Appalachian stream. Ecology 61:108–118.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of protein utilizing the principle of dye-protein binding. Anal Biochem 72:248–254.

Castagna M, Vincenti S, Marciani P, Sacchi VF. 2002. Inhibition of the lepidopteran amino acid co-transporter KAAT1 by phenylglyoxal: role of arginine 76. Insect Mol Biol 11:283–289.

Giordana B, Parenti P. 1994. Determinants for the activity of the neutral amino acid/K^+ symport in lepidopteran larval midgut. J Exp Biol 196:145–155.

Giordana B, Sacchi VF, Hanozet GM. 1982. Intestinal amino acid absorption in lepidopteran larvae. Biochim Biophys Acta 692:81–88.

Giordana B, Leonardi MG, Casartelli M, Consonni P, Parenti P. 1998. K+/neutral amino acid symport of *Bombyx mori* larval midgut operates in extreme environmental conditions. Am J Physiol 274:R1361–R1371.

Giordana B, Forcella M, Leonardi MG, Casartelli M, Fiandra L, Hanozet GM, Parenti P. 2002. A novel regulatory mechanism for amino acid absorption in lepidopteran larval midgut. J Insect Physiol 48:585–592.

Giordana B, Milani A, Grimaldi A, Farneti R, Casartelli M, Ambrosecchio MR, Digilio MC, Leonardi MG, de Eguileor

M, Pennacchio F. 2003. Absorption of sugars and amino acids by the epidermis of *Aphidius ervi* larvae. J Insect Physiol 49:1115–1124.

Grant DF, Minshall GW. 1984. Intestinal protein dynamics in the stonefly *Pteronarcys californica*. J Insect Physiol 30:441–449.

Hanozet GM, Giordana B, Sacchi VF. 1980. K^+-dependent phenylalanine uptake in membrane vesicles isolated from the midgut of *Philosamia cynthia* larvae. Biochim Biophys Acta 596:481–486.

Haszonits O, Crailsheim K. 1990. Uptake of L-leucine into isolated enterocytes of the honeybee *Apis mellifera* L. depending on season. J Insect Physiol 36:835–842.

Hennigan BB, Wolfersberger MG, Harvey WR. 1993. Neutral amino acid symport in larval *Manduca sexta* midgut brush border membrane vesicles deduced cation-dependent uptake of leucine alanine and phenylalanine. Biochim Biophys Acta 1148:216–222.

Hong YS, Reuveni M, Neal JJ. 1995. A sodium- and potassium-stimulated tyrosine transporter from *Leptinotarsa decemlineata* midguts. J Insect Physiol 41:527–533.

Kesler DH. 1982. Cellulase activity in four species of aquatic insect larvae in Rhode Island USA. J Freshwater Ecol 1:559–562.

Lamberti GA, Moore JW. 1984. Aquatic insects as primary consumers. In: Resh VH, Rosenberg DM, editors. The ecology of aquatic insects. New York: Praeger Publishers. p 164–195.

Leonardi MG, Casartelli M, Parenti P, Giordana B. 1998. Evidence for a low affinity, high-capacity uniport for amino acids in *Bombyx mori* larval midgut. Am J Physiol 274: R1372–R1375.

Martin MM, Martin JS, Kukor JJ, Merritt RW. 1980. The digestion of protein and carbohydrate by stream detritivore *Tipula abdominalis* Diptera Tipulidae. Oecologia 46:360–364.

Neal JJ, Wu D, Hong YS, Reuveni M. 1996. High affinity transport of histidine and methionine across *Leptinotarsa decemlineata* midgut brush border membrane. J Insect Physiol 42:328–325.

O'Riordan AM. 1969. Electrolyte movement in the isolated midgut of the cockroach (*Periplaneta americana*). J Exp Biol 51:699–714.

Palacin M, Estévez R, Bertran J, Zorzano A. 1998. Molecular biology of mammalian plasma membrane amino acid transporters. Biol Rev 78:968–1054.

Parenti P, Sacchi FV, Hanozet GM, Giordana B. 1986. Na-dependent uptake of phenylalanine in the midgut of a cockroach *Blabera gigantea*. J Comp Physiol B 156:549–556.

Parenti P, Villa M, Hanozet GM. 1992. Kinetics of leucine transport in brush border membrane vesicles from lepidopteran larvae midgut. J Biol Chem 267:15391–15397.

Parenti P, Forcella M, Pugliese A, Casartelli M, Giordana B, Leonardi MG, Hanozet GM. 2000. Substrate specificity of the brush border K^+-leucine symport of *Bombyx mori* larval midgut Insect. Biochem Mol Biol 30:243–252.

Parenti P, Forcella M, Pugliese A, Giacchini R, Rossaro B, Hanozet GM. 2001. Leucine transport in membrane vesicles from *Chironomus riparius* larvae displays a mélange of crown-group features. Arch Insect Biochem Physiol 48:51–62.

Parthasarathy R, Xie T, Wolfersberger MG, Harvey WR. 1994. Substrate structure and amino acid/K^+ symport in brush-border membrane vesicles from larval *Manduca sexta* midgut. J Exp Biol 197:237–250.

Resh V, Rosenberg D. 1984. The ecology of aquatic insects. New York: Praeger Publishers. 625 p.

Reuveni M, Dunn PE. 1994. Proline transport into brush border membrane vesicles from the midgut of *Manduca sexta* larvae. Comp Biochem Physiol 107A:685–691.

Reuveni M, Hong YS, Dunn PE, Neal JJ. 1993. Leucine transport into brush border membrane vesicles from guts of *Leptinotarsa decemlineata* and *Manduca sexta*. Comp Biochem Physiol 104A:267–272.

Sacchi VF, Parenti P, Perego C, Giordana B. 1994. Interaction between Na^+ and the K^+-dependent amino acid transport in midgut brush border membrane vesicles from *Philosamia cynthia* larvae. J Insect Physiol 40:69–74.

Silva CP, Terra WR. 1994. Digestive and absorptive sites along the midgut of the cotton seed sucker bug *Dysdicercus peruvianus* Hemiptera: Pyrrhocoridae. Insect Biochem Mol Biol 24:493–505.

Smith L. 1955. Spectrophotometric assay of cytochrome c oxi-

dase. In: Glick T, editor. Methods in biochemical analysis, Vol. 2. New York: Interscience. p 427–434.

Terra WR, Ferreira C. 1994. Insect digestive enzymes: properties compartmentalization and function. Comp Biochem Physiol 109B:1–62.

Tomlin E, McLean H, Caveney S. 1993. Active accumulation of glutamate and aspartate by insect epidermal cells. Insect Biochem Mol Biol 23:561–569.

Tuppy H, Weisbauer U, Wintersberger E .1962. Aminosäure-p-nitroanilide als substrate für aminopeptidasen und andere proteolytische fermente. Hoppe-Seyler's Z Physiol Chem 329:278–288.

Wheeler WC, Whiting M, Wheeler QD, Carpenter JM. 2001. The phylogeny of the extant hexapod orders. Cladistics 17:113–169.

Wolfersberger MG. 2000. Amino acid transport in insects. Ann Rev Entomol 45:111–120.

Wolfersberger MG, Luethy P, Maurer A, Parenti P, Sacchi FV, Giordana B, Hanozet GM. 1987. Preparation and partial characterization of amino acid transporting brush border membrane vesicles from the larval midgut of the cabbage butterfly *Pieris brassicae*. Comp Biochem Physiol 86A:301–308.

Wright SH, Ahearn GA. 1997. Nutrient absorption in invertebrates In: Dantzler WH, editor. Handbook of physiology. New York: Oxford University Press. p.1137–1205.

Zhuang ZP, Linser PJ, Harvey WR. 1999. Antibody to H^+ V-ATPase subunit E colocalizes with portasomes in alkaline larval midgut of a freshwater mosquito *Aedes aegypti* L. J Exp Biol 202:2449–2460.