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Cation-dependent leucine, alanine, and phenylalanine uptake at pH 10 in brush-border membrane vesicles from larval *Manduca sexta* midgut

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Using the rapid filtration technique, cation gradient driven leucine, alanine and phenylalanine uptake by brush-border membrane vesicles (BBMV) from the highly studied model insect, *Manduca sexta*, is characterized at the physiological pH of 10. The vesicles are sealed and nonspecific binding is small. Almost identical initial time courses of leucine uptake are obtained whether the vesicles are osmotically balanced initially or at equilibrium. The maximum accumulation values are also similar and the equilibrium values are identical with either treatment. Equilibrium is reached by 60 min. Amino acid accumulation is cation gradient dependent and is abolished by 18 μM valinomycin. Uptake of all three amino acids occurs over a broad pH range with maximum rates at approximately pH 10 and lower rates at pH 7.5. The cation selectivity of phenylalanine and alanine uptake changes with pH; the sequence is $K^+ > Na^+ > Cs^+ \gg Rb^+ = Li^+$ at pH 10.0, whereas $K^+ = Na^+$ at pH 8.0; the selectivity of leucine uptake is $K^+ = Na^+ > Cs^+ \gg Rb^+ = Li^+$ at pH 10. Maximum K^+ driven accumulation of all three amino acids decreases with anions in the order: $SCN^- > NO_3^- > Cl^- = CO_3^{2-} = SO_4^{2-} = HPO_4^{2-} >$ gluconate$^-$. V_{max} values are similar for all three amino acids. There are large differences in initial uptake rates (leucine > phenylalanine = alanine), and maximum accumulation values (leucine > phenylalanine > alanine).

Introduction

The lepidopteran midgut has long been a model for invertebrate epithelial ion homeostasis [1–8]. Potassium ions move passively from blood to columnar and goblet cells via kinetically identified channels in the basal membrane [9,10]; they are pumped from goblet cells to apical goblet cavity via an electrogenic, protonmotive V-ATPase, which has been well characterized biochemically [8] in parallel with a biochemically identified, electrophoretic potassium ion/proton antiporter [6]; they reach the lumen accompanied by carbonate ions rendering the lumen highly alkaline [11–13]. The > 150 mV (lumen positive) electrical potential difference drives K^+ along with amino acids across the brush-border back into the columnar cells by symport systems which have been well characterized kinetically [14–20]. Isolation and sequencing of amino acid/K^+ symporter proteins will be facilitated by their detailed characterization in a transport model such as *M. sexta* midgut from which other transport proteins have already been isolated [21] and sequenced [22,23].

Early studies on an isolated lepidopteran (*Hyalophora cecropia*) larval midgut demonstrated that amino acids are transported from the lumen side to the blood side [24]. The transport was shown to be dependent on K^+ and oxygen but not directly dependent on K^+ transport across the midgut. Rapid filtration studies of K^+ gradient driven phenylalanine uptake by isolated brush-border membrane vesicles (BBMV) from *Philosamia cynthia* midguts identified the first insect amino acid/K^+ symporter [15]. Several amino acid symport systems have been identified subsequently in lepidopteran midgut BBMV, e.g., *P. cynthia*, *Bombyx mori*, and *Pieris brassicae* [17]. K^+-dependent phenylalanine uptake has also been demonstrated in BBMV from midguts of *Lymantria dispar* and *M. sexta* [25,26]. Although cation gradients are commonly used to drive amino acid uptake by vesicles in vitro little or no such gradient exists in vivo [2] where uptake is thought to be driven by the electrical gradient [17,27].

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The lepidopteran larval midgut maintains a physiological pH in the range of 8 to 12 [2]; nevertheless, previous studies of cation-dependent amino acid uptake by midgut BBMV have usually been conducted at pH 7.5 with more recent measurements at pH 8.8–8.9 [18,20]. In the only previous study on *M. sexta*, which was conducted at pH 8.0, phenylalanine uptake was driven by K^+ and Na^+ but, unexpectedly, not by Rb^+ or Li^+ [26]. Recently we have identified a K^+-dependent neutral amino acid symport system in larval *M. sexta* midgut BBMV. Inhibition of uptake both with and without an ion gradient as well as countertransport accumulation of leucine, alanine, and phenylalanine by the twenty naturally occurring amino acids and two analogues at pH 10 suggests that these three amino acids, along with a dozen others, are transported by a broad spectrum symporter (see the accompanying paper by Hennigan et al. [35]) that is similar to the B-type

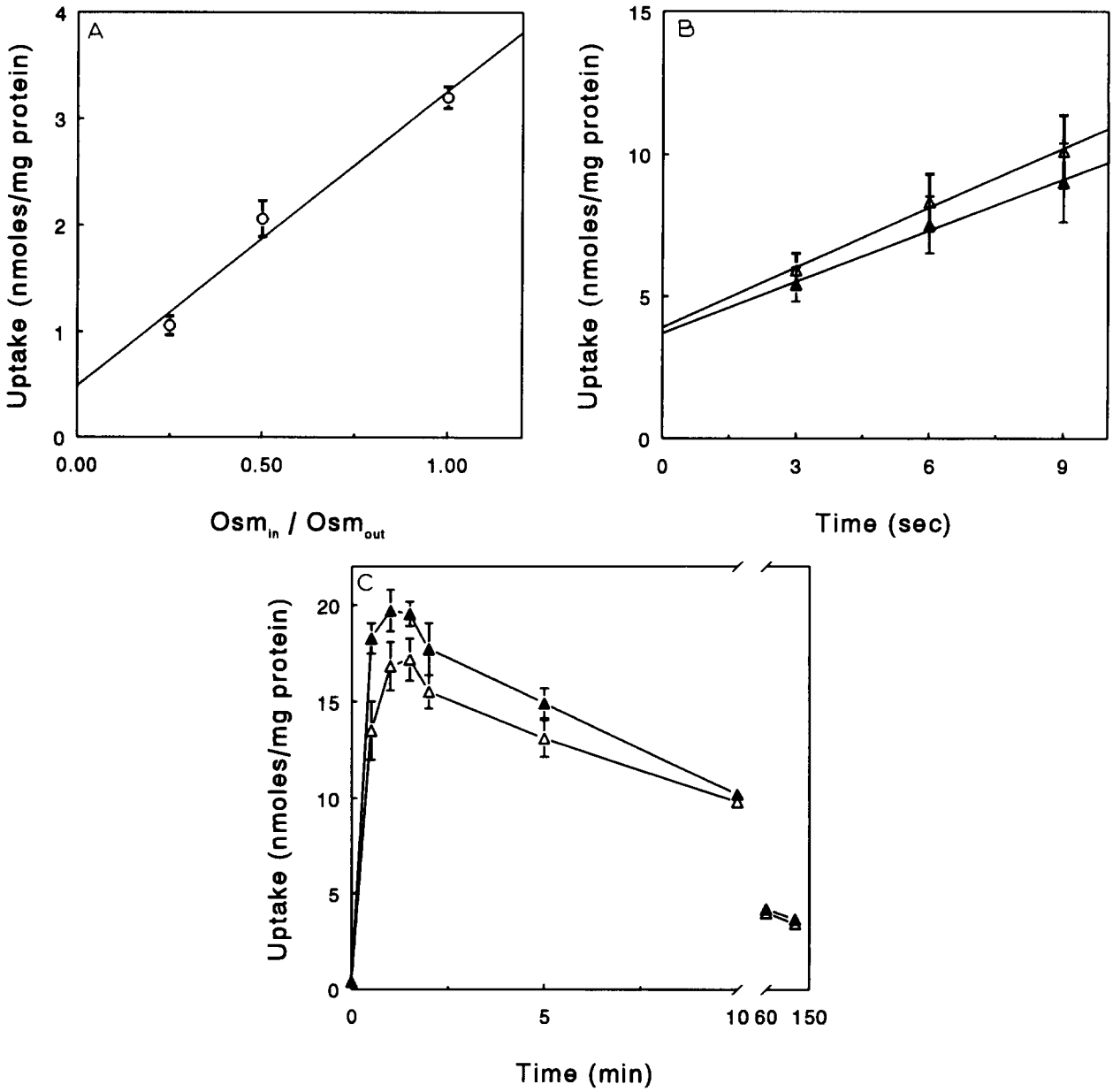

Fig. 1. Effects of osmotic balance on leucine uptake by BBMV. (A) Plot of equilibrium (1 h incubation) L-leucine (1 mM) uptake, in the presence of 50 mM KSCN, against inside/outside osmolarity ratio. The vesicles were loaded with 50 mM AMPD and 100 mM sucrose. The transport buffer contained 50 mM AMPD, 1 mM labelled leucine, 50 mM KSCN and sucrose as needed to establish the osmolarity gradient. Initial rate (B) and typical time course (C) of uptake of leucine under (a) *final isosmolar* (△) and (b) *initial isosmolar* (▲) conditions at pH 10. Vesicles were resuspended in 50 mM AMPD and 100 mM mannitol for (a) and in 50 mM AMPD and 200 mM mannitol for (b). The transport buffer contained 50 mM AMPD, 100 mM mannitol, 100 mM KSCN and 1 mM labelled leucine for (a); it contained 50 mM AMPD and 120 mM KSCN and 1 mM labelled leucine for (b) (mean ± S.E., $n = 3$, when not given the S.E. bars are smaller than the symbols used).

system identified by Giordana et al. [17]. The uptakes of leucine, alanine and phenylalanine, typical substrates of this system, are further characterized here at the physiological pH of 10.

Materials and Methods

Rearing of insects. Fifth instar *M. sexta* larvae, weighing 5.5 ± 0.5 g, were used in all experiments. They were reared at 27°C under constant light using eggs and larval diet from Carolina Biological Supply (Burlington, NC).

BBMV preparation. BBMV were prepared by the differential magnesium precipitation method of Biber et al. [28] as modified by Wolfersberger et al. [19]. The preparation yields mean enrichment factors (specific activity of BBMV/specific activity of homogenate) for aminopeptidase (EC 3.4.11.2) and cytochrome-*c* oxidase (EC 1.9.3.1) of 12.84 and 0.42, respectively [29].

Amino acid uptake measurement. Uptake experiments were performed at 25 ± 1°C using the rapid filtration technique [19,30]. Unless otherwise noted, freshly prepared BBMV pellets were resuspended at a protein concentration of 0.5 mg/ml in resuspension buffer (100 mM mannitol, 50 mM aminomethylpropanediol [AMPD], adjusted to the desired pH – usually 10 – with HCl) and allowed to equilibrate for one hour on ice. The BBMV were collected by centrifugation at approx. $31\,000 \times g$ for 30 min and again suspended, at a protein concentration of approx. 5 mg/ml, in resuspension buffer using a syringe equipped with a 1.5 cm × 25 gauge needle. For *final isosmolar* conditions incubations were started by mixing 10-μl aliquots of BBMV with 10-μl aliquots of transport buffer (2 mM radioactively labeled amino acid, 100 mM mannitol, 100 mM KSCN, 50 mM AMPD adjusted to pH 10 with HCl). For *initial isosmolar* conditions the mannitol concentration in the resuspension buffer was increased to 200 mM. Alternative compositions of transport buffers are given in the appropriate table or figure legends.

Incubations were terminated by the addition of 2 ml of ice-cold stop solution (100 mM mannitol, 100 mM KSCN, 50 mM AMPD at pH 10). The diluted mixtures were filtered immediately through a prewetted cellulose nitrate filter (0.65 μm pore size, Sartorius No. 11305, Hayward, CA). All filters were washed with two 4-ml aliquots of ice-cold stop solution, placed in a vial with 10 ml of scintillation fluid (ScintiVerse E or BD, Fisher Scientific, Pittsburgh, PA), and counted in a liquid scintillation spectrometer (Model 2000CA, Packard Instruments, Downers Grove, IL). 10 μl-aliquots of the transport buffer containing the radioactively labeled amino acid were counted as standards to convert radioactivity in cpm into moles of amino acid [19].

Protein determinations. The protein concentrations of homogenates and BBMV preparations were determined by the method of Bradford [31] using a Bio-Rad kit (Richmond, CA) with bovine serum albumin as a standard.

Chemical reagents. L-[3,4,5-^{3}H]Leucine, L-[2,3-^{3}H]alanine, and L-[*side chain*-^{3}H]phenylalanine were from ICN Biochemicals (Costa Mesa, CA). Nonradioactive amino acids were from Sigma (St. Louis, MO). Aminomethylpropanediol was from Eastman Kodak (Rochester, NY). All other reagents were analytical grade products from either Fisher or Mallinckrodt (St. Louis, MO).

Results

Osmotic behavior of BBMV

The equilibrium accumulation of leucine increased linearly as the ratio of osmolarity inside to outside was increased (Fig. 1A) demonstrating that the vesicles are sealed. The small intercept at an infinite osmotic gradient (Fig. 1A) indicates that nonspecific binding of leucine is small. These results are similar to those obtained in earlier studies on lepidopteran midgut BBMV [15,26]. Both the initial uptake (Fig. 1B) and accumulation time course (Fig. 1C) were similar under *initial* and *final* isosmolar conditions.

Initial uptake velocity is high at pH 10

All three amino acids are taken up rapidly into BBMV over a broad pH range with a maximum at approximately pH 10 (Fig. 2). The rapid uptake range extends to 10.5, the pH normally found in the midgut of the intact larva. The uptake decreases as the pH is lowered to 8.0. With Hepes buffer at pH 7.0 the uptake was the same as at pH 8.0 (data not shown). Therefore, AMPD buffer at pH 10.0 was used for further uptake experiments. All three amino acids have similar K_m and V_{max} values for K^+ gradient driven uptake at pH 10 (Table I). However, the initial uptake rate is greater for leucine than for either alanine or phenylalanine.

Maximum accumulation of leucine, alanine, and phenylalanine

The greater initial velocity of leucine uptake is also reflected in the maximum accumulation values (Fig. 3). Both the 15-s and the 90-s uptake values for leucine are higher than those for either alanine or phenylalanine. The maximum K^+ driven accumulation of these amino acids at pH 10 occurs at 90 s. The K^+ gradient fails to bring about an accumulation of leucine into vesicles in the presence of 18 μM valinomycin (Fig. 3) thus demonstrating the requirement for the driving force of the K^+ gradient in the uphill transport of these amino acids.

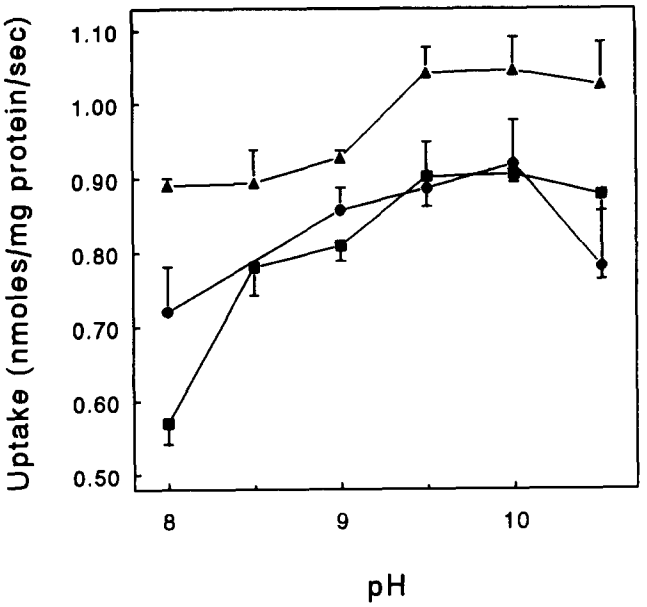

Fig. 2. Effect of pH on initial rates of amino acid uptake by brush-border membrane vesicles from larval *M. sexta* midgut. BBMV, resuspended in 100 mM mannitol and 50 mM AMPD at the appropriate pH, were mixed with an equal volume of medium containing 100 mM mannitol, 50 mM AMPD, 100 mM KSCN, and 2 mM of radioactively labeled leucine (▲), phenylalanine (■), or alanine (●), at the appropriate pH. Reaction mixtures were stopped at 3, 5, and 7 s and the rate of initial uptake was determined by linear regression. (Mean ± S.E., $n = 3$, when not given, the S.E. bars are smaller than the symbols used).

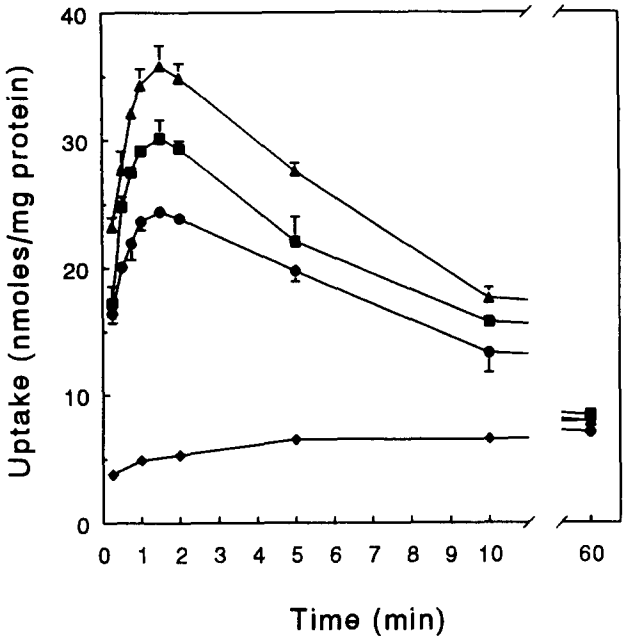

Fig. 3. Time course of amino acid uptake by BBMV. BBMV, resuspended in 100 mM mannitol and 50 mM AMPD at pH 10.0, were mixed with an equal volume of pH 10 medium containing 100 mM mannitol, 50 mM AMPD, 100 mM KSCN, and 2 mM of radioactively labeled leucine (▲), phenylalanine (■), or alanine (●). BBMV, incubated for 60 min with 8 μg valinomycin/mg protein, were mixed with an equal volume of pH 10 medium containing 100 mM mannitol, 50 mM AMPD, 100 mM KSCN and 2 mM radioactively labeled leucine (♦). (Mean ± S.E., $n = 3$, when not given, the S.E. bars are smaller than the symbols used).

Effectiveness of cations for driving amino acid accumulation

The ability of the alkali metal cations, all as chloride salts, to drive leucine accumulation by larval *M. sexta* midgut BBMV at pH 10 is compared in Fig. 4. K^+ and Na^+ gradients both drive maximum accumulation of leucine to more than three times its equilibrum con-

TABLE I

Kinetic parameters for amino acid uptake in the presence of an inwardly directed potassium gradient

Vesicles in 100 mM mannitol and 50 mM AMPD at (pH 10) were added to uptake solutions containing 100 mM mannitol, 100 mM KSCN, 50 mM AMPD (pH 10) plus an appropriate concentration of amino acid ranging from 0.1 mM to 10 mM. Reactions were stopped at 3, 5, and 7 s. The rate of uptake was determined by linear regression. The K_m and V_{max} were calculated by using a Lineweaver-Burk double reciprocal plot. The K_m values are in mM; V_{max} values are in nmol/s per mg protein. Reported values are means ± S.E. for three determinations; they are not corrected for a small diffusional component of uptake which does not significantly alter the K_m and V_{max} values.

Amino acid	K_m	V_{max}
L-Leucine	0.26 ± 0.07	1.45 ± 0.05
L-Alanine	0.38 ± 0.06	1.38 ± 0.17
L-Phenylalanine	0.46 ± 0.02	1.59 ± 0.12

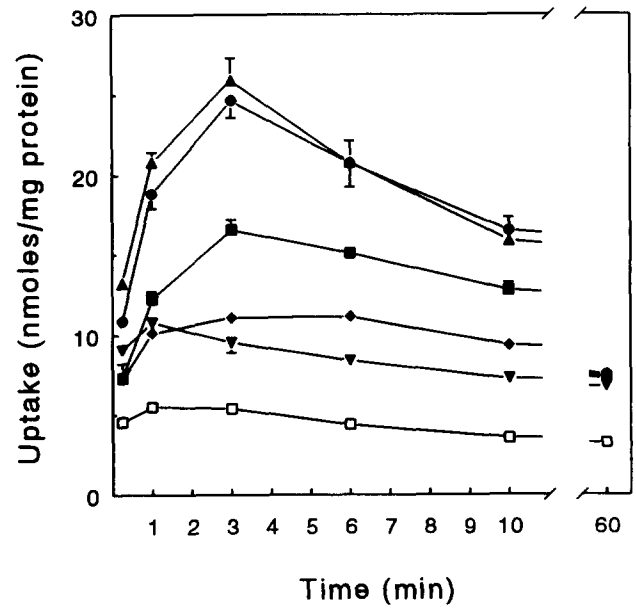

Fig. 4. Cation specificity of leucine uptake by BBMV. BBMV, resuspended in 100 mM mannitol and 50 mM AMPD at pH 10.0, were mixed with an equal volume of pH 10.0 medium containing 100 mM mannitol, 50 mM AMPD, 2 mM [^{3}H]leucine, and either: 100 mM KCl (▲), 100 mM NaCl (●), 100 mM CsCl (■), 100 mM RbCl (♦), 100 mM LiCl (▼), or 100 mM choline chloride (□). (Mean ± S.E., $n = 3$, when not given, the S.E. bars are smaller than the symbols used).

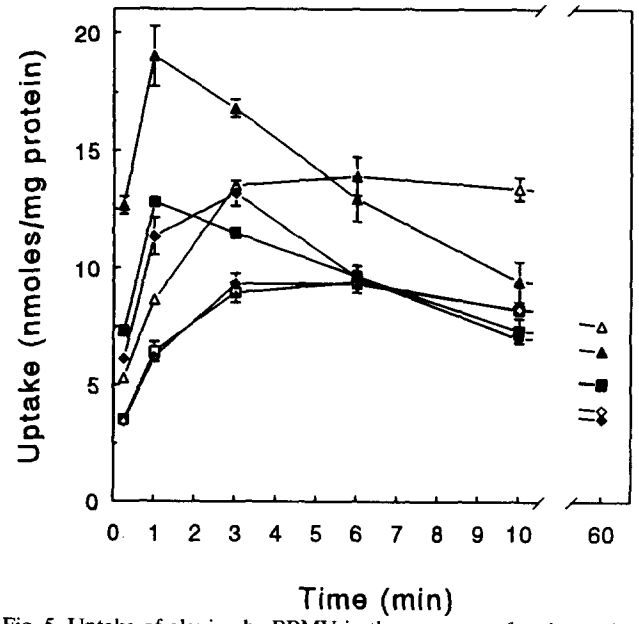

Fig. 5. Uptake of alanine by BBMV in the presence of various salts. BBMV, resuspended in 100 mM mannitol and 50 mM AMPD at pH 10.0, were mixed with an equal volume of pH 10 medium containing 100 mM mannitol, 50 mM AMPD, 2 mM [^{3}H]alanine, and either: 100 mM KSCN (▲), 100 mM KCl (■), 50 mM K_2SO_4 (♦), 100 mM NaSCN (△), 100 mM NaCl (□), or 50 mM Na_2SO_4 (◇). (Mean ± S.E., $n = 3$, when not given, the S.E. bars are smaller than the symbols used).

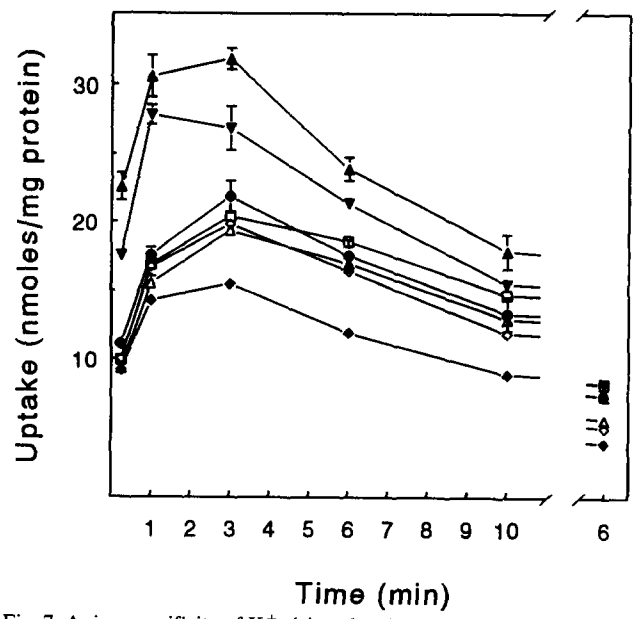

Fig. 7. Anion specificity of K^+ driven leucine uptake by brush-border membrane vesicles from larval *M. sexta* midgut. BBMV, resuspended in 100 mM mannitol and 50 mM AMPD at pH 10.0, were mixed with an equal volume of pH 10 medium containing 100 mM mannitol, 50 mM AMPD, 2 mM [^{3}H]leucine and either: 100 mM KSCN (▲), 100 mM KNO_3 (▼), 100 mM KCl (●), 50 mM K_2CO_3 (□), 50 mM K_2SO_4 (◇), 50 mM K_2HPO_4 (△), 100 mM potassium gluconate (♦). (Mean ± S.E., $n = 3$, when not given, the S.E. bars are smaller than the symbols used).

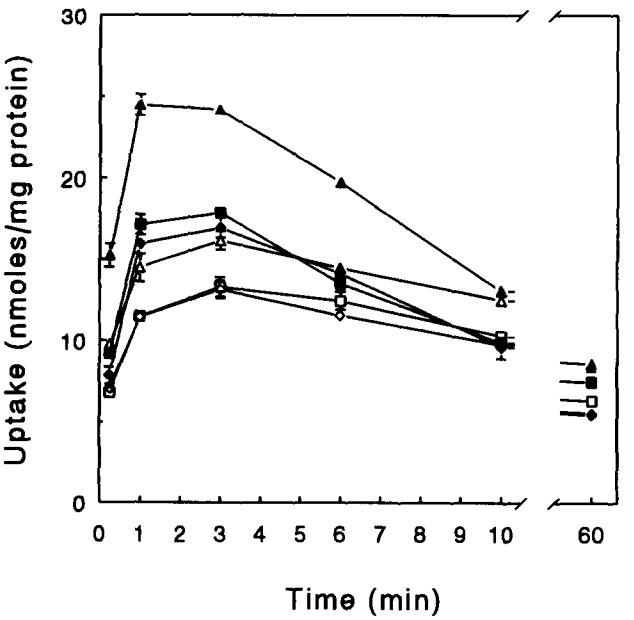

Fig. 6. Uptake of phenylalanine by BBMV in the presence of various salts. BBMV, resuspended in 100 mM mannitol and 50 mM AMPD at pH 10.0, were mixed with an equal volume of pH 10 medium containing 100 mM mannitol, 50 mM AMPD, 2 mM [^{3}H]phenylalanine, and either: 100 mM KSCN (▲), 100 mM KCl (■), 50 mM K_2SO_4 (♦), 100 mM NaSCN (△), 100 mM NaCl (□), or 50 mM Na_2SO_4 (◇). (Mean ± S.E., $n = 3$, when not given, the S.E. bars are smaller than the symbols used).

centration. Cs^+ gradients drive maximum accumulation of leucine to twice its equilibrum concentration. Neither a Li^+, Rb^+, nor choline$^+$ gradient drives more than a marginal leucine accumulation. K^+ is much more effective than Na^+ in driving both initial uptake and maximum accumulation of alanine and phenylalanine at pH 10 (Figs. 5 and 6).

Effects of anions on amino acid cotransport

The effects of several anions on K^+ gradient driven leucine uptake by larval *M. sexta* midgut BBMV are shown in Fig. 7. Thiocyanate is the most permissive anion for leucine uptake followed closely by nitrate with chloride, carbonate, phosphate and sulfate being less permissive; gluconate is the least permissive anion and yields the lowest equilibrium values. KSCN gradients drive both alanine and phenylalanine uptake more effectively than KCl or K_2SO_4 gradients. Furthermore, equilibrium values for alanine and phenylalanine uptake are highest when driven by KSCN and lowest when driven by K_2SO_4 gradients (Figs. 5 and 6).

Discussion

Osmolarity of resuspension buffer

In many studies of cation gradient driven amino acid uptake, *initial isosmotic* conditions across the vesicle

membrane are established by adding sufficient impermeant solute, such as mannitol, to the resuspension buffer to provide an initial internal osmolarity calculated to be equal to that on the outside (e.g., Refs. 32, 20). The driving cation is usually added as a salt of a permeant anion such as thiocyanate to minimize electrical potential difference buildup across the membrane. Consequently, as the symported cation and accompanying anion enter the vesicle its interior must become hyperosmotic. By contrast in the earlier studies of amino acid uptake by mammalian intestinal BBMV [33] and by lepidopteran midgut BBMV [14–19], the external salt was not balanced by internal mannitol and the vesicles were hyposmolar initially; presumably water left and salt entered because *final osmotic* equilibrium was reached. To evaluate the importance of *initial* versus *final* isosmotic conditions the time course of leucine uptake was determined with aliquots of the same preparation under both conditions. Both the initial uptake rates (Fig. 1B) and the maximum accumulation values (Fig. 1C) were similar and the equilibrium values were identical in both cases. Under both conditions equilibrium was reached by 60 min (Fig. 1C). For the present study the *final isosmolar* protocol was used to facilitate comparisons with earlier studies on lepidopteran BBMV.

The effect of pH on initial uptake rates

The pH ranges from 9.8 to 11.3 in the larval midgut of *M. sexta* and is similarly high in most lepidopteran larvae [2]. Nonetheless, prior to this report, all studies of K^+/amino acid cotransport by lepidopteran midgut BBMV had been conducted at pH values between 7 and 9. In the present characterization of midgut amino acid uptake at high pH, the initial rates of leucine, alanine, and phenylalanine uptake are all significantly greater at pH 9.5 or pH 10 than at pH 8 (Fig. 2). Therefore, the symport system(s) operating for leucine, alanine, and phenylalanine are well suited for uptake of these amino acids into midgut cells under conditions present in the intact organism. The accumulations seen in Fig. 3 confirm the ability of the symport system(s) to transport these amino acids against their own concentration gradients even at pH 10.

In many respects amino acid uptake at pH 10 is similar to that found earlier at nearly neutral pH. The maximum accumulation ratio for leucine exceeds that for phenylalanine both at pH 10 in *M. sexta* (Fig. 3) and at pH 7.4 in *P. cynthia* [15,18]. The initial rate of phenylalanine uptake approximates that of alanine uptake at pH 10 in *M. sexta* (Fig. 2) although the maximum accumulation is greater for phenylalanine than for alanine (Fig. 3). In *P. brassicae* larval midgut BBMV the initial rate of alanine uptake at pH 7.4 is slighter greater than that of phenylalanine although the maximum accumulations of phenylalanine and alanine are equal [19].

Effects of cations on amino acid uptake

At pH 7.4 K^+ is more effective than Na^+ in driving phenylalanine uptake by *P. cynthia* larval midgut BBMV [15]. At pH 10 K^+ is also more effective than Na^+ in driving phenylalanine accumulation by *M. sexta* BBMV (Fig. 2); however, at pH 8.0 K^+ and Na^+ are equally effective [26]. This K^+ preference at high pH seems appropriate since K^+ is the major alkali ion present in the lumen of the midgut [1,2]. Nonetheless, K^+ and Na^+ are equally effective for driving leucine uptake in *M. sexta* BBMV at pH 10 (Fig. 4).

Neither Li^+ nor Rb^+ gradients are able to drive appreciable phenylalanine or leucine accumulation into *M. sexta* BBMV at either pH 8 or pH 10 (Ref. 26 and Fig. 4). That K^+ and Na^+ drive uptake whereas Rb^+ and Li^+ do not is unexpected. In many physiological systems Rb^+ can replace K^+ and Li^+ can replace Na^+ whereas K^+ cannot replace Na^+ [34]. However, Li^+ can drive appreciable phenylalanine uptake [16] and modest leucine uptake [18] into *P. cynthia* BBMV. $Choline^+$ is a very poor substrate for the amino acid symporter. It fails to drive the accumulation of leucine and also causes shrinkage of the BBMV as shown by a decreased equilibrium (60 min) uptake value (Fig. 3).

Effects of anions on amino acid accumulation

The order of anion permeability of the brush-border membrane can be deduced from the maximum accumulation and equilibrium values for the salt gradient driven uptake of leucine, alanine, and phenylalanine (Figs. 4, 5 and 6). A low equilibrium value is obtained with potassium gluconate as the driving salt for leucine uptake, intermediate values are obtained with potassium sulfate or potassium phosphate, and the same high values are obtained with all other tested potassium salts (Fig. 7). These results indicate that gluconate, and to a lesser degree phosphate and sulfate permeate the BBMV less well than thiocyanate, nitrate, carbonate, or chloride.

Anions which do not permeate the membrane may diminish accumulation of amino acids for both electrical and osmotic reasons. Leucine, alanine, and phenylalanine carry predominantly a single net negative charge at pH 10. If more than one K^+ is transported with each amino acid then the buildup of net positive vesicular charge, expected from the transport of K^+ and the amino acid, will not be neutralized if anions are impermeant; the charge buildup will slow uptake. Moreover, impermeant anions will maintain external hyperosmolarity leading to vesicle shrinkage. Such shrinkage probably accounts for the decreased equilibrium values for the uptake of amino acids driven by

salts containing relatively impermeant anions (Figs. 4–7).

Kinetic parameters of amino acid uptake

The apparent K_m values for the K^+ driven uptake of leucine, alanine, and phenylalanine into BBMV of *M. sexta* at pH 10 (Table I) are almost identical to the K_m values for these same amino acids transported into BBMV of *P. cynthia* at pH 7.4 [17]. However, the V_{max} values for uptake of leucine, alanine, and phenylalanine into *M. sexta* BBMV at pH 10 (Table I) are greater than the V_{max} values for uptake of these three amino acids into *P. cynthia* BBMV at pH 7.4 [17]. The V_{max} of leucine transport into *P. cynthia* BBMV at pH 8.9 is higher than that at pH 7.4 [18]. The values of V_{max} for potassium-gradient driven leucine, alanine, and phenylalanine cotransport into *M. sexta* BBMV at pH 10 are not statistically different from each other (Table I) whereas the V_{max} values for potassium-gradient driven amino acid cotransport into *P. cynthia* BBMV at pH 7.4 decrease in the order leucine > phenylalanine > alanine [17]. The higher V_{max} values at higher pH again demonstrate that these lepidopteran symport systems operate more effectively at the high pH characteristic of midgut lumen than at the lower pH commonly used for in vitro studies.

Acknowledgements

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