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α-AMINOISOBUTYRIC ACID TRANSPORT IN THE MIDGUT OF TWO LEPIDOPTERAN LARVAE

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SUMMARY

1. A net absorption of α-aminoisobutyric acid (AIB) takes place *in vitro* in the midgut of two lepidopteran larvae, *Philosamia cynthia* Drury and *Bombyx mori* L.
2. In *P. cynthia* the midgut epithelium accumulates AIB from the lumen, while in the same conditions AIB accumulation is not observed in *B. mori* midgut cells.
3. In *P. cynthia*, when the lumen is bathed with a low K solution, the net absorption of AIB is reduced and the intracellular accumulation from the lumen is abolished.
4. Brush border membrane vesicles, prepared from the midgut of both species, show a transient K-dependent concentrative uptake of AIB.
5. The relationship between AIB uptake and AIB concentration in the presence of a transmembrane K gradient was studied in *B. mori* vesicles and the kinetic constants were calculated.
6. These results confirm that there is a K-amino acid co-transport system on the brush border of columnar cells in the midgut of both larvae.

INTRODUCTION

The midgut of lepidopteran larvae plays a key role in the ionic homoeostasis of the animal, since it extrudes potassium ions from the haemolymph to the lumen at the expense of metabolic energy (Harvey & Nedergaard, 1964; Wolfersberger, Harvey & Cioffi, 1982). The activity of the potassium pump (Zerahn, 1977), which seems to be located in the apical membrane of the goblet cells (Blankemeyer & Harvey, 1978), generates the high, lumen-positive transepithelial potential difference (PD) observed in several lepidopteran larvae, i.e. *Hyalophora cecropia* (Harvey & Nedergaard, 1964), *Philosamia cynthia, Bombyx mori, Macrothylatia rubi* (Giordana & Sacchi, 1977), *Manduca sexta* (Blankemeyer & Harvey, 1978; Moffet, 1979), and *Pieris brassicae* (unpublished results).

The transmucosal potential difference with the negative pole in the cell side (Wood, Farrand & Harvey, 1969; Blankemeyer & Harvey, 1978; Moffet, Hudson, Moffett & Ridgway, 1982; Monticelli & Giordana, 1982), together with the high K concentration in the lumen contents (Harvey, Wood, Quatrale & Jungreis, 1975; Giordana &

Key words: Insect midgut, α-aminoisobutyric acid absorption, K-amino acid co-transport.

Sacchi, 1978) establish *in vivo* a steep electrochemical potential difference of K ($\Delta\bar{\mu}_K$) across the brush border of columnar cells, which favours a downhill entrance of this cation into the absorptive cells. These features, the virtual absence of Na (Giordana & Sacchi, 1978), and the presence of a co-transport between amino acids and K in a purified preparation of brush border membrane vesicles (Hanozet, Giordana & Sacchi, 1980), have led to a suggested model for amino acid absorption in lepidopteran larvae. According to this model, a co-transport system is located on the luminal membrane of columnar cells and the $\Delta\bar{\mu}_K$ across this membrane provides the driving force for the entrance of the amino acid into the cell (Giordana, Sacchi & Hanozet, 1982).

The aim of this work is to study the absorption of α-aminoisobutyric acid (AIB) in the midgut of *P. cynthia* and *B. mori* in the light of this model. This amino acid is a suitable tool for studying a co-transport system, since both *H. cecropia* (Nedergaard, 1977) and *P. cynthia* midguts show a concentrative uptake of AIB from the lumen side.

MATERIALS AND METHODS

Experimental animals

Larvae in the fifth instar of either *P. cynthia* or *B. mori* were used. The larvae were reared in the laboratory and fed on *Ailanthus glandulosa* and *Morus alba* leaves respectively. The midgut was dissected from the larvae as a cylinder and the peritrophic membrane with enclosed intestinal contents was removed.

Flux measurements, AIB intracellular pool determinations and cation concentration assays in the whole midgut

The midgut excised from the larva was mounted as a cylinder on an apparatus similar to that described by Nedergaard & Harvey (1968). The solutions used for perfusion are given in Table 1. The standard solutions reproduce the cation composition and the osmolarity as determined in the haemolymph of the used larvae (Giordana & Sacchi, 1978). Both luminal and haemolymph solutions were stirred and aerated by forcing a gas mixture of 95 % O_2–5 % CO_2 through the inlets; pH was 7·4. 2 μCi ml^{-1} of (U-^{14}C)-labelled amino acid was added to the mucosal solution to measure the influx (lumen to haemolymph) and 1 μCi ml^{-1} of labelled amino acid was added to the haemolymph solution to measure the efflux (haemolymph to lumen).

Table 1. *Composition of perfusion solutions*

		$KHCO_3$	KCl	$MgSO_4$	$CaCl_2$	Sucrose	$ChHCO_3$	ChCl	AIB
Philosamia cynthia	Standard	25	—	37	9	168	—	—	5–10
	Low K	2	—	37	9	168	23	—	5
	Standard+choline	25	—	37	9	122	—	23	5
Bombyx mori	Standard	25	21	44	9	110	—	—	10

Concentrations are given in mM.
Ch, choline.

Samples were withdrawn from mucosal or haemolymph solutions after an equilibration period of 30 min, which was necessary to obtain steady fluxes, and after another period of 30 min. Radioactivity was measured by means of a liquid scintillation spectrometer (Packard Tri-Carb 300). At the end of the experiments, the exposed tissue was removed, gently blotted on filter paper, homogenized, put in a tared tube and weighed. Distilled water (0·5 ml) was then added, the suspension was frozen, thawed, resuspended and centrifuged for 30 min. The supernatants were assayed for radioactivity, and intracellular AIB transport pools were calculated as previously reported (Sacchi, Cattaneo, Carpentieri & Giordana, 1981). Intracellular concentration of K was determined by use of a flame photometer and tissue values were corrected for ion concentration in the extracellular space as reported by Giordana & Sacchi (1978). The tissues were then stored in an oven (110 °C) overnight to obtain the dry weight. Transepithelial electrical potential difference was recorded by calomel electrodes connected *via* agar-KCl (3 M) bridges to the solutions bathing both sides of the isolated midgut. PD values were read on a Keithley voltmeter (Mod. 177 DMM).

Brush border membrane vesicle preparation and transport experiments

Vesicles from *P. cynthia* and *B. mori* midguts were prepared by means of Ca precipitation, following the procedure of Schmitz *et al.* (1973), modified by Kessler *et al.* (1978), as described in a previous paper (Giordana *et al.* 1982). Transport experiments were performed by a rapid filtration technique (Kessler *et al.* 1978). Vesicles were incubated in a mixture containing 100 mM-mannitol, 10 mM-HEPES Tris (pH 7·5), with the labelled amino acid and salt gradients as indicated in the legends of figures.

Determination of enzyme specific activities

The purification of brush border membrane vesicles obtained from *B. mori* was tested by measurements of marker enzyme activities. Small aliquots of the crude homogenate and of the vesicle suspension were withdrawn and disaccharidase activity was assayed according to Semenza & Von Balthazar (1974) with sucrose, maltose and trehalose as substrates, using the glucose oxidase-peroxidase method (Boehringer Test-combination). Alkaline phosphatase, acid phosphatase, aminopeptidase and γ-glutamyltransferase were determined with Boehringer kits, using respectively *p*-nitrophenylphosphate, L-leucine-*p*-nitroanilide and L-glutamyl-3-carboxy-4-*p*-nitroanilide as substrates. Lactate dehydrogenase was assayed according to Bergmeyer & Bernt (1974). Cytochrome oxidase activity was assayed according to Smith (1955). Protein determination was carried out according to Bradford (1976) using a Bio-Rad kit.

RESULTS

α-Aminoisobutyric acid is a rare, neutral amino acid, resistant to degradation in most organisms (Christensen, 1979), and it is often used in transport studies as an analogue of alanine, so that metabolic alterations can be avoided. In the midguts of

Table 2. (A) *Transepithelial unidirectional and net fluxes of AIB (10 mM) in the isolated midgut of* Bombyx mori *and* Philosamia cynthia. (B) *Intracellular AIB pools*

	A			B		
	J_{l-h}	J_{h-l} (μmol g^{-1} dry weight h^{-1})	J_{net}	Luminal pool	Haemolymph pool (mmol l^{-1} cell water)	Total pool
Bombyx mori	116·3 ± 14·8 (4)	16·5 ± 2·6 (4)	99·8 ± 15·0	4·2 ± 1·2 (4)	19·5 ± 0·9 (4)	18·7 ± 2·1 (4)
Philosamia cynthia	79·2 ± 9·8 (4)	3·8 ± 0·2 (4)	75·4 ± 9·8	17·4 ± 1·6 (4)	24·1 ± 2·3 (4)	34·1 ± 5·1 (4)

J_{l-h}: lumen to haemolymph; J_{h-l}: haemolymph to lumen; J_{net}: $J_{l-h} - J_{h-l}$.
Luminal pool: labelled AIB in th lumen.
Haemolymph pool: labelled AIB in the haemolymph.
Total pool: labelled AIB in both compartments.
AIB 10 mM in the bathing solutions.
Means ± S.E. Number of experiments in parentheses.

both *P. cynthia* and *B. mori* there is a net movement of this amino acid from the lumen to the haemolymph, in the absence of any chemical gradient across the tissue (Table 2A), as observed also in *H. cecropia* (Nedergaard, 1972). It should be noted that all the transport experiments were carried out in the presence of the transepithelial electrical potential difference. However, at the pH of the bathing solutions, AIB does not bear any net charge, and so one should not expect any direct influence of the PD on the amino acid movement.

AIB accumulates within the cells of the midgut of *P. cynthia* and *B. mori* when the labelled amino acid is present both in the lumen and haemolymph solutions (Table 2B). Under these conditions, the intracellular AIB concentration is almost twice the concentration of the bathing solutions in *B. mori*, and more than three times that in *P. cynthia*. The difference is essentially due to the fact that the midgut accumulates AIB from the lumen in *P. cynthia*, while in *B. mori* it does not. It should be noted that, while the total pool is the real concentration of AIB in the cells, the luminal and haemolymph pools take into account only the AIB molecules coming from the lumen or from the haemolymph compartment respectively.

Since AIB accumulates within the cells, this means that an uphill movement of the amino acid takes place across the mucosal membrane. The presence of a concentrative uptake across the apical barrier gives some information about the absorptive process, i.e. it suggests that a primary or secondary (a co-transport mechanism) active process is present on this membrane. It should also be pointed out that the cell concentration and the pool values are expressed as mmol l^{-1} cell water, but the tissue is composed of different kinds of cells (columnar, goblet, regenerative, muscle and tracheal cells) of which only the columnar ones are absorptive. Furthermore King, Sepulveda & Smith (1981) have suggested that only a fraction of the rabbit absorptive enterocytes possess the full transport capacity. Therefore, the AIB concentration and pool values could be underestimated.

Both in *P. cynthia* and *B. mori* midguts, AIB is accumulated in the cells from the haemolymph side (Table 2B); the same result has been obtained in *H. cecropia*

Table 3. Philosamia cynthia *midgut: effect of luminal low K (2 mM) on lumen to haemolymph unidirectional AIB (5 mM) flux, luminal pool, transepithelial electrical potential difference (PD) and intracellular K concentrations*

Luminal solution	J_{l-h} (μmol g^{-1} dry weight h^{-1})	Luminal pool (mmol l^{-1} cell water)	PD (mV)	[K] (mmol l^{-1} cell water)
Standard	36·8 ± 3·9* (13)	7·7 ± 0·8† (14)	72·7 ± 2·8 (14)	121·7 ± 7·3 (8)
Low K	18·8 ± 2·8* (6)	4·5 ± 0·9† (6)	81·0 ± 3·1 (6)	119·1 ± 1·9 (8)
Standard + choline	32·6 ± 5·0 (7)	8·5 ± 1·0 (7)	76·5 ± 4·4 (7)	—

PD values were measured during flux experiments: mean values between initial and final recordings have been used to calculate the reported mean.

Means ± S.E. Number of experiments in parentheses.

*$P < 0.01$.

†$P < 0.02$.

According to Nedergaard (1981), this accumulation may depend on an amino acid exchange mechanism in the basolateral membrane of the midgut epithelium of these larvae.

As would be expected if AIB is co-transported with K at the luminal border of columnar cells, the unidirectional lumen to haemolymph flux (J_{l-h}) is reduced when K is removed from the mucosal solution, and AIB does not accumulate in the luminal pool (Table 3). These results are consistent with the presence, at the luminal membrane of the enterocyte, of a co-transport mechanism which requires not only the transepithelial electrical potential difference (Nedergaard, 1973), but also the presence of luminal K, as extensively discussed in previous papers (Hanozet *et al.* 1980; Giordana *et al.* 1982). Choline is the cation used as a substitute for K; experiments performed with both K (25 mM) and choline (23 mM) show that the cation itself does not alter either the AIB unidirectional flux or the luminal pool (Table 3). No difference between control and experimental conditions can be observed as far as the intracellular K concentrations and PD are concerned.

In order to ascertain the presence of a co-transport between AIB and K at the luminal surface of columnar cells, transport experiments were performed on brush border membrane vesicles prepared from the midgut of the two larvae. These isolated membranes retain the transport properties of membranes in intact cells and allow the investigation of transport characteristics under strictly controlled conditions: that is to say, it is possible to know the composition of both the environments separated by the vesiculated membrane fragments, and all the phenomena observed are not dependent on metabolism. Fig. 1A shows the time course of AIB uptake in brush border membrane vesicles obtained from *P. cynthia* midguts, in the presence and in the absence of a K gradient. A four-fold and a 2·8-fold transient accumulation of AIB occur in the presence of an initial gradient of KSCN or KCl respectively. As has been previously reported (Hanozet *et al.* 1980), this difference is due to the anions that, owing to their different permeabilities across the membrane, cause diffusional transmembrane

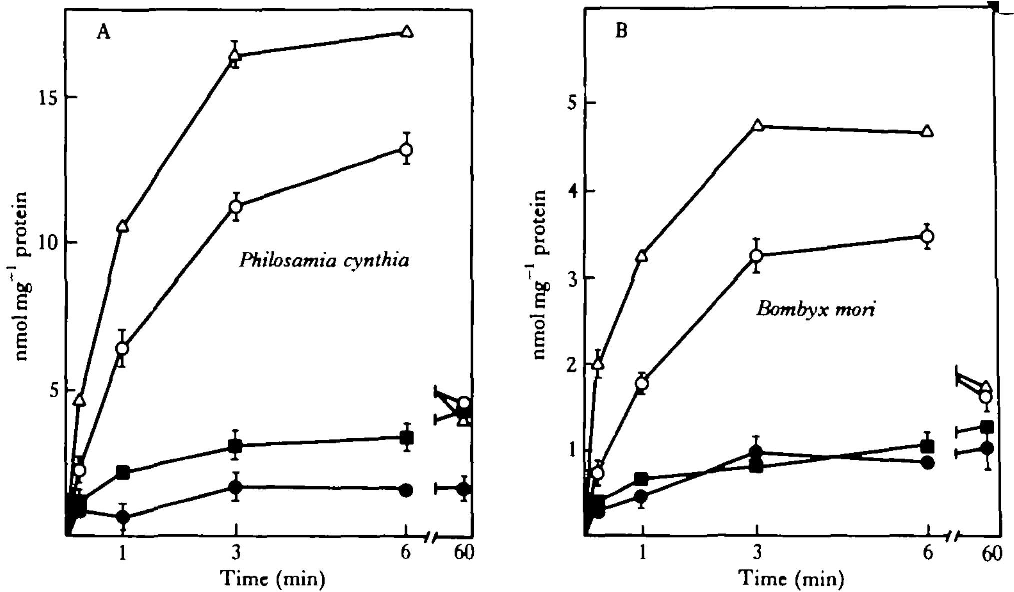

Fig. 1. (A) Uptake of 1 mM-AIB in brush border vesicles prepared from *Philosamia cynthia* midgut. △, Initial gradient of KSCN (0 mM inside, 100 mM outside); ○, initial gradient of KCl (0 mM inside, 100 mM outside); ●, initial gradient of choline Cl (0 mM inside, 100 mM outside); ■, initial gradient of mannitol (100 mM inside, 200 mM outside). Each experiment was performed in triplicate. Means ± S.E., when not given S.E. values were smaller than the symbols used. (B) Uptake of 1 mM-AIB in brush border vesicles prepared from *Bombyx mori* midgut. Symbols indicate experimental conditions as reported in (A). Each experiment was performed in triplicate. Means ± S.E., when not given S.E. values were smaller than the symbols used.

electrical potential differences of unequal values. These electrical potential differences in turn determine different $\Delta\tilde{\mu}_K$ values.

The uptake is merely equilibrative either with mannitol or choline gradients: in the absence of K and without any electrical potential difference across the membrane in the first case, and in the absence of K, but in the presence of an electrical potential difference when the choline Cl gradient is present.

Qualitatively the same results are obtained with *B. mori* vesicles (Fig. 1B). It has been pointed out in a previous paper (Giordana *et al.* 1982) that purified brush border membranes are difficult to isolate from *B. mori* midguts. For this preparation the midguts were previously rinsed with isotonic saline plus 5 mM-EDTA, and Ca precipitation was performed after a homogenization more vigorous than the one used before. To assess the purity of the vesicle suspension, marker enzymes were assayed (Table 4). A good enrichment of brush border marker enzymes, especially of γ-glutamyltransferase, was obtained (Fig. 2). As already stated for *P. cynthia* (Giordana *et al.* 1982) it is confirmed that disaccharidases are not restricted to the luminal membrane. A different distribution pattern of membrane-bound enzymes was found in a preparation of brush border membrane obtained from midgut and midgut caeca of *Rhynchosciara americana* (Diptera) (Ferreira & Terra, 1980, 1982), where the enrichment factor was higher for disaccharidases than for aminopeptidase; trehalase was also poorly recovered in the microvillar fraction.

Table 4. *Enzyme activities measured in the homogenate and in brush border vesicles from* Bombyx mori *midgut*

	Homogenate	Vesicles
γ-glutamyltransferase (γ-GT)	2·286 ± 0·023 (3)	44·850 ± 1·829 (3)
Leucine aminopeptidase (LAP)	0·365 ± 0·039 (3)	4·037 ± 0·199 (3)
Alkaline phosphatase (AlP)	0·730 ± 0·024 (3)	6·618 ± 0·840 (3)
Acid phosphatase (AcP)	9·600 ± 1·000 (3)	42·300 ± 2·000 (3)
Cytochrome oxidase (Cyt. ox.)	1·240 ± 0·196 (3)	0·417 ± 0·009 (3)
Lactate dehydrogenase (LDH)	6·200 ± 0·200 (3)	6·800 ± 0·300 (3)
Sucrase (Suc)	0·548	0·236
Maltase (Malt)	0·070	0·018
Trehalase (Tre)	0·074	0·020

Activities are expressed as μmol min^{-1} mg^{-1} protein.
γ-GT, AcP and LDH are expressed as nmol min^{-1} mg^{-1} protein.
Cyt. ox. is expressed as first order rate constant (min^{-1} mg^{-1} protein).
Means ± S.E. Number of experiments in parentheses.

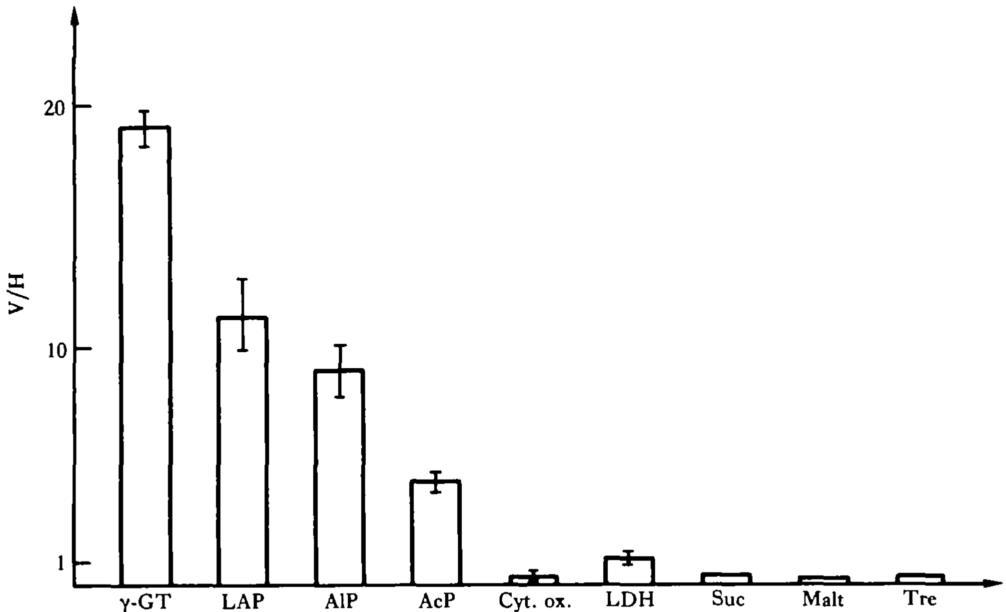

Fig. 2. Enrichment of brush border marker enzymes and of contaminants as a ratio between specific activity in the vesicles and in midgut homogenate (V/H). Abbreviations as in Table 4. Means ± S.E., number of experiments as in Table 4.

Both lactate dehydrogenase and cytochrome oxidase activities show that a slight contamination by other cell compartments is present in the final pellet. Special attention should be given to acid phosphatase activity: an enrichment factor of four was found

for this enzyme, which is usually considered a lysosomal marker. However it has been shown (Akai, 1969) that acid phosphatase is not restricted to lysosome-like structures in the columnar cells of *B. mori* midgut, but it is present also in blebs protruding from the apical region of the cells. Moreover Biber, Stieger, Haase & Murer (1981) have also found an enrichment of this enzyme in brush border vesicles from rat kidney, and they have demonstrated that this enzyme is not followed by other lysosomal marker enzymes.

In conclusion, membranes or aggregates other than brush border vesicles are present in the *B. mori* midgut final pellet: this is also supported by the fact that from 1 g of midgut fresh tissue, 0·8 mg and 2·7 mg of proteins are obtained in the final pellet of *P. cynthia* and *B. mori* respectively. Nevertheless Fig. 1 shows that AIB probably does not enter the contaminating membranes, either because they are poorly permeable or because they are not in vesicle form, since the two preparations differ quantitatively but not qualitatively as far as the AIB uptake is concerned.

Since the uptake of AIB could be the result of binding to membranes and not of transport into a vesicle space, we have ascertained if the amount of solute taken up by the vesicles at equilibrium is dependent upon the vesicular volume. The intravesicular space was reduced by increasing the amounts of the impermeant solute trehalose in the incubation mixture. The amount of AIB associated with the vesicles under equilibrium conditions decreases with increasing medium osmolarity, as shown in Fig. 3, so that AIB is really transported into an osmotically sensitive compartment. Extrapolation to infinite medium osmolarity, i.e. at a negligible intravesicular space, shows that the binding of AIB to membranes, if any, is very small.

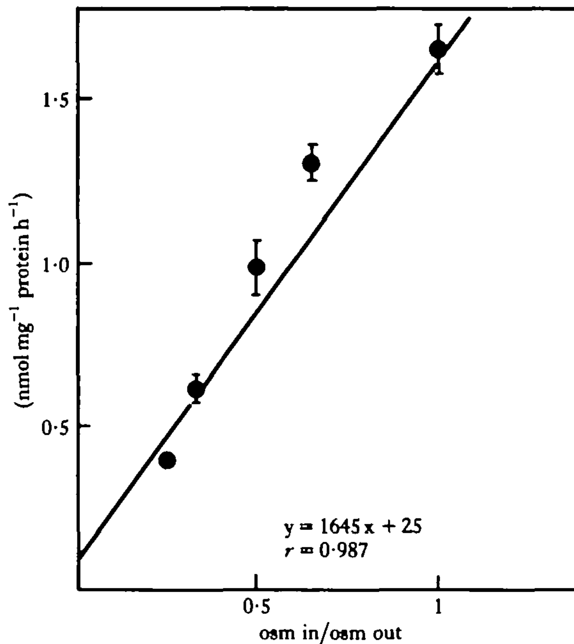

Fig. 3. Equilibrium uptake (after 60 min incubation) of AIB (1 mM) plotted as a function of the inside/outside osmolarity ratio (osm in/osm out), set by varying the external concentration of trehalose. Uptake was measured in the presence of an initial gradient of 20 mM KSCN. *Bombyx mori* vesicles.

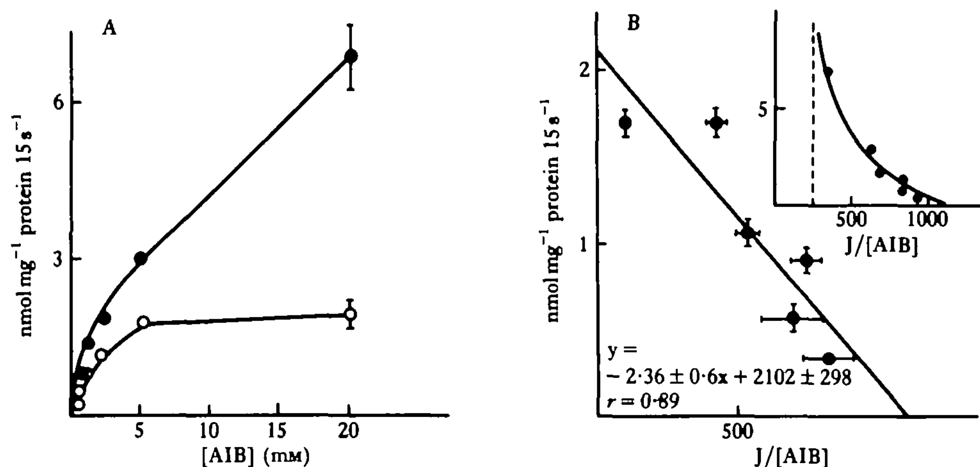

Fig. 4. (A) AIB uptake in brush border vesicles of *Bombyx mori* midgut as a function of AIB concentration in the incubation medium. Uptake measured in the presence of a KCl gradient (0 mM inside, 100 mM outside). ●, Total uptake; ○, carrier-mediated uptake. Experiment in triplicate. Means ± S.E., when not given S.E. values were smaller than the symbols used. (B) Eadie-Hofstee plot of the carrier-mediated uptake reported in A (open circles). The inset shows the Eadie-Hofstee plot of the total uptake reported in A (dark circles). Means ± S.E., when not given S.E. values were smaller than the symbols used.

The relationship between AIB uptake and AIB concentration in the presence of a transmembrane K gradient has been studied, and the kinetic constants have been calculated. Fig. 4A shows the overall AIB uptake measured at concentrations varying from 0·5 to 20 mM: the curve obtained does not reach saturation and is consistent with a saturation component plus a linear one, i.e. is described by the following equation:

$$J = \frac{J_{max} \cdot [S]}{K_t + [S]} + k_d \cdot [S],$$

where J is the total influx, J_{max} is the carrier-mediated maximal influx, [S] is the concentration of AIB, and K_t is the transport constant. Simple diffusion is described by $J_d = k_d \cdot [S]$, where k_d is the permeability coefficient of AIB. The value of k_d can be determined graphically by the slope of the linear component, and when each value of the total AIB uptake is corrected for the linear component, a saturable AIB transport into the vesicles is obtained. The linear component is presumably due to a diffusion of the amino acid, even if a carrier-mediated transport with a low affinity constant cannot be excluded. The linear component is also evident from the Eadie-Hofstee plot (Fig. 4B, inset). From this plot it can be concluded that AIB is taken up by a single saturable system, and from linear regression analysis a J_{max} of 2102 ± 298 pmol mg^{-1} protein (mean ± S.E., experiment in triplicate) and a K_t of $2{\cdot}36 \pm 0{\cdot}6$ mM can be calculated.

DISCUSSION

The data reported in this paper confirm the model recently proposed for amino acid absorption in the midgut of lepidopteran larvae (Giordana *et al.* 1982): AIB is transported through the brush border *via* a co-transport mechanism with K, the driving

force of this process being the $\Delta\tilde{\mu}_K$ between lumen and columnar cells maintained by the lumen-directed K pump. This co-transport seems to be responsible for AIB accumulation in the midgut cells of *P. cynthia*, since the accumulation from the lumen does not occur and the lumen to haemolymph flux is reduced when the midgut lumen is bathed in a low K solution.

In these larvae the carrier protein has acquired a potassium specificity, while maintaining a degree of sodium specificity (Giordana *et al.* 1982). Berteloot, Khan & Romaswamy (1981) have recently demonstrated the existence of a sodium- and potassium-dependent transport of L-phenylalanine in brush border vesicles of mouse enterocytes, which is the sole mammalian system studied to date showing a K-dependent amino acid transport.

It is noteworthy that in the intact midgut a low K concentration (2 mM) in the lumen causes a 50 % reduction of the influx, and net flux is not abolished under this experimental condition. Actually, the potassium concentration in the midgut lumen, especially in the proximity of the microvilli, is probably much higher than 2 mM, as a consequence of the activity of the K pump. Therefore, there could still be an electrochemical gradient, favourable to the entrance of potassium into the columnar cells, sustained by the high transmucosal electrical potential difference (V_m). Assuming a V_m of 114 mV (cell negative to the lumen) and an intracellular potassium activity of 120 mM, $\Delta\tilde{\mu}_K$ becomes zero only with a luminal K concentration of about 1 mM. In some experiments performed to test the potassium enrichment in the bulk lumen solution, a K concentration as high as $6 \cdot 15 \pm 0 \cdot 75$ mM (mean ± S.E., 4 experiments) has been measured in *B. mori* after an incubation period of 90 min.

In any case, from the data reported in this work, the presence of a potassium-independent AIB absorption cannot be excluded, and therefore its possible contribution to the absorptive process should be taken into account.

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