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CHARACTERISTICS OF DIPEPTIDE TRANSPORT IN NORMAL AND PAPAIN-TREATED BRUSH BORDER MEMBRANE VESICLES FROM MOUSE INTESTINE

II. UPTAKE OF GLYCYL-L-LEUCINE

A. BERTELOOT, A.H. KHAN AND K. RAMASWAMY

Division of Gastroenterology, Department of Medicine, University of South Carolina School of Medicine, Veterans Hospital Enclave, Columbia, SC 29201 (U.S.A.)

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The characteristics of glycyl-L-leucine uptake have been studied using normal and papain-treated brush border membrane vesicles prepared from mouse small intestine. Our results show that: (1) glycyl-L-leucine is taken up into an osmotically reactive intravesicular space; (2) intact peptide transport can be studied up to 5 min without interference of hydrolytic events when using papain-treated vesicles; (3) the time course curves of glycyl-L-leucine uptake by normal and papain-treated vesicles are identical whatever the composition of the media (mannitol, NaSCN or KSCN) and do not show any overshoot phenomenon in the presence of an electrochemical gradient of Na^+ (extravesicular > intravesicular); (4) a linear relationship exists between initial rates of dipeptide uptake and peptide concentrations; (5) peptide uptake is weakly inhibited by other di- and tri-peptides at a 60 mM concentration. We can conclude that intact peptide transport occurs down a concentration gradient by a non-Na^+-dependent process and that passive and facilitated diffusion mechanisms, the latter either by a high affinity-low capacity system or a low affinity-high capacity system, are involved in this transport. It also appears that γ-glutamyltransferase and the γ-glutamyl cycle are not involved in peptide absorption.

Introduction

In the preceeding paper [1], we have reported that the treatment of brush border membrane vesicles from mouse intestine with papain results in a useful model to study the transport of intact peptides without interference from that of free amino acids released by membrane hydrolysis. With this new model, it has been clearly shown that glycyl-L-phenylalanine was taken up intact into an osmotically reactive intravesicular space by a non-Na^+-dependent process while superficial hydrolysis followed by uptake of free amino acids liberated was also involved in peptide absorption with normal vesicles. Further studies on the analysis of the nature of the intact peptide transport process were consistent with both passive and facilitated diffusion mechanisms of uptake, the latter occurring by either a high affinity-low capacity or a low affinity-high capacity system. In this paper, we report further attempts to characterize the nature of the mechanism(s) involved in intact peptide transport using another dipeptide, glycyl-L-leucine, a substrate that has been the subject of many in vitro and in vivo studies [2,3]. Our results failed to show any Na^+-dependent active transport of intact peptide but are consistent mostly with passive diffusion mechanism of uptake.

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid.

Materials and Methods

1. Preparation of brush border membrane vesicles. Control mice (strain C57-BL/Ks J-db/m) were killed by decapitation. The whole intestine was then removed and processed as previously described [1]. Brush border membranes were purified by the calcium chloride precipitation method of Schmitz et al. [4] and brush border membrane vesicles were obtained by the method of Hopfer [5] as already described [6].

2. Transport studies. The method employed has been fully described previously [1,6]. Briefly, incubation media contained, in a 250 μl final volume, 100 mM Tris-Hepes buffer (pH 7.5), 0.1 mM $MgSO_4$, 300 mM mannitol or 100 mM mannitol plus 100 mM NaSCN or KSCN, 0.25 mM, 0.38 mM or 1 mM glycyl-L-leucine and 1.22 μCi of [1-^{14}C]glycyl-L-leucine (Amersham, spec. act. 20 mCi/mmol). For transport studies of glycine, a concentration of 0.1 mM glycine with 1.16 μCi of [U-^{14}C]glycine (Amersham, spec. act. 118 mCi/mmol) were used. When the substrate concentration was varied or when the effect of various compounds on the uptake of glycyl-L-leucine was studied, the osmolarity of the buffer was maintained at 300 mM by appropriately adjusting the concentration of mannitol. Transport studies were initiated by the addition of 250–750 μg proteins of brush border membrane vesicles resuspended as described in Ref. 1 and were conducted at room temperature. At time intervals, 50 μl of the reaction mixture (50–150 μg of protein) were transferred to the stop solution, filtered and washed as previously described [1]. Filters were then processed for scintillation counting [6].

The effect of medium osmolarity on the uptake of glycyl-L-leucine was studied as described previously [1] with cellobiose as the impermeant solute [5].

Treatment of brush border membrane vesicles with gel-complexed papain was performed as previously described [1,6].

Linear regressions have been performed using the desk calculator HP-97 and the curve-fitting program (standard pack). Coefficients of determination, r^2, are shown in the legends of the figures when appropriate.

3. Assays. Brush border membrane-catalyzed hydrolysis of glycyl-L-leucine was studied in the same incubation system employed for analysis of uptake as described previously [1].

Papain was assayed by a titrimetric determination of the acid produced during the hydrolysis of benzoyl-arginyl ethyl ester (BAEE) as described previously [6].

Protein was assayed by the method of Lowry et al. [7] using bovine serum albumin as standard.

Results

1. Uptake of [1-^{14}C]glycyl-L-leucine by normal vesicles

Uptake of [1-^{14}C]glycyl-L-leucine by normal vesicles is shown in Figs. 1A and 1B for substrate concentrations of 0.38 and 1 mM, respectively. At the lower substrate concentration (Fig. 1A), equilibrium was reached within 5 min and the time course curves were identical in the presence of NaSCN or KSCN gradients (extravesicular > intravesicular). The same results were also obtained with the higher substrate concentration (Fig. 1B). No appreciable overshoot phenomenon, characteristic of active transport against an electrochemical gradient, has been detected at either peptide concentration.

2. Uptake of [1-^{14}C]glycyl-L-leucine by papain-treated vesicles

Uptake of [1-^{14}C]glycyl-L-leucine by papain-treated vesicles is shown in Figs. 2A and 2B for substrate concentrations of 0.38 and 1 mM, respectively. Similar uptake curves were obtained either in the absence or the presence of salt gradients. Equilibrium values were reached within 5 and 1 min for the respective concentrations of 0.38 (Fig. 2A) and 1 mM (Fig. 2B) and no overshoot was present. However, higher uptake values were obtained with papain-treated vesicles as compared to those obtained with normal vesicles in Fig. 1. As estimated from initial uptake values at 0.15 and 0.45 min, this increase after papain treatment perfectly matches the extent of protein removal as ratios of 1.66 (0.38 mM) and 1.58 (1 mM) between papain-treated and normal vesicles are closely related to the theoretical ratio of 1.64 corresponding to the 39% removal of protein by papain digestion [6].

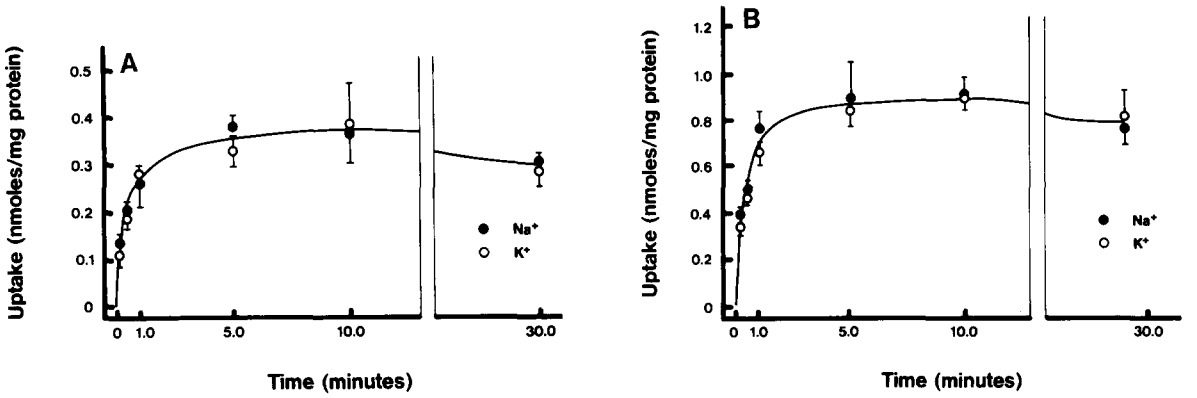

Fig. 1. Time course study of [1-^{14}C]glycyl-L-leucine uptake by normal mouse intestinal brush border membrane vesicles at peptide concentrations of 0.38 mM (A) and 1 mM (B), respectively. Uptake studies were performed as discussed under Materials and Methods. Two different media prepared in 10 mM Tris-Hepes buffer (pH 7.5) containing 0.1 mM $MgSO_4$, 100 mM mannitol, and either 100 mM NaSCN (●——●) or 100 mM KSCN (○——○) were used. Values represent the mean ±1 S.E. for at least three different preparations of vesicles, and duplicate assays at 0.15 and 0.45 min.

. *Brush border membrane-catalyzed hydrolysis of glycyl-L-leucine*

Figs. 3A and 3B show the time course of peptide hydrolysis by brush border membrane oligopeptidases during uptake experiments at substrate concentrations of 0.38 and 1 mM, respectively. With normal vesicles, there was no detectable hydrolysis during the first minute of incubation with the lower substrate concentration (Fig. 3A) and only 3% of the peptide was hydrolyzed during the same time at the higher concentration (Fig. 3B). After papain treatment, free leucine was not detectable in the incubation media until 5 min of incubation at both concentrations. It was also observed that hydrolysis of glycyl-L-leucine was independent of the composition of the incubation media (results not shown).

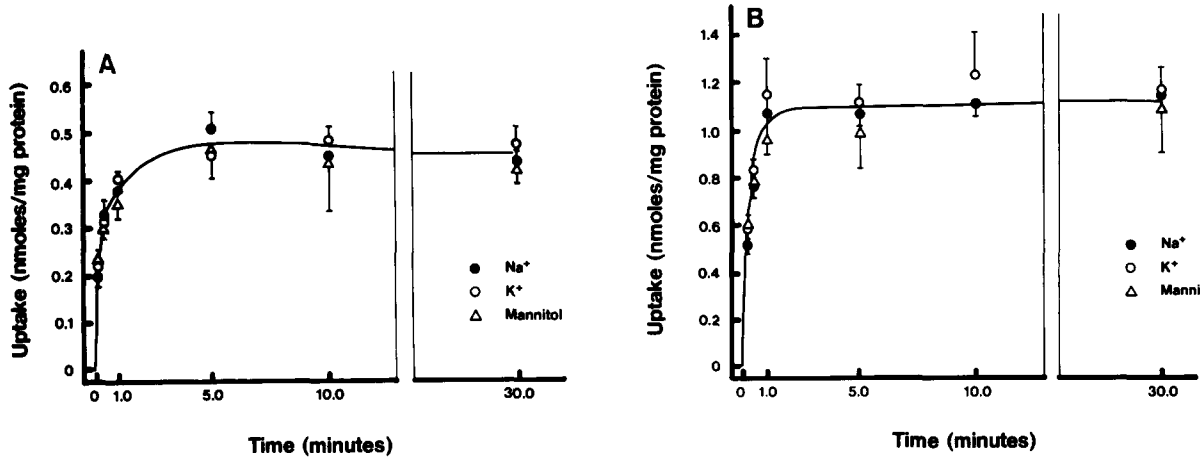

Fig. 2. Time course study of [1-^{14}C]glycyl-L-leucine uptake by papain-treated mouse intestinal brush border membrane vesicles at peptide concentrations of 0.38 mM (A) and 1 mM (B), respectively. Papain treatment and uptake studies were performed as discussed under Materials and Methods. Three different media prepared in 10 mM Tris-Hepes buffer (pH 7.5) containing 0.1 mM $MgSO_4$ were used: △——△, 300 mM mannitol; ●——●, 100 mM mannitol + 100 mM NaSCN; ○——○, 100 mM mannitol + 100 mM KSCN. Values represent the mean ±1 S.E. for at least three different preparations of vesicles, and duplicate assays at 0.15 and 0.45 min.

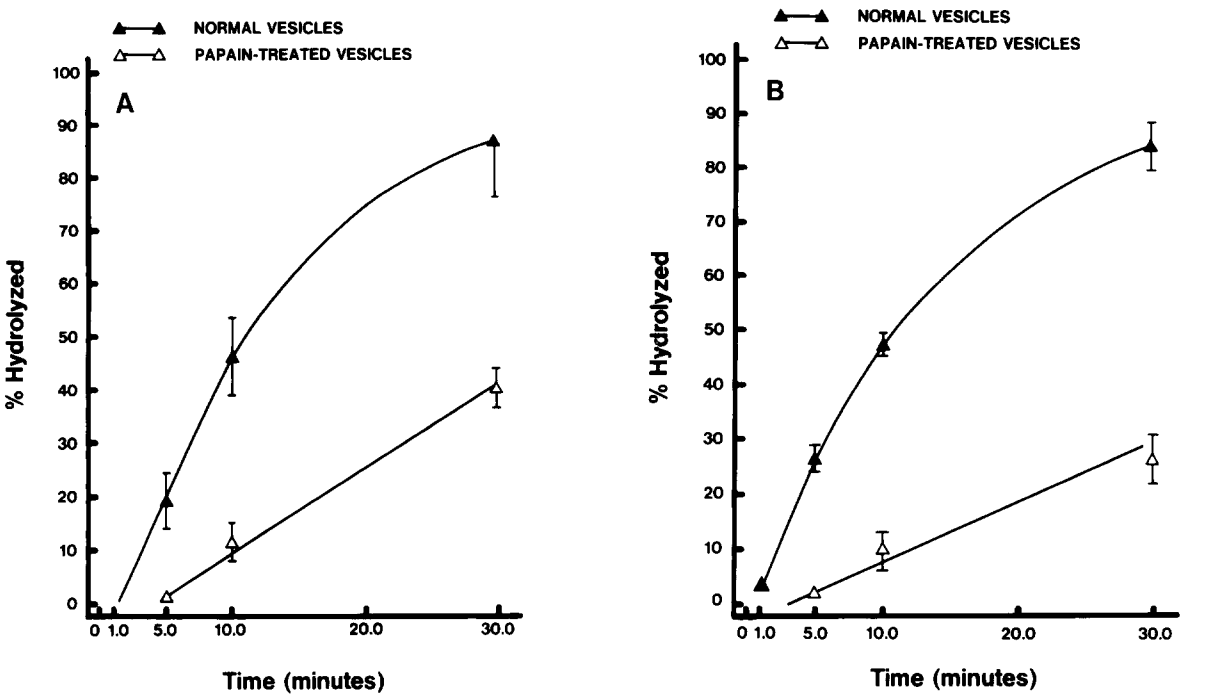

Fig. 3. Time course study of brush border membrane catalyzed hydrolysis of glycyl-L-leucine under conditions of uptake studies. Peptide concentrations were 0.38 mM (A) and 1 mM (B), respectively. The vesicle preparations used were the same as those used in Figs. 1 and 2. Values represent the mean ± 1 S.E. of three different experiments in either mannitol, NaSCN or KSCN media as it has been found that ions do not influence rates of peptides hydrolysis.

4. Uptake of [U-^{14}C]glycine by normal vesicles

Uptake of [U-^{14}C]glycine by normal vesicles is shown in Fig. 4 at a substrate concentration of 0.1 mM. Glycine was found to be actively transported by a sodium-dependent carrier-mediated mechanism as evidenced by the presence of a slight overshoot phenomenon in the presence of an Na^+-gradient (extravesicular > intravesicular). However, it has to be noted that glycine is poorly absorbed and weakly Na^+-dependent as compared to phenylalanine at the same concentration [1].

5. Uptake as a function of osmolarity

Uptake of radiolabel from [1-^{14}C]glycyl-L-leucine at equilibrium (5 min incubation) as a function of medium osmolarity is demonstrated in Fig. 5. This uptake was quite sensitive to changes in medium osmolarity, sharply decreasing with increases in osmolarity. When extrapolated to infinite medium osmolarity, uptake was negligible.

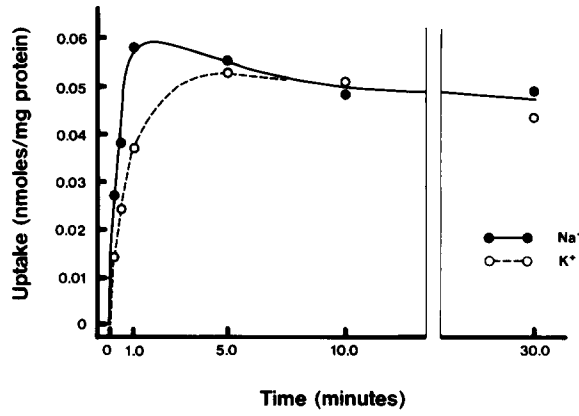

Fig. 4. Time course study of [U-^{14}C]glycine uptake by normal mouse intestinal brush border membrane vesicles. Uptake studies were performed as discussed under Materials and Methods. Media contained 10 mM Tris-Hepes buffer (pH 7.5), 0.1 mM $MgSO_4$ and either 100 mM mannitol + 100 mM NaSCN (●——●) or 100 mM mannitol + 100 mM KSCN (○ - - - ○). Values represent the mean for two different preparations of vesicles.

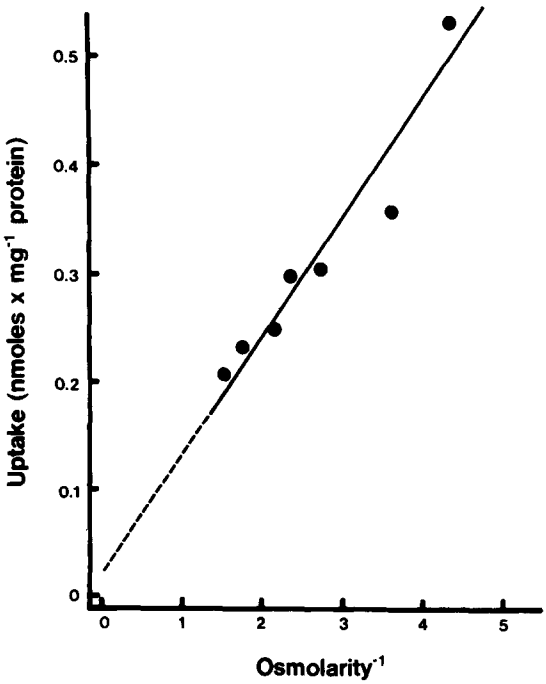

Fig. 5. Uptake of [1-^{14}C]glycyl-L-leucine by mouse intestinal brush border membrane vesicles as a function of the osmolarity of the incubation medium. Uptake studies were performed as discussed under Materials and Methods in media containing 10 mM Tris-Hepes buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM NaSCN, 0.38 mM [1-^{14}C]glycyl-L-leucine and variable concentrations of cellobiose to give the desired medium osmolarity. Values represent the mean of duplicate assays at 5 min incubation. A linear regression program has been used to fit the data and a coefficient of determination $r^2 = 0.958$ has been found.

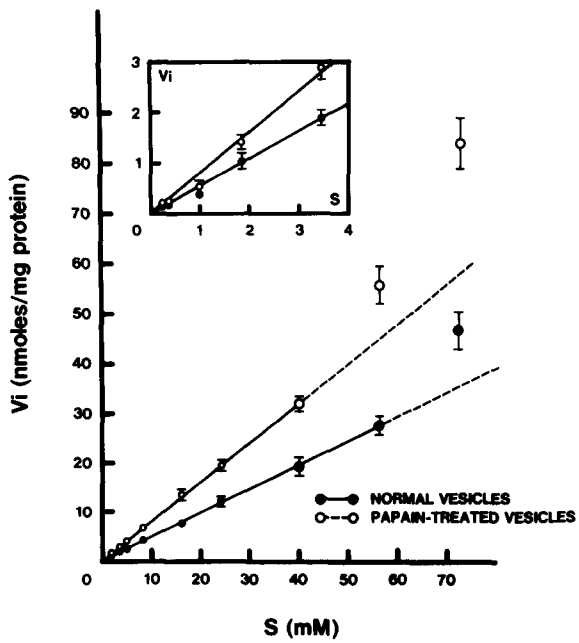

Fig. 6. Concentration dependence of glycyl-L-leucine initial uptake by normal (●———●) and papain-treated vesicles (○———○). Uptake studies were performed as discussed under Materials and Methods in media containing 10 mM Tris-Hepes buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM NaSCN, and variable concentrations of substrate and mannitol as to keep a 100 mM total concentration. Initial uptake has been measured at 0.15 min. Values represent the mean ±1 S.E. for duplicate assays on two different preparations of vesicles. A linear regression program has been used to fit the data and coefficients of determination $r^2 = 0.9993$ and $r^2 = 0.9996$ have been found for normal and papain-treated vesicles, repectively, for concentrations varying from 0 to 40 mM. Inset shows the absence of saturation in the low range of concentrations. Coefficients of determination $r^2 = 0.991$ and $r^2 = 0.988$ have been found by linear regression analysis for normal and papain-treated vesicles, respectively.

The above indicates uptake into an osmotically reactive intravesicular space without appreciable binding to the surface of the vesicles.

6. Concentration dependence of glycyl-L-leucine transport

The concentration dependence of glycyl-L-leucine uptake is shown in Fig. 6. Initial velocities have been estimated from uptake values at 0.15 min for dipeptide concentrations varying from 0.25 mM up to 72 mM. In these conditions, there was a linear relationship between initial uptake and substrate concentrations whether using normal ($r^2 = 0.9993$) or papain-treated ($r^2 = 0.9996$) vesicles. However, there was an upward deviation from linearity for the highest peptide concentrations, this effect being amplified when using papain-treated vesicles. Absence of saturation in the range of 0.25–4 mM dipeptide concentrations is also shown in the inset of Fig. 6. A ratio of 1.61 has been found between the slopes obtained for papain-treated and normal vesicles. This values agrees with the 39% removal of protein by papain digestion [6] and shows that papain treatment did not remove any protein essential for peptide transport.

7. Effects of peptides and free amino acids on glycyl-L-leucine uptake

A variety of peptides and free amino acids have

TABLE I

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

Uptake measurements at 0.15 min were performed as discussed under Materials and Methods in a medium containing 10 mM Tris-Hepes buffer (pH 7.5), 0.1 mM $MgSO_4$, 100 mM NaSCN, 0.38 mM of radioactive substrate, 40 mM mannitol and 60 mM of the different compounds tested. The given values represent the mean ± 1 S.E. for duplicate assays on two different preparations.
a, relative uptake as compared to glycyl-L-leucine 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 the three amino acids.

Test compound	Peptide uptake (nmol/mg protein	Relative uptake	
		a	b
None	0.150±0.006	100	100
Glycine	0.127±0.006	85 [a]	100
L-Leucine	0.120±0.008	80 [a]	100
L-Phenylalanine	0.136±0.005	91	100
Glycylglycine	0.128±0.007	85 [a]	96
Glycylsarcosine	0.124±0.008	83 [a]	93
Glycyl-L-proline	0.127±0.007	85 [a]	96
Glycyl-L-phenylalanine	0.122±0.009	81 [a]	92
L-Prolylglycine	0.133±0.008	89	100
Carnosine	0.110±0.008	73 [a]	83 [a]
β-Alanylglycine	0.120±0.009	80 [a]	90
Triglycine	0.101±0.011	67 [a]	76 [a]
Glycylglycylsarcosine	0.118±0.007	79 [a]	89 [a]
Glycyl-L-leucyl-L-tyrosine	0.119±0.008	79 [a]	90
Glycyl-L-prolyl-L-alanine	0.230±0.031	153 [a]	190 [a]
L-Leucylglycylglycine	0.134±0.005	89 [a]	101
L-Phenylalanylglycylglycine	0.120±0.013	80 [a]	90
Glutathione	0.119±0.010	79 [a]	90

[a] Indicates statistical significance relative to control values ($P<0.05$) as estimated by Student's t test.

been tested for their ability to inhibit glycyl-L-leucine uptake in normal vesicles and the results are described in Table I. Glycyl-L-leucine uptake was not significantly affected by the presence of either L-phenylalanine or L-prolylglycine in the incubation media. However, glycine, leucine, and various dipeptides and tripeptides tested had an inhibitory effect on glycyl-L-leucine uptake except for glycyl-L-prolyl-L-alanine that produced a stimulation. It has to be noted that the inhibition of glycyl-L-leucine uptake never exceeded 33%, this maximum value being obtained with triglycine. However, if the inhibitory effect of amino acids is considered as non-specific for peptide uptake, it then appears that only glycylglycylsarcosine, carnosine and triglycine produced significant inhibitions of glycyl-L-leucine uptake with respective values of 11, 17 and 24%.

Discussion

Our studies with papain-treated brush border membrane vesicles have been designed to investigate the characteristics of dipeptide transport in almost complete absence of peptide hydrolysis. It has been shown recently that controlled-papain digestion of intestinal brush border membrane vesicles did not modify the characteristics of sugar and amino acid transport [6]. In the previous report [1], we have also shown that papain digestion allowed the removal of approx. 70% of membrane oligopeptidase activities, thus permitting uptake studies of intact glycyl-L-phenylalanine without interference from free amino acids up to 1 min incubation, a time at which equilibrium uptake values were already reached. From the results of Fig. 3, it appears that this time can be

extended up to 5 min with glycyl-L-leucine as substrate, strengthening our earlier conclusion that peptide transport per se can be investigated using papain-treated vesicles. It also has to be noted that glycyl-L-leucine was not extensively hydrolyzed even by normal vesicles as compared to similar preparations from rat intestine [8]. This result is consistent with the localization of the majority of glycyl-L-leucine hydrolase activity in the cytosol fraction of monkey enterocytes [9,11].

An examination of the time course of [1-^{14}C]-glycyl-L-leucine uptake by normal (Fig. 1) and papain-treated (Fig. 2) vesicles revealed the absence of an overshoot phenomenon and the independence of uptake on the composition of the media at both concentrations of substrate used for these studies. However, higher uptake values were obtained after papain treatment, the increase closely matching the extent of protein removal during digestion, and showing that papain digestion did not remove any protein essential in peptide transport. These results rule out the involvement of γ-glutamyltransferase and oligopeptidases in group translocation mechanisms [2,25,26], as papain-treated vesicles showed reduced oligopeptidase activity and no detectable γ-glutamyltransferase activity [1]. The conclusion of these studies is then that glycyl-L-leucine was taken up intact down a concentration gradient by a non-Na^+-dependent mechanism, thus confirming our previous conclusion with glycyl-L-phenylalanine [1]. However, the presence of another mechanism, namely uptake of free amino acids following hydrolysis by the brush border membrane oligopeptidases, cannot be ruled out in normal vesicles. Glycine is poorly absorbed and weakly Na^+-dependent (Fig. 4) as compared to phenylalanine [1], and, therefore, this second mechanism would have remained undetectable. The conclusion that glycyl-L-leucine uptake is independent of the presence of an Na^+-gradient is at variance with the results obtained with rat intestinal brush border membrane vesicles [8] but agrees with more recent perfusion studies in the anuran small intestine [12–15]. It has to be noted that the high rates of glycyl-L-leucine hydrolysis found in rat vesicles [8] may have impaired a correct estimation of Na^+-dependency of intact peptide uptake. Our conclusion also agrees with more recent studies in rabbit intestinal and kidney vesicles [16] with glycyl-L-proline as substrate.

Further studies on the analysis of the nature of the intact peptide transport process revealed a linear relationship between initial uptake and substrate concentrations up to 40 mM (Fig. 6). However, above this concentration, an upward deviation from linearity has been found. Also, inhibition studies by a variety of peptides and amino acids showed significant reductions of glycyl-L-leucine uptake by amino acids tested (except L-phenylalanine) and peptides (except L-prolylglycine). However, the inhibition never exceeded 33% and only glycylglycylsarcosine, carnosine and triglycine were found inhibitory when the effect of amino acids was considered as non-specific. It also has to be noted that 53% stimulation was found when glycyl-L-prolyl-L-alanine was present in the media. Such results are not easy to interpret and some comments appear necessary. It has already been pointed out previously [1] that linear kinetics do not exclude mediation of transport by either a high affinity-low capacity system or a low affinity-high capacity system. However, the lack of important inhibitory effects by other peptides at a 60 mM concentration, though still compatible with the presence of the two carrier-mediated systems mentioned above, also points out the presence of either an important diffusible component in dipeptide uptake or several systems with overlapping specificities. Important simple diffusional components in transport of dipeptides have been reported recently [17,18] and no evidence for saturation was obtained for β-alanylglycylglycine [19] and prolylhydroxyproline [10] in hamster small intestine. However, the possibility of the involvement of several systems with overlapping specificities in peptide transport is compatible with the apparent lack of correlation among the inhibitory actions of di- and tri-peptides on the uptake of glycyl-L-phenylalanine [1] and glycyl-L-leucine. More puzzling is the observation that an apparent *cis*-stimulation effect on glycyl-L-leucine uptake was obtained with high concentrations of either glycyl-L-leucine or glycyl-L-prolyl-L-alanine. Such an effect could be produced by the formation of dimeric and oligomeric complexes between permeant molecules, either spontaneous or protein-mediated, thus reducing the possibility

of hydrogen bonding with water molecules and increasing the apparent permeability [21–24]. However, it has to be noted that such an effect is probably unphysiological considering the high concentrations of peptide involved (> 50 mM).

In conclusion, the results obtained with glycyl-L-phenylalanine and glycyl-L-leucine show that these peptides are taken up intact by mouse intestinal brush border membrane vesicles by a non-Na^+-dependent process but also that they undergo a certain amount of superficial hydrolysis followed by uptake of the free amino acids liberated. These results are compatible with both passive and facilitated diffusion mechanisms of uptake, the latter occurring either by low affinity-high capacity or high affinity-low capacity systems. However, the existence of more than one carrier-mediated system for transport of peptides has to be considered as uptake of these two peptides showed a few different characteristics. More studies using other peptides and peptides with higher specific radioactivities will be required to further clarify these points. Finally, our results, showing a lack of an effect of removal of γ-glutamyltransferase and oligopeptidases by papain-treatment on peptide transport, are proof against the involvement of these enzymes in group translocation mechanisms [2,25,26].

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