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L-Canavanine inhibits L-arginine uptake by broiler chicken intestinal brush border membrane vesicles

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Abstract   1. Intestinal brush border membrane vesicles (BBMV) were prepared from 3-week-old broiler chickens.

2. Electron microscopy of the BBMV fraction showed single membrane vesicles of different sizes with no electron dense material inside. No other organelles were observed. The sucrase and maltase activities were enriched by factors of 16 and 18, respectively, in the BBMV fraction in comparison with the homogenate. On the other hand, the Na^+/K^+-ATPase sensitivity to ouabain was increased by a factor of 0·8.

3. The BBMV showed a maximum L-$[^{14}C]$-arginine uptake ($944·9 \pm 22·9$ pmoles/mg protein) at 45 s and thereafter it declined slowly. In the presence of 0·5 mM L-canavanine, the L-$[^{14}C]$-arginine uptake by BBMV was reduced by 43·6% at 45 s.

4. It is concluded that L-canavanine inhibits L-arginine Na^+-dependent transport across the enterocyte apical membrane in a highly purified intestinal BBMV from broiler chickens.

INTRODUCTION

L-Canavanine (2-amino-4-(guanidinooxy) butyric acid) is a non-protein amino acid whose structure resembles that of L-arginine, an essential amino acid for growing chicks. L-Canavanine is present in appreciable amounts in the raw seeds of *Canavalia ensiformis* which have received considerable attention as an alternative feedstuff for commercial chickens, with poor results (D'Mello *et al.*, 1989; Mendez *et al.*, 1998). Rosenthal (1977) has reported a variety of antimetabolic effects of L-canavanine in micro-organisms and on invertebrate animals. There is also an indication that L-canavanine may affect the absorption of basic amino acids that compete for the same transport system in the small intestine (Herzberg *et al.*, 1971). Michelangeli and Vargas (1994) showed significant reductions in plasma concentrations of arginine, lysine and histidine when a diet containing 1% L-canavanine was fed to broiler chicks. The mechanisms whereby L-canavanine exert these effects in chicks are largely unknown. Therefore, the present study was undertaken to investigate the effect of L-canavanine on the L-$[^{14}C]$-arginine uptake by intestinal brush border membrane vesicles (BBMV) isolated and purified from broiler chicks.

MATERIALS AND METHODS

Chemicals

The $[^{14}C]$-arginine (320 mCi/mmole) was purchased from New England Nuclear Research Product (Boston, MA, USA). All the other chemicals were of analytical grade and purchased from Sigma Chemical (St Louis, MO, USA).

L-Canavanine purification and identification

L-Canavanine was purified from *Canavalia ensiformis* seeds, supplied by the Instituto de Genética de la Facultad de Agronomia de la Universidad Central de Venezuela, following the procedures outlined by Bass *et al.* (1995). Proton and ^{13}C nuclear magnetic resonance (NMR) were performed to assess L-canavanine purity.

Animals

One-day-old Arbor Acres chicks were housed in wire mesh cages and fed on a standard diet for broiler chickens formulated according to the recommendations of the National Research Council (1994). Chickens had access to food and water on an *ad libitum* basis. After a fasting

period of 12 h, the 3-week-old chicks were killed by cervical dislocation and the small intestine was rapidly removed and washed with 0·14 M NaCl.

Preparation of intestinal brush border membrane vesicles

BBMV were prepared according to Hopfer et al. (1983) with some modifications. All procedures were carried out at 4°C. A portion of the jejunum, from the end of the duodenal loop to Meckel's diverticulum, was removed and flushed with ice-cold saline. The cleaned small intestine was opened longitudinally, the inner surface scraped with a glass slide in order to obtain the mucosa, which were suspended in 5 mM EDTA, pH 7·4. The suspension was homogenised using a Waring blender and centrifuged at $400\,g$ for 10 min. The pellet was resuspended in 5 mM EDTA, pH 7·4 and centrifuged at $500\,g$ for 10 min. The pellet was again resuspended in 0·1 M mannitol, 10 mM MgCl$_2$, 1 mM Tris-HEPES, pH 7·4, slowly stirred for 10 min, allowed to stand for another 20 min and later centrifuged at $500\,g$ for 10 min. The pellet was resuspended in 0·1 M mannitol, 0·41 M NaSCN, 1 mM Tris-HEPES, pH 7·4, using 12 strokes of a Potter Elvehjem homogeniser, diluted 1:1 with 0·1 M mannitol, 10 mM MgCl$_2$, 1 mM Tris-HEPES and centrifuged at $6000\,g$ for 10 min. The supernatant was centrifuged at $34\,000\,g$ for 25 min. The pellet was resuspended in the same buffer and centrifuged as above. The resulting pellet was considered to be the BBMV fraction.

Relative purity of the BBMV fraction

The BBMV fraction purity was assayed both by electron microscopy and the activities of marker enzymes.

1   (a) *Electron microscopy*. The BBMV fraction was fixed in glutaraldehyde, post-fixed in osmium tetraoxide and embedded in Embed 812. Ultrathin slices were stained with uranyl acetate and lead citrate, and scanned with transmission electron microscopy (TEM) using a Phillips model EM-208.

2   (b) *Marker enzymes*. The relative purity of BBMV was routinely evaluated through the measurement of the activity of three marker enzymes. Maltase [EC 3.2.1.20] and sucrase [EC 3.2.1.48] were used as the BBMV marker, and were assayed according to Dahlqvist (1968) with some modifications. The incubation time was 15 min for maltase and 30 min for sucrase. The reaction tubes were boiled for 20 s at the end of the incubation time and the glucose produced was determined by the glucose oxidase

method (Trinder, 1969). The third marker enzyme was the Na$^+$/K$^+$-ATPase [EC 3.6.1.3], sensitive to ouabain, chosen as a marker for the basolateral membrane and was assayed following the method of Proverbio and Del Castillo (1981). The Na$^+$/K$^+$-ATPase activity was calculated as the difference between ATP hydrolysis in the absence and in the presence of ouabain. Phosphorus released was measured as described by Burchell et al. (1988).

Protein concentration of BBMV was measured by the method of Bradford (1976) using BSA as standard.

Measurement of L-[^{14}C]-arginine uptake

The assay was carried out following the fast filtration method of Hopfer et al. (1973), using freshly prepared vesicles. The BBMV were passed 10 times through a 28G needle in 100 mM mannitol, 100 mM KCl, 0·2 mM MgSO$_4$, 20 mM Tris-HEPES, pH 7·4, stood for 10 min at 25°C to allow vesicle formation (Black et al., 1989), centrifuged at $27\,000\,g$ for 30 min and resuspended in 50 mM mannitol, 2 mM Tris-HEPES, pH 7·4. The L-[^{14}C]-arginine uptake was started by the addition of 10 µl of BBMV to 250 µl of 100 mM mannitol, 100 mM NaCl, 2 mM MgSO$_4$, 2 mM L-arginine, 0·1 µCi L-[^{14}C]-arginine and 40 mM Tris-HEPES, pH 7·4. At the indicated times, the reaction was stopped by the addition of 2 ml of ice-cold 150 mM NaCl, 10 mM Tris-HEPES, pH 7·4, fast filtered through 0·22 µm nitrocellulose membrane, and washed three times with 3 ml of the same solution. To measure the effect of L-canavanine on L-arginine uptake by BBMV, the former was added to the incubation buffer at a concentration of 0·5 mM. The filters were dried and the radioactivity counted using a scintillation counter (Beckman Model LS 6000 IC). The results were expressed as arginine pmoles uptake/mg BBMV protein.

Statistical analysis

Data in the figures and tables are expressed as means ± SE. Comparisons between group means were performed by Student's *t*-test. A significant difference between means was considered to be present when $P < 0.05$.

RESULTS

L-Canavanine purity

The ^{13}C NMR spectrum of the L-canavanine (Figure 1) allowed identification of the presence
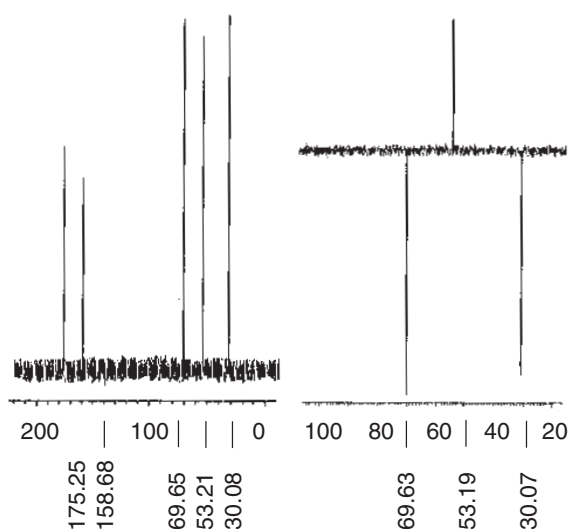
Figure 1. *L-Canavanine ^{13}C NMR. Twenty milligrams of L-canavanine were dissolved in D_2O. The spectrum was obtained using a JEOL Eclipse 270 with a superconductor magnet of 6345 T ($V_{resonance}$ $^{13}C = 67.93\,MHz$).*

Table 1. *Values of chemical displacement (δ) and allocation of carbon atoms of the L-canavanine analysed by ^{13}C NMR*

δ (ppm)[1]	Integration DEPT 135°[2]	Allocation[3]
30·0855	–CH$_2$–	–CH$_2$–
53·2086	–CH–	–N–CH–
69·6533	–CH$_2$–	–O–CH$_2$–
158·6770	–C–	–N–C=N–
		N–
175·2512	–C–	–C=O
		OH

[1]δ (ppm) = chemical displacement in parts per million.
[2]Integration DEPT 135° = number of hydrogen atoms bound to the same carbon atom.
[3]Allocation = allocation according to theoretical values of chemical displacement.

Figure 2. *L-Canavanine ^{1}H NMR. Twenty milligrams of L-canavanine were dissolved in D_2O. The spectrum was obtained using a JEOL Eclipse 270 with a superconductor magnet of 6345 T ($V_{resonance}$ $^{1}H = 270\,MHz$).*

Table 2. *Values of chemical displacement (δ) and allocation of hydrogen atoms of the L-canavanine analysed by ^{1}H NMR*

δ (ppm)[1]	Integration DEPT 135°[2]	Allocation[3]
2·04–2·17	2	–CH$_2$–
3·74–3·75	1	–CH–
3·83–3·87	2	–CH$_2$–

[1]δ (ppm) = chemical displacement in parts per million.
[2]Integration DEPT 135° = number of hydrogen atoms bound to the same carbon atom.
[3]Allocation = allocation according to theoretical values of chemical displacement.

of 5 carbon atoms with the chemical displacement values shown in Table 1. For the allocation of each of the referred carbons a DEPT-135° spectrum was used.

The ^{1}H NMR spectrum (Figure 2) provided the information presented in Table 2. The spectrum indicates that all the protons are very near to each other in the structure. The values of chemical displacement and allocation of hydrogen atoms correspond exactly with the allocation of carbon atoms derived from Table 1. The determined molecular weight corresponds to 175·091 g/mole and its structure $C_5H_{12}NO_3$. In the spectrum, there is no evidence of the presence of any other contaminating organic compound, thus establishing that the analysed sample was indeed L-canavanine.

Figure 3 shows an electron micrograph of BBMV. The preparations were essentially composed by almost spherical vesicles, of rough
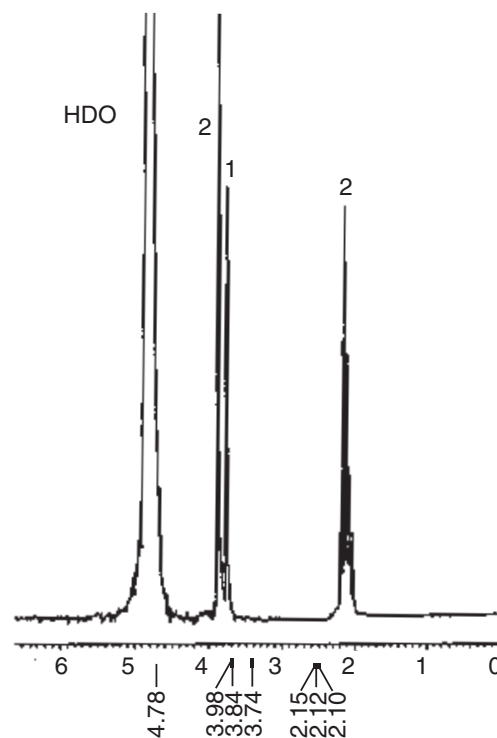
surface closed membranes, with an approximate diameter of 202·5 nm and with little or no electron dense material inside the vesicles. No other subcellular organelles were observed. Few unclosed membrane fragments and small vesicles enclosed in others of greater size were observed.

Table 3 shows a marked increase in the specific activities of the brush border membrane marker enzymes (sucrase and maltase) in the BBMV fraction in comparison with the activities of the same enzymes in the original homogenate. On the other hand, there was a 1·2 times decrease in the specific activity of the basolateral membrane marker enzyme (Na$^+$/K$^+$-ATPase).

Figure 4 shows the L-[^{14}C]-arginine uptake by BBMV as a function of time, measured in conditions of an initial gradient of 100 mM NaCl, in the absence and presence of L-canavanine. The BBMV L-[^{14}C]-arginine uptake increased from
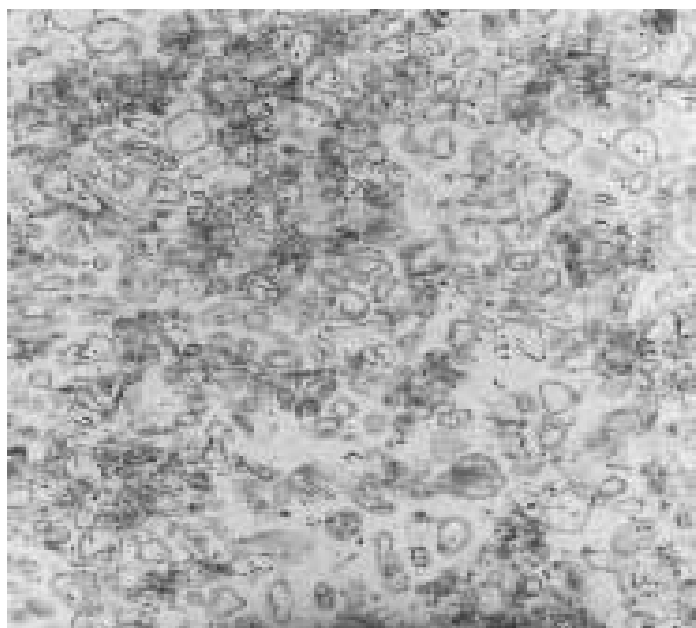
Figure 3. *BBMV electron microscopy. Intestinal BBMV were purified from 3-week-old chickens according to Hopfer et al. (1983), fixed in glutaraldehyde and processed for electron microscopy as described in Materials and Methods. The sections were observed using a Phillips EM 208 transmission electron microscopy. The bar corresponds to 1 μm.*

Table 3. *Marker enzyme activities in homogenate and BBMV isolated from broiler chickens*

Marker enzyme[1]	Homogenate[2]	BBMV[2]	Enrichment factor[3]
Sucrase	$50 \cdot 5 \pm 10 \cdot 1$	$809 \cdot 3 \pm 78 \cdot 9$	$16 \cdot 0$
Maltase	$596 \cdot 1 \pm 82 \cdot 8$	$10\,650 \cdot 9 \pm 845 \cdot 4$	$17 \cdot 9$
Na^+/K^+-ATPase	$37 \cdot 4 \pm 9 \cdot 4$	$31 \cdot 1 \pm 17 \cdot 2$	$0 \cdot 8$

[1]Sucrase and maltase activity are expressed as nmoles of glucose/min/mg protein. The Na^+/K^+-ATPase as nmoles of Pi/min/mg protein.
[2]The values are expressed as the means ± SD of 6 determinations by triplicate using 2 to 4 chickens.
[3]The enrichment factor was calculated as the relation: enzyme activity in BBMV fraction/enzyme activity in homogenate.

376 pmoles/mg protein at 15 s incubation time to reach a maximum of 944·9 pmoles/mg protein at 45 s incubation and later on, it declined. In the presence of 0·5 mM L-canavanine, L-[14C]-arginine uptake was drastically reduced at all times studied, inhibition at 30 s of 85·5% being the highest recorded. On average, the rate of L-[14C]-arginine uptake was reduced by 63·8% during 150 s of incubation.

All the differences found in L-arginine uptake due to the presence of L-canavanine were statistically significant ($P < 0 \cdot 05$).

DISCUSSION

The electron microscopy of the BBMV fraction (Figure 3) revealed that considering form, average size, lack of electron dense material in its interior and absence of contaminating organelles, the isolated BBMV constituted a highly
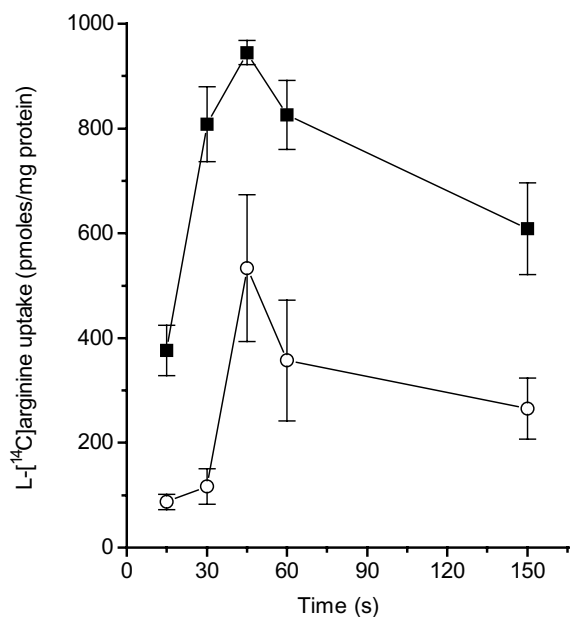

Figure 4. *L-[14C]-arginine uptake in the absence or presence of 0·5 mM L-canavanine, by BBMV from broiler chickens. Intestinal BBMV purified by the method of Hopfer et al. (1983) were used to carry out L-[14C]-arginine uptake in the absence (■) or in the presence of 0·5 mM L-canavanine (○) by the method of Hopfer et al. (1973). Each point corresponds to the mean of 5 determinations by triplicate ± SD. Differences in L-arginine uptake between the absence and the presence of L-canavanine were statistically significant at $P < 0 \cdot 05$.*

purified fraction which was similar to that reported for chickens by Max *et al.* (1978) and Black *et al.* (1989).

The enrichment of the maltase and sucrase activities as marker enzymes of the apical enterocyte membrane also confirmed the high

purity of the BBMV fraction. The sucrase specific activity obtained was higher than the values reported for BBMV from jejunum of 4- to 5-week-old chicks (Coleto *et al.*, 1998) and that of 6-week-old chicks (Torras-Llort *et al.*, 1996). The sucrase enrichment factor of the present work is lower than the 21·1 and 22·8 reported by Coleto *et al.* (1998) and Max *et al.* (1978) and higher than the 10·8 enrichment factor found by Torras-Llort *et al.* (1996). For the enzyme maltase no information was found in the literature to make any comparison.

It is known that ATPase activity in the basolateral membrane is inhibited by 1 mM ouabain (Fujita *et al.*, 1971). The Na^+/K^+-ATPase specific activity reported in the present work is similar to that obtained by Christiansen and Carlsen (1981). The enrichment factor of Na^+/K^+-ATPase was lower than that reported by Coleto *et al.* (1998), indicating a very low contamination with basolateral membrane in the preparation used. However, the consistent presence of certain levels of Na^+/K^+-ATPase activity in the brush border membrane fraction could be associated with some minor residues of basolateral membrane adhering to the brush border membrane during the cellular fractioning, as suggested previously by Quigley and Gotterer (1969). In general, the high enrichment factors of sucrase and maltase along with a reduction of the Na^+/K^+-ATPase activity, strongly suggest that the brush border membrane fraction prepared had a high purity and allowed these BBMV fractions to be used in the studies of amino acid transport across membranes.

The BBMV L-arginine uptake curve obtained is similar in shape to that reported for L-lysine uptake by chicken jejunal BBMV (Torras-Llort *et al.*, 1996). The greater L-arginine uptake observed in the present work, in comparison to that obtained with L-lysine (Torras-Llort *et al.*, 1996), might have been due, among other factors, to the different age of the chickens used since there is indication that in younger birds the uptake of cationic amino acids might be greater than in older birds (Obst and Diamond, 1992).

This study demonstrated that the L-canavanine, at a concentration of 0·5 mM, strongly inhibited L-arginine uptake by BBMV under the conditions tested and offers an explanation for the results found by Michelangeli and Vargas (1994). Herzberg *et al.* (1971) reported that L-canavanine also inhibited the transport of L-lysine and L-methionine indicating that the inhibitory effect of L-canavanine may affect not only the uptake of cationic amino acids but also those of the neutral amino acids which share, under certain conditions, similar transport systems. Additionally, it has been shown that L-canavanine inhibits arginine and lysine transport in *Neurospora crassa* (Bauerle and Garner, 1964), in *Saccharomyces cerevisiae* (Grenson *et al.*, 1966) and in *Escherichia coli* (Wilson and Holden, 1969).

In conclusion, the data obtained show that L-canavanine inhibits the Na^+-dependent L-arginine transport at the enterocyte apical membrane. The L-arginine uptake inhibition in BBMV by L-canavanine may reduce the intestinal absorption of the former amino acid and eventually change the pool of the cationic amino acids in chickens.

ACKNOWLEDGEMENTS

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