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# TRANSPORT OF CARNOSINE BY MOUSE INTESTINAL BRUSH-BORDER MEMBRANE VESICLES

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**The characteristics of carnosine (β-alanyl-L-histidine) transport have been studied using purified brush-border membrane vesicles from mouse small intestine. Uptake curves did not exhibit any overshoot phenomena, and were similar under $Na^+$, $K^+$ or choline$^+$ gradient conditions (extravesicular > intravesicular). However, uptake of histidine showed an overshoot phenomenon in the presence of a $Na^+$-gradient. There was no detectable hydrolysis of carnosine during 15 min of incubation with membrane vesicles under conditions used for transport experiments. Analysis of intravesicular contents further showed the complete absence of the constituent free amino acids of carnosine, and indicates that intact carnosine is transported. Studies on the effect of concentration on peptide uptake revealed that transport occurred by a saturable process conforming to Michaelis-Menten kinetics with a $K_m$ of 9.6 ± 1.4 mM and a $V_{max}$ of 2.9 ± 0.2 nmol/mg protein per 0.4 min. Uptake of carnosine was inhibited by both di- and tripeptides with a maximum inhibition of 68% by glycyl-L-leucyltyrosine. These results clearly demonstrate that carnosine is transported intact by a carrier-mediated, $Na^+$-independent process.**

## Introduction

Our recent studies on the intestinal transport of peptides using normal and papain-treated brush-border membrane vesicles showed that glycylphenylalanine and glycylleucine were transported by $Na^+$-independent and carrier-mediated systems [1,2]. The phenomenon of overshoot in response to a $Na^+$-gradient (extravesicular > intravesicular), characteristic of $Na^+$-gradient dependent active transport, demonstrated for monosaccharide and free amino acid transport [3–6], was not observed for either peptide. We further showed that there was a major diffusional component of the transport of these peptides and that other di- and tripeptides exerted only moderate inhibition. In the present study, carnosine (β-alanyl-L-histidine), a relatively non-hydrolyzable peptide, was used as a substrate for transport by brush-border membrane vesicles in order to compare the transport characteristics of hydrolyzable and non-hydrolyzable peptides. The transport of carnosine has been studied earlier using tissue slices [7–9] and renal brush-border membrane vesicles [10] but these studies have not yielded conclusive results on the transport characteristics

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Abbreviations: Tris, tris(hydroxymethyl)aminomethane; Hepes, 4(2-hydroxyethyl)-1-piperazineethane sulfonic acid.

of the intact peptide. Our studies show that carnosine is transported intact by a carrier-mediated process that is not dependent on the presence of $Na^+$.

## Materials and Methods

*Method*

*(1) Preparation of brush-border membrane vesicles.* The whole small intestine from control mice (strain C57 BL KSJ/dbm) was removed and processed as described previously [1,2]. Brush-border membranes were isolated by the calcium chloride precipitation method of Schmitz et al. [11] and brush-border membrane vesicles were obtained by the method of Hopfer et al. [3], as already described [12].

*(2) Transport studies.* The method employed has been fully described [1,12]. Briefly, the vesicles were resuspended to a final protein concentration of 5–15 mg/ml in 10 mM Tris-Hepes buffer (pH 7.5), 300 mM mannitol and 0.1 mM $MgSO_4$. Incubation media contained, in a 250 $\mu$l final volume: 10 mM Tris-Hepes buffer (pH 7.5), 0.1 mM $MgSO_4$, 300 mM mannitol, or 100 mM mannitol plus 100 mM of the sodium or potassium salts used or choline chloride, and 3.94 mM [*ring*-2-$^{14}$C]carnosine (1.25 $\mu$Ci). For transport studies with histidine and $\beta$-alanine, concentrations of 61.6 $\mu$M and 100 $\mu$M, respectively, were used. When the substrate concentration was varied or when the effects of various compounds on the uptake of carnosine were studied, the osmolarity of the buffer was adjusted to 300 mosM by appropriately adjusting the concentration of mannitol. Transport studies were initiated by the addition of vesicles containing 250–750 $\mu$g of membrane protein and were conducted at room temperature. At time intervals, 50-$\mu$l aliquots of the reaction mixture were transferred to the stop solution, filtered and washed as described previously [1,2]. Filters were then processed for scintillation counting [12]. All the experiments presented here were repeated at least three times and were always performed in duplicates. Using different membrane preparations, qualitatively identical results were obtained for the same experiments but in view of the significant variations in the equilibrium uptake values, only results of typical experiments are shown. The variations in duplicates for the experiments shown were always less than $\pm 5\%$ of the mean value.

The kinetic constants for transport ($K_m$ and $V_{max}$) were estimated by the weighted linear regression method of Wilkinson [13].

*(3) Membrane hydrolysis of carnosine.* The incubation medium used was the same as above except that unlabeled carnosine (3.94 mM) was used. At various time intervals, 50-$\mu$l aliquots of the reaction mixture were added to 1.0 ml of 80% ethanol. A control was included where incubation medium and vesicles were added directly to ethanol without incubation. The samples were evaporated to dryness under $N_2$ atmosphere and used for amino acid analysis using a Beckman amino acid analyzer.

*(4) Assay of intravesicular contents.* The membrane filters obtained from transport experiments at 0.4 and 1 min were immediately transferred to 25 ml of boiling water to stop all peptide hydrolysis. A control was included, where the incubation mixture without carnosine was filtered, and filter and an amount of unlabeled carnosine calculated to be equal to the transported amount were added to 25 ml of boiling water. For each time point, 25 filters were used. The water extracts were centrifuged at $35400 \times g$ for 60 min to remove all membrane particles and debris. The supernatant solutions were freeze-dried and used for amino acid analysis.

Protein was assayed by the method of Lowry et al. [14] using bovine serum albumin as standard.

*Materials*

[*ring*-2-$^{14}$C]Carnosine (spec. act. 1.27 mCi/mmol) was obtained from Amersham, Arlington Heights, IL. L-[U-$^{14}$C]Histidine, $\beta$-[1-$^{14}$C]alanine and D-[1-$^3$H(n)]mannitol were obtained from New England Nuclear Corp., Boston, MA. The membrane filters (pore size, 0.45 $\mu$m) were obtained from Sartorius. Unlabeled carnosine was obtained from Calbiochem and all other chemicals were obtained from Sigma and were of the highest purity available.

## Results

*(1) Uptake of carnosine, histidine and $\beta$-alanine*

The uptake of carnosine by brush-border mem-

brane vesicles in the presence of NaSCN, KSCN or choline chloride gradients (extravesicular > intravesicular) is presented in Fig. 1. Equilibrium was reached within 5 min and the rate of uptake was the same with all the three salt gradients. The overshoot phenomenon, a characteristic feature of active transport in response to an electrochemical gradient of $Na^+$, was not observed. Uptake of L-[U-$^{14}$C]histidine by membrane vesicles is shown in Fig. 2 at a substrate concentration of 61.6 $\mu$M and it is clear that the transport of histidine is stimulated by $Na^+$. Histidine was actively transported by a sodium-dependent carrier-mediated system as shown by the overshoot of uptake in the presence of a $Na^+$ gradient. The equilibrium values were the same in the presence of NaSCN or KSCN gradients. However, the uptake of $\beta$-alanine did not exhibit an overshoot phenomenon (results not shown), confirming the results of Stevens et al. [6] with rabbit jejunal brush-border vesicles.

*(2) Hydrolysis of carnosine*

The hydrolysis of carnosine by brush-border membrane vesicles was examined under conditions used for transport. The constituent amino acids, $\beta$-alanine and histidine, were not measurable in the incubation media during the time of the study

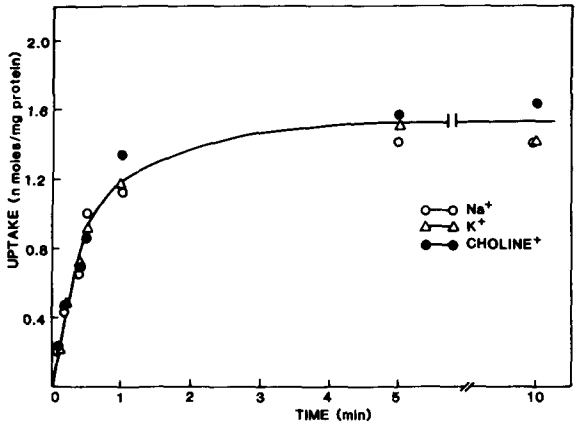


Fig. 1. Time-course study of [*ring*-2-$^{14}$C]carnosine uptake by mouse intestinal brush-border membrane vesicles. Uptake studies were performed as discussed under Experimental Procedures. Three different media prepared in 10 mM Tris-Hepes buffer (pH 7.5) containing 0.1 mM $MgSO_4$ were used ○——○, 100 mM mannitol + 100 mM NaSCN; △——△, 100 mM mannitol + 100 mM KSCN; ●——●, 100 mM mannitol + 100 mM choline chloride.

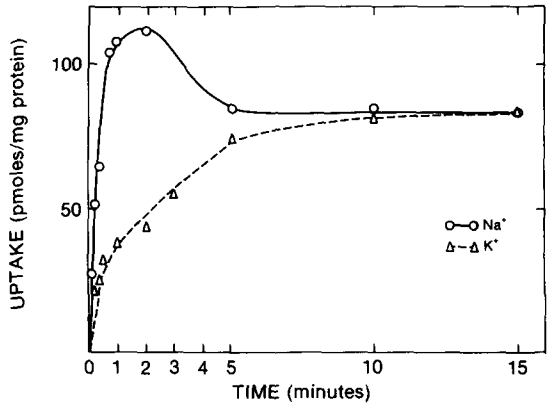


Fig. 2. Time-course study of L-[U-$^{14}$-C]histidine uptake by mouse intestinal brush-border membrane vesicles. Uptake studies were performed as discussed under Experimental Procedures. Two different media prepared in 10 mM Tris-Hepes buffer (pH 7.5) containing 0.1 mM $MgSO_4$ were used. ○——○, 100 mM mannitol + 100 mM NaSCN; △——△, 100 mannitol + 100 mM KSCN.

(15 min). The recovery of carnosine was between 88.4 to 95.5% of the expected values and there was no decrease in the amount of carnosine during incubation up to 15 min. These results clearly show that carnosine was not hydrolyzed during the transport study. Analysis of intravesicular contents further showed that $\beta$-alanine and histidine were not detectable inside the vesicles. These results, therefore, confirm that carnosine was transported intact across the brush-border membrane.

*(3) Concentration dependence of carnosine and histidine transport*

The effect of increasing concentration on transport of carnosine was investigated by determining initial velocities from uptake values at 0.4 min for carnosine concentrations varying from 1.5 mM up to 30 mM. The uptake of carnosine was saturable and conformed to Michaelis-Menten kinetics. Analysis of the results using the Lineweaver-Burk plot and weighted linear regression according to Wilkinson [13] gave a $K_m$ value of 9.6 $\pm$ 1.4 mM, and a $V_{max}$ value of 2.9 $\pm$ 0.2 nmol/mg protein per 0.4 min. The Lineweaver-Burk plot yielded a straight line through the point, indicating the presence of a single transport system. Similar studies on the uptake of histidine showed that uptake occurred by a saturable process with a $K_m$ value of

9.34 ± 1.23 mM and a $V_{max}$ of 5.16 ± 0.26 nmol/mg protein per 0.2 min.

*(4) Effects of free amino acids, di- and tripeptides on carnosine uptake*

Various free amino acids, dipeptides and tripeptides were tested for their inhibitory effect on uptake of carnosine and the results are shown in Table I. Among the free amino acids tested, L- and D-histidine inhibited the uptake of carnosine by about 30% while β-alanine did not exert any inhibition. The inhibition by other free amino acids varied from 10 to 20%. Among the dipeptides tested, glycyl-D-leucine, prolylglycine and β-alanylglycine did not show any appreciable inhibitory effect, while the extent of inhibition by glycyl-D-phenylalanine, glycyl-L-phenylalanine, glycyl-L-leucine, glycylsarcosine, glycyl-L-proline varied from 37 to 65%. Among the tripeptides, glycyl-L-leucyl-L-tyrosine showed the most inhibition (68%). It is noteworthy that the substitution of L-leucine by D-leucine in glycyl-L-leucine abolished the inhibition of uptake observed with glycyl-L-leucine. If the inhibitory effect of amino acids is considered non-specific for peptide uptake, it then appears that glycyl-L-leucyl-L-tyrosine, glycyl-L-leucine, glycyl-L-phenylalanine were strongly inhibitory (> 50%) while glycylsarcosine, glycyl-L-proline, glycyl-D-phenylalanine, L-leucylglycylglycine showed moderate inhibition (20–40%).

TABLE I

EFFECTS OF PEPTIDES AND FREE AMINO ACIDS ON CARNOSINE UPTAKE BY MOUSE INTESTINAL BRUSH-BORDER MEMBRANE VESICLES

Uptake measurements at 0.4 min were performed as discussed under Experimental Procedures in a medium containing 10 mM Tris-Hepes buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM NaCl, 3.94 mM radioactive carnosine, 40 mM mannitol and 60 mM of the different compounds tested. The given values represent the mean ± S.E. for duplicate assays on at least three different preparations. (a) Relative uptake as compared to carnosine uptake in the absence of test compounds. (b) Relative uptake when considering the effect of tested amino acids as non-specific. In this case, values in the presence of added peptides were compared to the mean between controls and uptake in the presence of amino acids except β-alanine (1.71 ± 0.09).

| Test compound | Peptide uptake (nmol/mg protein) | Relative uptake | |
|---|---|---|---|
| | | a | b |
| None | 2.09 ± 0.07 | 100 | 100 |
| L-Histidine | 1.46 ± 0.05 | 70 | 100 |
| D-Histidine | 1.39 ± 0.01 | 66 | 100 |
| Glycine | 1.88 ± 0.17 | 90 | 100 |
| L-Proline | 1.75 ± 0.08 | 84 | 100 |
| L-Phenylalanine | 1.68 ± 0.14 | 80 | 100 |
| L-Leucine | 1.68 ± 0.04 | 80 | 100 |
| β-Alanine | 2.22 ± 0.08 | 107 | – |
| Glycylglycine | 1.58 ± 0.26 | 75 | 93 |
| Glycylsarcosine | 1.25 ± 0.11 | 60 | 73 |
| Glycyl-L-proline | 1.01 ± 0.02 | 48 | 59 |
| Glycyl-L-leucine | 0.74 ± 0.06 | 35 | 43 |
| Glycyl-D-leucine | 2.06 ± 0.07 | 99 | 121 |
| Glycyl-L-phenylalanine | 0.76 ± 0.08 | 36 | 44 |
| Glycyl-D-phenylalanine | 1.31 ± 0.02 | 63 | 77 |
| L-Prolylglycine | 1.90 ± 0.25 | 91 | 111 |
| β-Alanylglycine | 2.20 ± 0.28 | 105 | 129 |
| Glycylglycylglycine | 1.64 ± 0.21 | 78 | 96 |
| Glycyl-L-leucyl-L-tyrosine | 0.66 ± 0.09 | 32 | 39 |
| Glycyl-L-prolyl-L-alanine | 1.68 ± 0.20 | 80 | 98 |
| L-Leucylglycylglycine | 1.79 ± 0.02 | 85 | 95 |
| L-Phenylalanylglycylglycine | 1.29 ± 0.22 | 62 | 76 |

## Discussion

Our recent studies on peptide transport by brush-border membrane vesicles were designed to investigate the characteristics of dipeptide transport in the absence of peptide hydrolysis. Previous studies with everted sacs and intestinal segments were complicated by the hydrolysis of peptides by cytosolic peptidases [15,16]. The use of papain-treated brush-border membrane vesicles was necessary for studies of the transport of glycylphenylalanine and glycylleucine, as these two peptides were shown to be hydrolyzed by brush-border membrane peptidases [1,2]. In our present studies with carnosine, we observed that there was no hydrolysis of carnosine by the brush-border membrane vesicles. Further, the absence of constituent free amino acids of carnosine in the intravesicular medium indicated that carnosine was not hydrolyzed during or after transport. Our results differ from those of Ganapathy and Leibach [10] with renal brush-border membrane vesicles. These investigators showed that about 30% of carnosine was hydrolyzed within the vesicles at 1 min of incubation, while free amino acids were not detected in the incubation medium. These results led to the hypothesis that the carnosine-hydrolyzing

peptidase was located in the brush-border membrane with an active site accessible to the inner side of the membrane [10]. Our results, however, show that intact carnosine was transported across the mouse intestinal brush-border membrane.

An examination of the time course of uptake (Figs. 1 and 2) revealed the absence of an overshoot phenomenon and the independence of uptake on the composition of the media for carnosine, and an active $Na^+$ gradient-dependent process for histidine. Our results, therefore, show that carnosine was transported intact down a concentration gradient by a $Na^+$-independent process. The absence of a concentrative mechanism for carnosine has also been shown with renal brush-border membrane vesicles [10] and anuran small intestine [17]. However, our results are in contrast to those of Matthews et al. [7] who reported active $Na^+$-dependent transport of carnosine by in vitro hamster jejunal segments. It should be pointed out that, when equal concentrations of carnosine were present in the initial mucosal and serosal fluids of hamster everted sacs, much of carnosine in the serosal fluid was hydrolyzed and that its concentration fell below that in the mucosal fluid at the end of the experiment [7]. Further, the replacement of $Na^+$ by $choline^+$ resulted in only moderate reductions in carnosine uptake by hamster intestinal segments, and hydrolysis of the peptide during absorption was indicated by the presence of the constituent free amino acids. Methews [16] had recently pointed out the need for further studies on $Na^+$-dependency and nature of the transport process. Our studies using brush border membrane vesicles show $Na^+$-dependent active transport for histidine but fail to show either $Na^+$-requirement or active transport for carnosine. Further, our results are similar to those of our earlier studies with glycylphenylalanine [1] and glycylleucine [2] using mouse intestinal brush-border membrane vesicles and to the studies of Ganapathy et al. [18–20] with glycylproline in renal as well as intestinal brush-border membrane vesicles. Our findings, therefore, strengthen the conclusion that dipeptide transport occurs by a non-active and $Na^+$-independent mechanism. It should be stressed that our results are unique in demonstrating the characteristics of intact peptide transport by brush-border membrane vesicles and differ significantly from the studies of Ganapathy and Leibach [10,19] and Sigrist-Nelson [21] with brush-border membrane vesicles from rabbit and rat, respectively, where significant hydrolysis of the tested peptides has been observed.

Further studies on the nature of carnosine transport process showed that uptake occurred by a saturable mechanism conforming to Michaelis-Menten kinetics. These results differ from our earlier studies with glycylphenylalanine and glycylleucine [1,2], where linearity of uptake with concentration was observed. A comparison of the kinetic characteristics of carnosine and histidine transport showed that histidine transport exhibits a higher $V_{max}$ than that of carnosine.

Studies on inhibition of carnosine uptake by free amino acids and other peptides clearly show that carnosine uptake was inhibited to a significant extent (up to 68%) by various peptides, in contrast to our earlier studies with glycylphenylalanine and glycylleucine, where inhibition did not exceed 43%. It can, therefore, be concluded that most of carnosine uptake was carrier-mediated and inhibited by di- and tripeptides. A closer examination of the extent of inhibition by various free amino acids and peptides revealed that the interactions are very complex. For example, the free amino acids L-histidine and D-histidine inhibited the uptake of carnosine by about 30% while free amino acids tested earlier on uptake of glycylphenylalanine and glycylleucine did not show any significant inhibition. Studies on the nature of inhibition, competitive or non-competitive, are necessary to fully understand the interactions between free amino acids and carnosine. The substitution of L-leucine by D-leucine in the peptide glycylleucine abolished the inhibition seen with glycylleucine. However, the extent of inhibition by glycyl-D-phenylalanine was similar to that by glycyl-L-phenylalanine; therefore, definite conclusions on the stereospecificity of the peptide uptake process cannot yet be drawn. Similar results, i.e. inhibition by glycyl-D-phenylalanine and lack of inhibition by glycyl-D-leucine of glycylproline transport, have been observed in our recent studies (unpublished observations). Inhibition of carnosine transport by tripeptides indicated the presence of a peptide uptake process with wide specificity for di- and tripeptides. More work on

the transport of tripeptides and interactions between di- and tripeptides for peptide transport is needed to elucidate the specificity of the uptake process.

In conclusion, the results obtained with carnosine show that it is taken up intact by mouse intestinal brush-border membrane vesicles by a $Na^+$-independent, carrier-mediated process. The demonstration of a major carrier-mediated process for carnosine is also different from our recent observations with hydrolyzable peptides like glycylphenylalanine and glycylleucine, where a major diffusional component was demonstrated. It is, therefore, probable that non-hydrolyzable peptides are transported mostly by carrier-mediated, $Na^+$-independent processes. More studies using various other di- and tripeptides will be required to further clarify these points.

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