J. Physiol. (1980), **302**, *pp.* 155–169
With 6 *text-figures*
Printed in Great Britain

MECHANISMS OF GLYCYL-L-LEUCINE UPTAKE BY GUINEA-PIG SMALL INTESTINE: RELATIVE IMPORTANCE OF INTACT-PEPTIDE TRANSPORT

BY M. HIMUKAI* AND T. HOSHI†

*From the Department of Pediatrics,**
Tohoku University School of Medicine, Sendai 980,
and the Department of Physiology,† Faculty of Medicine,
University of Tokyo, Tokyo 113, Japan

(*Received 23 July 1979*)

SUMMARY

1. The characteristics of glycyl-L-leucine influx across the mucosal border of isolated guinea-pig ileum have been investigated. The influx of the peptide was measured with glycine-labelled or leucine-labelled compounds (Gly*-Leu or Gly-Leu*) and compared with that of constituent amino acids under various experimental conditions.

2. Gly-Leu* influx was twofold greater than Gly*-Leu influx over a wide range of peptide concentrations. The latter obeyed simple Michaelis–Menten kinetics whereas the former could be described in terms of two saturable components.

3. Total replacement of medium Na with mannitol had no effect on Gly*-Leu influx, while it markedly reduced Gly-Leu* influx to a level slightly greater than Gly*-Leu influx. L-Leucine influx was partially dependent on Na in contrast to glycine influx which was absolutely dependent on Na.

4. Gly*-Leu influx was not inhibited by the simultaneous presence of glycine or L-isoleucine, while Gly-Leu* influx was strongly inhibited by L-leucine and L-isoleucine. Gly-Leu* influx under submaximal inhibition by L-isoleucine was about the same as Gly*-Leu influx. Di- or tri-glycine did not inhibit glycyl-L-leucine influx, while glycyl-L-leucine markedly inhibited diglycine influx, the inhibition being not competitive but of the mixed type.

5. A Michaelis–Menten type relation was observed for the increment in the transmural potential induced by glycyl-L-leucine, L-leucine or the mixture of the dipeptide and L-leucine. In all cases, the values of the maximum potential change were identical, suggesting that a single electrogenic transfer mechanism was operating in these cases.

6. It is concluded that about a half of glycyl-L-leucine influx is mediated by a carrier system for intact glycyl-L-leucine which is independent of sodium, and the other half is transported as L-leucine after membrane surface hydrolysis, part of this component being sodium-dependent and electrogenic. It is also suggested that the carrier sites for glycyl-L-leucine and glycylglycine are very closely located but separated.

Address request for reprints to T.H.

0022-3751/80/8020-0398 $07.50 © 1980 The Physiological Society

INTRODUCTION

It has been established that dipeptides and tripeptides are transported intact across the intestinal brush border membrane (Matthews, 1975). The characteristics of the carrier-mediated transport of peptides have been studied by many investigators in isolated intestinal tissue preparations (Rubino, Field & Shwachman, 1971; Matthews, Addison & Burston, 1974; Addison, Burston, Payne, Wilkinson & Matthews, 1975; Burston, Marrs, Sleisenger, Sopanen & Matthews, 1977), *in situ* in the small intestines of man (Adibi & Soleimanpour, 1974) and frogs (Cheeseman & Parsons, 1976), and in isolated brush border membrane vesicles from rat small intestine (Sigrist-Nelson, 1975). It is also known that intestinal brush border membranes have dipeptidase activities (Peters, 1970; Fujita, Parsons & Wojnarowska, 1972) and some dipeptides are taken up as amino acids after membrane surface hydrolysis (Fern, Hider & London, 1969; Cheng, Navab, Lis, Miller & Matthews, 1971). The relative importance of these two uptake mechanisms appears to differ considerably among individual dipeptides. However, general rules determining the quantitative importance of these mechanisms are still unknown. Also, few studies have been made of developmental changes or comparative physiology of the relative importance of these mechanisms. For such studies, it seems of particular importance to know some standard dipeptides which are taken up by one of the following modes of uptake: (1) predominantly by the intact-peptide transport mechanism, (2) predominantly by amino acid transport after membrane surface hydrolysis, and (3) by an equivalent contribution of both mechanisms.

In a previous study, we demonstrated that, in guinea-pig small intestine, glycylglycine was transported almost entirely in the intact-peptide form (Himukai, Suzuki & Hoshi, 1978), confirming previous findings in other animal species (Fern *et al.* 1969; Adibi, 1971; Ugolev, 1974). In the present study, glycyl-L-leucine influxes across the mucosal border of guinea-pig ileum were examined in detail. It was found that this dipeptide was taken up by an almost equivalent contribution of the two mechanisms. Also, evidence is provided that the carrier sites for intact glycyl-L-leucine and glycylglycine are very closely located but separated. Developmental changes in the quantitative contributions will be dealt with for these dipeptides in a subsequent paper.

METHODS

Preparations. Everted segments of the ileum isolated from adult guinea-pigs were used in all experiments. The animals of either sex, weighing 400–550 g, were anaesthetized with urethane (1 g/kg body weight, I.P. injection), and the distal portion of the ileum, about 15 cm long, was excised. During the surgical operation, inhalation anaesthesia with ether was supplemented when the urethane anaesthesia seemed insufficient. The isolated portion was divided into small segments of 3 cm length, and each segment was everted in the usual way and fixed over a polyethylene tube of 5 mm outer diameter and about 10 cm length. Each polyethylene tube bore two red-line marks with a distance of 1·3 cm, and the everted intestine was tied with cotton threads at the levels of these marks. This procedure enabled us to obtain a constant area (2 cm^2) of the serosal surface. In the present study, all data obtained are presented in terms of unit area (cm^2) of the serosal surface. Such data can be converted to values per unit wet weight (mg) by using the conversion factor of $52 \pm 4{\cdot}1$ mg/cm^2 for the ileum of adult guinea-pigs (Himukai *et al.* 1978).

The samples thus prepared were preincubated in a standard buffer solution (composition described below) for 30 min at 37 °C. When the ionic composition of a test incubation medium was different from that of the standard solution, the samples were preincubated for an additional 1 min in a solution which had the same ionic composition as that of a test incubation medium. The following solution was used as the standard buffer solution in the present study: Na_2SO_4, 50 mM; mannitol, 160 mM; $KHCO_3$, 2·5 mM; KH_2PO_4, 0·25 mM; $CaSO_4$, 1·5 mM; $MgSO_4$, 1·0 mM; Tris/H_2SO_4, 20 mM, pH 7·4; and osmolality 290 m-osmole/kg H_2O. When concentrations of peptide or amino acid greater than 10 mM were employed, the concentration of mannitol in the incubation medium was correspondingly reduced. The reason for the use of a low Na^+/SO_4^{2-} Ringer solution was to prevent rapid decline of transport function of the preparations and to obtain larger transport-related potential changes across the intestinal wall. The relation between the magnitude of the transport-related potential change and the medium ionic composition was described elsewhere (Hoshi, Suzuki, Kusachi & Igarashi, 1976).

Uptake experiments. The tissue uptake of glycyl-L-leucine was measured by using either of two different radioactive tracers, [1-^{14}C]glycyl-L-leucine (Gly*-Leu) or glycyl-[U-^{14}C]-L-leucine (Gly-Leu*). Leucine influx from free leucine solution and glycine influx from free glycine solution were measured with [U-^{14}C]-L-leucine and [2-^{14}C]glycine, respectively. These tracers were added to the test media to a concentration of 0·05 or 0·1 μc/ml. Usually, D-[1-^{3}H]mannitol was also added to the media at 1 μc/ml. in order to correct for extracellular fluid adhering to the mucosal surface. The correction was made in the same way for both peptide and amino acid uptake. Upon completion of the test incubations, the tissues were washed in ice-cold isotonic mannitol solution (for about 5 sec), blotted on filter paper, then extracted in 3 ml. 3% trichloroacetic acid (TCA). The radioactivities of the extraction fluids were counted in a liquid scintillation counter (Aloka-LSC 602). All radioactive compounds were purchased from Radiochemical Centre, Amersham. The purity of radioactive dipeptides was examined by thin-layer chromatography before use. The following solvent systems were used for two-dimensional development: N-butanol:acetic acid:water (3:1:1 in volume) and phenol/water (3:1 in volume). After the development, the spots on the plate were collected and suspended in cab-o-sil toluene scintillator, and their radioactivities were counted in a liquid scintillation counter. The results showed that the purity was more than 96% for all dipeptides and no free glycine or L-leucine was detected. Further details of the experimental procedures were described in a previous paper (Himukai *et al.* 1978).

Efflux measurements. Efflux measurements were performed for glycine and L-leucine in order to assess whether there was a difference in the efflux rate constants of these two amino acids. The samples were equilibrated for 10 min with the standard solution containing one of the amino acids at 5 mM and its tracer. Subsequently, they were transferred into 4 ml. efflux solution (the standard solution) containing neither amino acid nor the tracer. The radioactivity of the efflux solution was measured at 0·25, 0·5, 1, 2, 4, 8 and 12 min. After completion of a series of sample collections, the amino acids remaining in the tissues were measured. From the data, the efflux curves, describing the amounts of amino acids remaining in the tissues against time, were obtained. The rate constants were calculated for each collection period according to Caldwell & Keynes (1969). In some experiments, the effect of addition of cold amino acid on its tracer efflux was examined. After control measurements of efflux for 12 min, one of the cold amino acids was added to the efflux solution to a final concentration of 1 mM in the case of L-leucine or 10 mM in the case of glycine, then the efflux measurements were followed for an additional 4–5 min.

Recording of the transport-related increments in the transmural potential. In this series of experiments, the everted preparations were fixed over a perforated polyethylene tube as described in a previous paper (Hoshi *et al.* 1976). The transmural potential difference was recorded by means of 1 M-KCl-agar bridges connected to half calomel electrodes and then to a high-sensitivity DC pen-recorder (National-VP 6541A). After the stabilization of the transmural potential, one of the constituent amino acids was added to the mucosal bathing solution and its concentration was raised in a step-wise fashion. The concentration-dependent increases in the potential were thus recorded. Secondly, a similar recording was made for glycyl-L-leucine, and then thirdly for the same amino acid as that tested first. Usually, the reproducibility was nearly complete. When the potential responses of the third record were smaller than those of the first, the mean values of the first and third records were compared with the second.

RESULTS

Time course of uptake of glycyl-L-leucine and its constituent amino acids

First, the time course of uptake was examined for glycyl-L-leucine and its constituent amino acids, glycine and L-leucine. The preparations were incubated at 37 °C in the standard buffer solutions containing one of the above substances at 5 mM. As shown in Fig. 1, the amounts of the substances taken up increased linearly

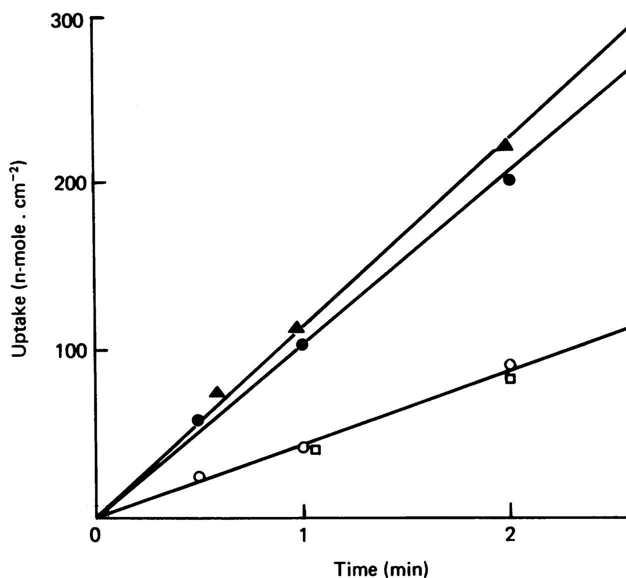

Fig. 1. The time course of uptake of glycyl-L-leucine, glycine and L-leucine. Standard medium conditions (37 °C). Open circles: Gly*-Leu uptake from 5 mM-glycyl-L-leucine solution; filled circles: Gly-Leu* uptake from 5 mM-glycyl-L-leucine; open squares: glycine uptake from free glycine solution (5 mM); filled triangles: L-leucine uptake from 5 mM-L-leucine solution. All values were corrected for the extracellular space.

with time at least up to 2 min in all cases. The rate of uptake of the leucine residue from glycyl-L-leucine was found to be about two times faster than that of the glycine residue and only slightly slower than that of L-leucine from free L-leucine solution. The uptake rate for the glycine residue was approximately the same as that for glycine from glycine solution. The observed difference in uptake rate between Gly*-Leu and Gly-Leu* is very similar to that seen in the monkey intestine (Das & Radhakrishnan, 1975), while it is in sharp contrast to identical rates observed in isolated brush border membrane vesicles from rat small intestine (Sigrist-Nelson, 1975).

Kinetic properties of uptake of glycyl-L-leucine and its constituent amino acids

As the tissue uptake of the substances was found to be linear with time up to 2 min, all influx measurements were performed by incubating the preparations for 1 min. Fig. 2 shows the relationship between the initial influx and medium concentration for glycyl-L-leucine and free L-leucine where the mean values obtained

from four or five experiments were plotted on a double-reciprocal plot. Glycyl-L-leucine influxes measured with Gly*-Leu and Gly-Leu* are indicated by different marks; open and closed circles, respectively. Again, Gly-Leu* influx is about two times higher than that of Gly*-Leu over a wide range of glycyl-L-leucine concentrations. It should be pointed out that the double-reciprocal plot for Gly*-Leu influx is linear over the concentration range from 0·5 to 60 mM, while the plot for

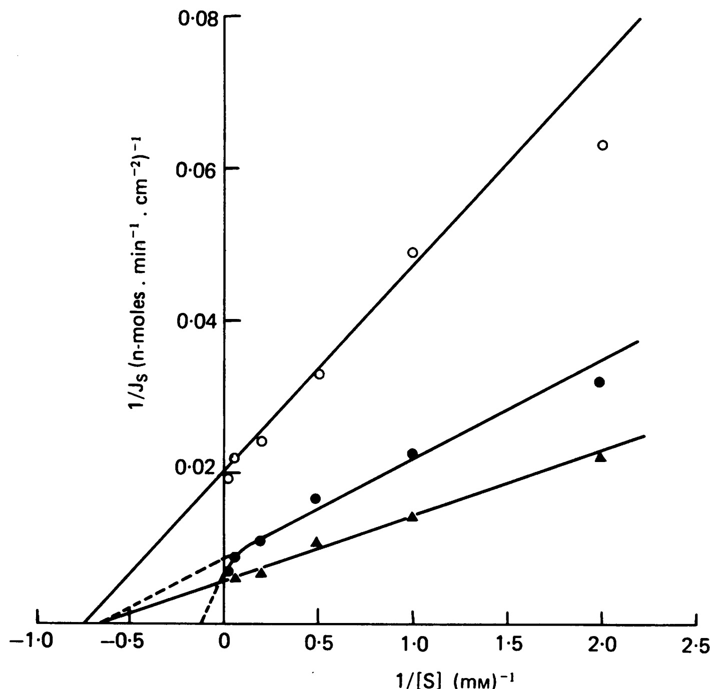

Fig. 2. Double reciprocal plots of the initial influxes of Gly*-Leu (open circles) and Gly-Leu* (filled circles) from glycyl-L-leucine solutions and of the initial influxes of L-leucine (triangles) from free L-leucine solutions. Standard medium conditions. Each value is the mean of four or six results from different animals. All values were corrected for extracellular space determined with [^{3}H]mannitol.

Gly-Leu* appears to show a two-component curve bending at around 10 mM. Indeed, the points fell on a single straight line in the range from 0·5 to 10 mM, but beyond this concentration, the influx values tended to become larger than expected from the straight line as the concentration was increased. The curve for Gly-Leu* at high concentrations appeared to cross the ordinate at the same point as the line for L-leucine (triangles). The plot for L-leucine was linear over the concentration range tested.

The value of the maximum influx (J_{max}) for glycyl-L-leucine measured with Gly*-Leu was 49·9 n-mole min^{-1} cm^{-2}, the value being much smaller than that for free L-leucine (175 n-mole min^{-1} cm^{-2}) and that for free glycine (290 n-mole min^{-1} cm^{-2}, Himukai *et al.* 1978). The values of half saturation concentration (K_t) for Gly*-Leu and L-leucine were 1·4 and 1·5 mM, respectively. The K_t value for leucine is about one eighteenth of that for glycine (27 mM, Himukai *et al.* 1978).

Na-dependence of glycyl-L-leucine influx

Total replacement of medium sodium with D-mannitol had no effect on glycyl-L-leucine influx when measured with Gly*-Leu. The data obtained at 5 mM-glycyl-L-leucine are shown in Table 1, but the same conclusion was reached at other peptide concentrations ranging from 1 to 60 mM (data not shown). In contrast, the influx measured with Gly-Leu* was reduced significantly by elimination of the external Na. However, Gly-Leu* influx in the absence of Na was still significantly greater than Gly*-Leu influx.

TABLE 1. Effect of total replacement of external sodium on the influxes of Gly*-Leu, Gly-Leu*, and L-leucine. Na was replaced by D-mannitol without changing the osmolality of the solutions. The data are given as means ± S.E.

Medium	Tracer	No. of observations	Influx (n-mole min^{-1} cm^{-2})
(*a*) Standard medium			
5 mM-glycyl-L-leucine	Gly*-Leu	6	38·6 ± 2·88
	Gly-Leu*	6	90·3 ± 7·02
0·5 mM-L-leucine	L-Leucine*	6	50·2 ± 4·35
(*b*) Na-free medium			
5 mM-glycyl-L-leucine	Gly*-Leu	6	39·2 ± 2·44
	Gly-Leu*	6	52·8 ± 3·73
0·5 mM-L-leucine	L-Leucine*	6	10·5 ± 0·88

L-Leucine influx was found to be partially Na-dependent. This is in contrast to glycine influx which is completely dependent on the presence of Na (Himukai *et al.* 1978). Among the data concerning leucine influx measured at various medium leucine concentrations, those obtained at 0·5 mM-leucine were of particular interest and suggestive of a contribution to one of the modes of glycyl-L-leucine uptake. In the presence of Na, leucine influx from 0·5 mM-leucine solution was 50 n-mole min^{-1} cm^{-2}, while it reduced to 10 n-mole min^{-1} cm^{-2} when Na was totally replaced. As shown in Table 1, the sum of free leucine influx from 0·5 mM-leucine and Gly*-Leu influx from 5 mM-glycyl-L-leucine is approximately the same as Gly-Leu* influx from 5 mM-glycyl-L-leucine, both in the absence and presence of Na. This seems to suggest that the leucine residue of the dipeptide is partly transported in intact peptide form and partly as free leucine after extracellular hydrolysis. The data also suggest that, when incubated with 5 mM-glycyl-L-leucine, L-leucine concentration in a local pool for leucine transport system rapidly increases to about 0·5 mM. Glycine concentration in the pool should increase concomitantly to the same level. However, glycine influx carried by the glycine transport is very small (5·6 ± 0·6 n-mole min^{-1} cm^{-2}, $n = 6$) at this concentration, even in the presence of Na, since the K_t value for glycine is very high (27 mM).

Inhibition experiments with free amino acids or di- or tri-glycine

In this series of experiments, observations were made of the effect on Gly*-Leu and Gly-Leu* influxes of the simultaneous presence of some neutral amino acids including glycine and leucine or di- or tri-glycine. The results of experiments with

glycine, leucine and isoleucine are summarized in Table 2. Gly*-Leu influx from 5 mM-glycyl-L-leucine solution was not affected by the addition of glycine or L-isoleucine at 10 mM. In contrast, Gly-Leu* influx was markedly inhibited by the presence of L-leucine at 5 mM or L-isoleucine at 10 mM. However, the values of inhibited influxes were still somewhat greater than or about the same as that of Gly*-Leu influx. It seems of particular importance that the inhibited value of

TABLE 2. Effect of Gly*-Leu and Gly-Leu* influxes of the simultaneous presence of glycine, L-leucine or isoleucine (*a*), and the effect of L-isoleucine on L-leucine influx (*b*)

Condition of influx measurement	Inhibitor (mM)	No. of observations	Influx (n-mole $min^{-1} cm^{-2}$)	% Control
(*a*) 5 mM-Glycyl-L-leucine				
Gly*-Leu	—	6	37.9 ± 2.75	
Gly*-Leu	Glycine (10)	6	39.8 ± 0.98	105
Gly*-Leu	L-Isoleucine (10)	6	37.7 ± 4.18	99
Gly-Leu*	—	4	99.0 ± 1.54	
Gly-Leu*	Glycine (10)	4	96.1 ± 10.9	97
Gly-Leu*	L-Leucine (5)	4	52.5 ± 4.65	53
Gly-Leu*	L-Isoleucine (10)	6	35.5 ± 5.75	36
(*b*) 0·5 mM-L-Leucine				
L-Leucine*	—	6	50.2 ± 4.35	
L-Leucine*	L-Isoleucine (10)	7	5.5 ± 0.2	11

TABLE 3. Effects of diglycine (*a*) and triglycine (*b*) on the influx of glycyl-L-leucine, and effect of triglycine on glycylglycine influx (*c*). *n*: number of observations

Condition of influx measurement	Inhibitor (mM)	*n*	Influx (n-mole $min^{-1} cm^{-2}$)	Statistical significance
(*a*) 5 mM-Glycyl-L-leucine				
Gly*-Leu	—	7	40.1 ± 3.39	
Gly*-Leu	Gly-Gly (10)	7	37.3 ± 4.00	n.s. ($0.3 < P$)
Gly-Leu*	—	7	86.4 ± 11.3	
Gly-Leu*	Gly-Gly (10)	7	84.3 ± 9.64	n.s. ($0.5 < P$)
(*b*) 5 mM-Glycyl-L-leucine				
Gly*-Leu	—	4	37.3 ± 5.25	
Gly*-Leu	Gly-Gly-Gly (10)	4	35.4 ± 8.88	n.s. ($0.5 < P$)
(*c*) 5 mM-Glycylglycine				
Gly*-Gly	—	4	30.9 ± 1.73	
Gly*-Gly	Gly-Gly-Gly (10)	4	16.8 ± 1.19	($P < 0.02$)

Gly-Leu* influx by 10 mM-isoleucine, a strong inhibitor of leucine transport, is nearly the same as control value of Gly*-Leu influx. The effect of 10 mM-isoleucine on leucine influx is shown in the same Table. Glycine at 10 mM had no effect on Gly-Leu* influx as in the case of Gly*-Leu.

The results of this series of experiments seem to indicate that Gly*-Leu influx is independent of both glycine and leucine transport systems and that the excess component of Gly-Leu* influx over Gly*-Leu influx is related to L-leucine transport.

The results of experiments in which the effects of glycylglycine and triglycine were examined are shown in Table 3. Both Gly*-Leu and Gly-Leu* influxes from 5 mM-glycyl-L-leucine solutions were not affected by the presence of 10 mM-glycyl-

glycine. The small differences seen in the mean values of influxes between control and experimental conditions were not statistically significant in either case. Glycylglycylglycine also had no effect on Gly*-Leu influx. For the purpose of comparison, the effect of the triglycine on glycylglycine (Gly*-Gly) influx was examined. In this case, a significant reduction, by about 46 %, was observed (Table 3).

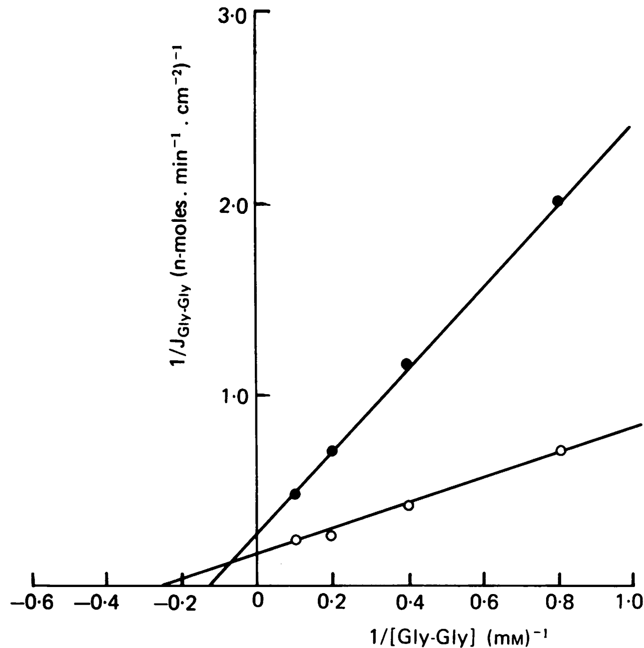

Fig. 3. Double reciprocal plots of glycylglycine influxes measured in the absence (open circles) and in the presence of 1·5 mM-glycyl-L-leucine. Each value is the mean of four results from different animals. All values were corrected for the extracellular space.

We previously demonstrated that glycylglycine transport was strongly inhibited by addition of glycyl-L-leucine (Himukai *et al.* 1978). This led us to suppose that both dipeptides share a common transport pathway. However, the results of the above inhibition study do not support this. Kinetically, the inhibitory action of glycyl-L-leucine on glycylglycine transport was found not to be competitive but of the mixed-type as shown in Fig. 3.

Efflux measurements

The data described above suggest that glycyl-L-leucine is taken up in two different ways, one involving transfer in the intact peptide form and the other as amino acids, preferentially as leucine, after extracellular membrane hydrolysis. However, there may be an alternative explanation for different uptake rates for Gly*-Leu and Gly-Leu*, as suggested by Das & Radhakrishnan (1975). They suggested that the peptide was taken up entirely in the intact form but glycine liberated by intracellular hydrolysis leaked out more rapidly than leucine. To test this possibility, efflux properties of glycine and leucine were compared.

The preparations preloaded with one of the amino acids for 10 min were transferred to the efflux solutions (the standard solution) containing no amino acids, and the effluxes of each amino acid were measured. Fig. 4 shows the time course of the decrease in amounts of the amino acids remaining in the tissues. The efflux curve for [^{3}H]mannitol is also shown in this Figure for the sake of comparison. As shown

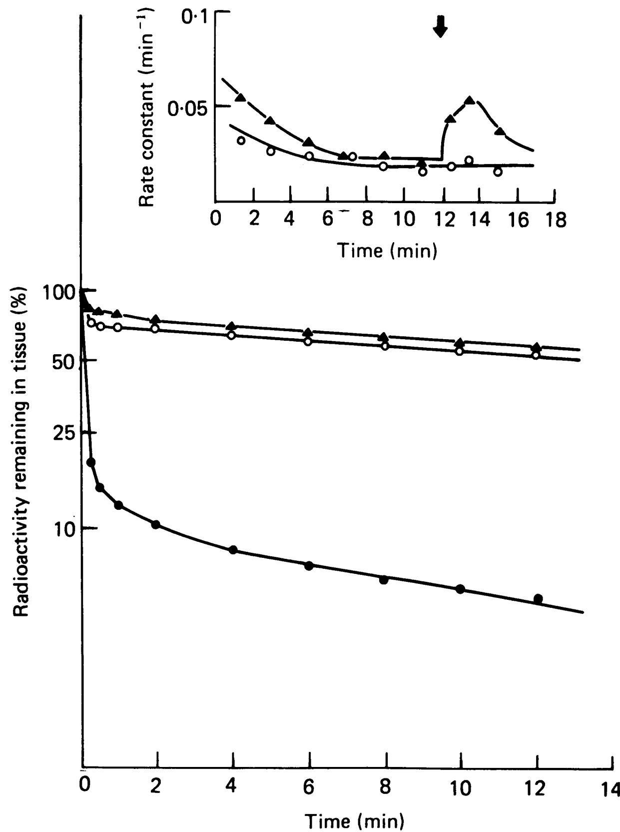

Fig. 4. Efflux curves for glycine (open circles) and L-leucine (filled triangles) from the everted intestines preloaded with respective amino acids. The preparations were preincubated for 10 min in the standard buffer solution containing one of the amino acids at 5 mM and its tracer (0·1 μc/ml.). [^{3}H]mannitol efflux (filled circles) is also shown for the purpose of comparison. In this case, the preparations were preincubated with [^{3}H]mannitol (1 μc/ml.) in the presence of 160 mM-cold mannitol. The efflux solution also contained 160 mM-mannitol. An inset shows the time course of the rate constants of effluxes of glycine (open circles) and L-leucine (triangles). At the time indicated by an arrow, cold glycine or L-leucine was added to the efflux solution. The data show that leucine efflux is markedly increased by the addition of cold leucine at 1 mM, while addition of glycine at 10 mM has no effect on glycine efflux. All values are means of results from four to six different animals.

in this Figure, no distinct difference was seen in the efflux curves between these two amino acids and the estimated values of the rate constants during a steady efflux stage were nearly identical.

An inset in Fig. 4 shows an example of the time course of changes in the efflux rate constants for both amino acids and the effect of addition of cold amino acids on

their efflux rate constants. The rate constants for each collection period declines with time before reaching a steady value. This indicates that there are at least two efflux components, one having a large rate constant (fast component) and the other a smaller one (slow component). In cases of transported amino acids, the efflux from the extracellular space usually has a much larger rate constant than that of the cellular efflux. Therefore, the change in rate constant after reaching a steady value may indicate mainly the change in the cellular efflux. The addition of cold

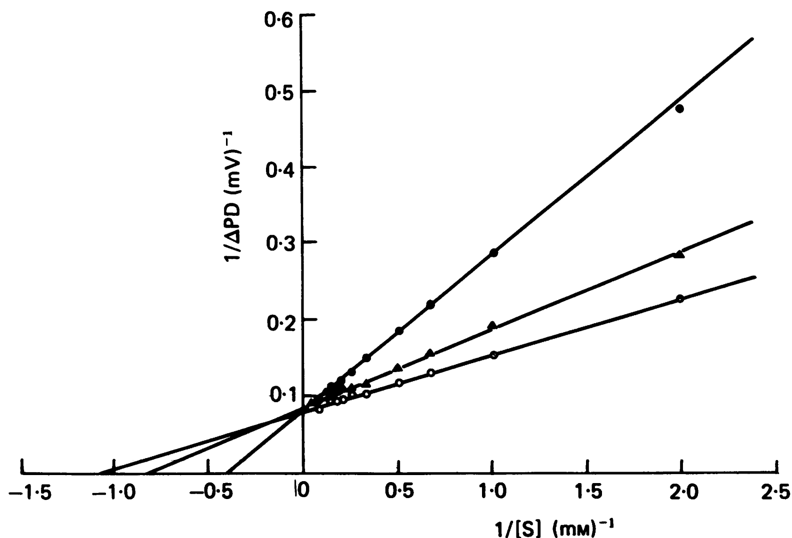

Fig. 5. Double reciprocal plots of the peptide- and amino acid-evoked changes in the transmural potential (ΔPD) against concentration ([S]). Closed circles: glycyl-L-leucine; triangles: L-leucine; open circles: the equimolar mixture of glycyl-L-leucine and L-leucine.

leucine, even at a low concentration, e.g. 1 mM, always caused a marked increase in the rate constant of leucine tracer efflux, while glycine tracer efflux was not affected by addition of cold glycine even at a high concentration, e.g. 10 mM. To explain such a great difference in trans-concentration effect is difficult at present. However, this finding indicates that leucine leaves the cell more readily than glycine.

Increments of the transmural potential difference caused by glycyl-L-leucine and its constituent amino acids

Na-linked transport of electrically neutral organic solutes across the brush border membrane is known to be electrogenic. A marked increase in the transmural potential (an increase in negativity of the mucosal side) has been shown to occur when various neutral amino acids, di- or tri-glycine are added to the mucosal bathing fluid (Kohn, Smyth & Wright, 1968). The magnitude of this transport-related potential change is directly proportional to Na-coupled influx of a transported organic solute across the mucosal border provided that the electrical conductance of the medium remains constant (Hoshi *et al.* 1976). The concentration-dependent increases in the transmural

potential were compared among L-leucine, glycyl-L-leucine and the equimolar mixture of L-leucine and glycyl-L-leucine. The data on the potential changes as a function of the concentration of the solute tested are shown in Fig. 5 where the data are plotted in the double-reciprocal manner. It is seen that the lines for the two single solutes and the mixture of them are all straight and intercepted the ordinate at a common point. This seems to suggest that the electrogenic components of transport in the three cases are mediated by a common mechanism, i.e. the sodium-dependent leucine transport mechanism.

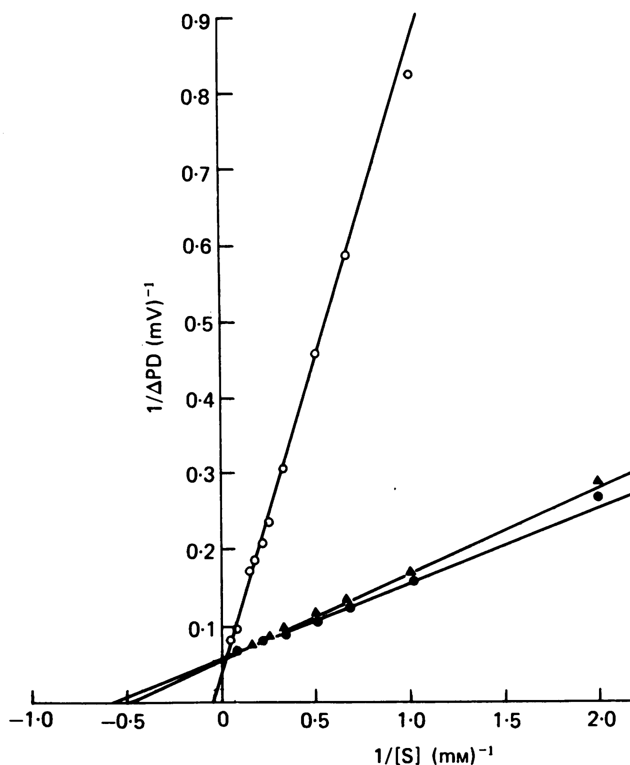

Fig. 6. Double reciprocal plots of the glycine- and leucine-evoked potentials *vs.* concentration. Open circles: glycine; triangles: L-leucine, and filled circles: the equimolar mixture of glycine and L-leucine.

Membrane hydrolysis of glycyl-L-leucine and consequent liberation of free glycine may also cause an increase in the transmural potential when Na is present in the medium. However, its contribution to the glycyl-L-leucine-induced potential changes seems to be negligibly small, since the concentration of glycine liberated into a local pool for the glycine transport system is relatively low and the K_t value for this amino acid is very high. A comparison of the glycine- and leucine-induced potential changes as a function of concentration is shown in Fig. 6. Also, a comparison was made for L-leucine and the equimolar mixture of glycine and L-leucine. As shown in this Figure, the lines for glycine and leucine are markedly different with regard

to K_t values and the intercepts on the ordinate. On the other hand, the lines for L-leucine and the mixture of the two amino acids are very similar, within the concentration range from 0·3 to 12 mM, supporting the view that the potential changes evoked by the equimolar mixture of glycine and L-leucine are caused predominantly by electrogenic leucine transport.

DISCUSSION

The results of the present study seem to indicate that, in guinea-pig small intestine, glycyl-L-leucine is taken up by intestinal epithelial cells through two different mechanisms: one is transport in intact peptide form and the other transport as amino acids, preferentially as leucine, after extracellular membrane hydrolysis. The relative importance of these mechanisms was found to be approximately the same over a wide range of the peptide concentration. The greater influx of Gly-Leu* than Gly*-Leu can be explained by the presence of a fraction of the dipeptide hydrolysed before the transport step and preferential transport of liberated leucine. This explanation is supported by the much smaller value of K_t for leucine (1·5 mM) than that for glycine (27 mM). It seems unlikely that glycine leaks out of cells more rapidly than leucine after cellular uptake of the intact peptide, as suggested by Das & Radhakrishnan (1975), since there is no significant difference in the efflux rate constants between glycine and leucine. Furthermore, the trans-stimulation of leucine but not of glycine efflux indicates that in fact leucine leaves the cell more readily than glycine, and not vice versa as required for the hypothesis of Das & Radhakrishnan (1975).

From the results described above the initial influx of glycyl-L-leucine (J) measured with Gly-Leu* can be described as

$$J = J_{\mathrm{G.L}} + J_{\mathrm{L}}, \quad (1)$$

where $J_{\mathrm{G.L}}$ is the influx component transferred in intact peptide form, J_{L} the component transferred as free leucine. The results of Na-replacement experiments and the inhibition study reveal that the peptide component, $J_{\mathrm{G.L}}$, is about the same as Gly*-Leu influx and free amino acid component is almost exclusively the leucine influx. As both Gly*-Leu and free leucine influxes conform to simple Michaelis–Menten kinetics, eqn. (1) can be rewritten as

$$J = J_{\mathrm{G.L}}^{\mathrm{max}} \frac{[\text{Gly-Leu}]_{\mathrm{m}}}{K_1 + [\text{Gly-Leu}]_{\mathrm{m}}} + J_{\mathrm{L}}^{\mathrm{max}} \frac{[\text{leu}]_{\mathrm{p}}}{K_2 + [\text{leu}]_{\mathrm{p}}}, \quad (2)$$

where $J_{\mathrm{G.L}}^{\mathrm{max}}$ and $J_{\mathrm{L}}^{\mathrm{max}}$ are the maximum influxes of the dipeptide and leucine, $[\text{Gly-Leu}]_{\mathrm{m}}$ the concentration of the peptide in the medium, K_1 and K_2 the values of K_t for Gly*-Leu and leucine, respectively, and $[\text{leu}]_{\mathrm{p}}$ the concentration of liberated leucine in a local pool for leucine transport system. The possible existence of a well-type local pool for hydrolysates of intestinal membrane hydrolysis has been suggested by many authors (Crane, 1967; Prichard, 1969; Parsons & Prichard, 1971; Igarashi, Saito, Himukai & Hoshi, 1976) and this has been assigned to the intermicrovillous space in the brush border (Hamilton & McMichael, 1968; Prichard, 1969). Calculation by making appropriate substitutions in eqn. (2) indicates that $[\text{leu}]_{\mathrm{p}}$ is about 0·5 mM when incubated with 5 mM-glycyl-L-leucine (also see the data

presented in Table 1). In contrast, actually estimated medium concentration of free leucine after 1 min of incubation with the peptide was less than 0·03 mM. The higher concentration of the hydrolysates in the local pool than in the medium and its role in efficient absorption of the hydrolysates have been clearly demonstrated for membrane digestion of disaccharides (Parsons & Prichard, 1971; Igarashi *et al.* 1976).

Sigrist-Nelson (1975) showed that, in isolated brush border membrane vesicles from rat intestines, Gly*-Leu and Gly-Leu* influxes did not differ significantly. The discrepancy between her findings and our observations is of particular interest because, in vesicular preparations, there is no local pool structure (the brush border structure). The amino acids liberated from the peptide by membrane hydrolysis will accumulate in the intermicrovillous spaces in intact cell preparations. In a separate series of experiments, we compared D-glucose transport from maltose between usual everted tissue preparations and purified brush border membrane vesicles isolated from rabbit ileum. In intact cell preparations glucose influxes from 10 mM-D-glucose and 5 mM-maltose solution were not so different, while in membrane vesicle preparations, the uptake curves were quite different. A marked overshoot uptake which forms a peak at 1–1·5 min was seen only in the case of uptake from free D-glucose. In the case of maltose, the uptake curve was nearly identical with that of D-mannitol or L-glucose (T. Hoshi & M. Himukai, unpublished observations). This supports the view that the brush border structure is essential for the efficient uptake of liberated amino acids or monosaccharides. The findings reported by Sigrist-Nelson, therefore, can be regarded as a phenomenon limited to the intact-peptide transport component.

One other important finding in the present study is that there are some differences in characteristics between transport of intact glycyl-L-leucine and intact glycyl-glycine. For example, the transport of intact glycyl-L-leucine is entirely independent of the presence of sodium and is non-electrogenic, whereas glycylglycine is electrogenic and partially dependent on Na. Glycine, glycylglycine and tri-glycine did not exert any inhibitory effect on transport of intact glycyl-L-leucine. Nevertheless, glycyl-L-leucine inhibited to a significant extent the transport of glycylglycine. Such a non-mutual inhibition cannot be explained in terms of competition unless a great difference in affinity for the binding site is present. In guinea-pig intestine, the value of K_t for glycylglycine is 3·4 mM, whereas the K_t value for intact glycyl-L-leucine (Gly*-Leu) is 1·5 mM. Therefore, another type of inhibition must be considered in this case. Kinetically, inhibitory action of glycyl-L-leucine was found to be of 'mixed-type'. This type of inhibition can arise from several situations (Segel, 1975). Among the possibilities, an allosteric interaction between two distinct sites for substrates, as proposed by Alvarado (1966, 1967), would be most probable. On the basis of this hypothesis, Alvarado (1967) explained the inhibitory action of phloretin on sugar transport which was somewhat different in nature from that of phlorizin, a true competitive inhibitor.

Our data suggest that the binding of glycyl-L-leucine to its binding site may cause some conformational distortion of glycylglycine binding site but the binding of glycylglycine has little effect on glycyl-L-leucine binding site. Robinson & Alvarado (1979) provided evidence that this type of interaction could occur even between

sugar and amino acid transport in the small intestine. In the case of peptide transport, it seems more probable that distinct binding sites for various di- or tri-peptides are located in the very proximity or on the same protein molecules within the brush border membrane.

It has been shown that the intestinal brush border membranes have dipeptidase activities which hydrolyse many kinds of dipeptides including glycyl-L-leucine (Peters, 1970; Fujita *et al.* 1972). Hydrolysis of leucine-containing dipeptides before the transport step has actually been demonstrated in everted rings of rat jejunum (Fern *et al.* 1969). Recent histological studies with a fluorescein-conjugated antibody of glycyl-L-leucine dipeptidase revealed that the fluorescence of the labelled antibody appeared as a thin rim on the outer surface of the brush border membrane (Norén, Dabelsteen, Sjöström & Josefsson, 1977). It is quite probable that such an outward-facing membrane-bound dipeptidase is responsible for the partial hydrolysis of glycyl-L-leucine during its uptake by the enterocytes.

REFERENCES

ADDISON, J. M., BURSTON, D., PAYNE, J. W., WILKINSON, S. & MATTHEWS, D. M. (1975). Evidence for active transport of tripeptides by hamster jejunum in vitro. *Clin. Sci.* **49**, 305–312.

ADIBI, S. A. (1971). Intestinal transport of dipeptides in man: relative importance of hydrolysis and intact absorption. *J. clin. Invest.* **50**, 2266–2275.

ADIBI, S. A. & SOLEIMANPOUR, M. R. (1974). Functional characterization of dipeptide transport system in human jejunum. *J. clin. Invest.* **53**, 1368–1374.

ALVARADO, F. (1966). Transport of sugars and amino acids in the intestine. Evidence for a common carrier. *Science, Lond.* **151**, 1010–1013.

ALVARADO, F. (1967). Hypothesis for the interaction of phlorizin and phloretin with membrane carriers for sugars. *Biochim. biophys. Acta* **135**, 483–495.

BURSTON, D., MARRS, T. C., SLEISENGER, M. H., SOPANEN, T. & MATTHEWS, D. M. (1977). Mechanisms of peptide transport. In *Peptide Transport and Hydrolysis*, Ciba Foundation Symposium 50, pp. 79–98. Amsterdam, Oxford, New York: Elsevier. Excerpta Medica.

CALDWELL, P. C. & KEYNES, R. D. (1969). The exchange of ^{22}Na between frog sartorius muscle and the bathing medium. In *Laboratory Techniques in Membrane Biophysics*, ed. PASSOW, H. & STÄMPFLI, R., pp. 63–68. Berlin, Heidelberg, New York: Springer.

CHENG, B., NAVAB, F., LIS, M. T., MILLER, T. N. & MATTHEWS, D. M. (1971). Mechanisms of dipeptide uptake by rat small intestine in vitro. *Clin. Sci.* **40**, 247–259.

CHEESEMAN, C. I. & PARSONS, D. S. (1976). The role of some small peptides in the transfer of amino nitrogen across the wall of vascularly perfused intestine. *J. Physiol.* **262**, 459–476.

CRANE, R. K. (1967). Structural and functional organization of an epithelial cell brush border. In *Intracellular Transport*, ed. WARREN, K. B., pp. 71–103. New York: Academic.

DAS, M. & RADHAKRISHNAN, A. N. (1975). Studies on a wide-spectrum intestinal dipeptide uptake system in the monkey and in the human. *Biochem. J.* **146**, 133–139.

FERN, E. B., HIDER, R. C. & LONDON, D. R. (1969). The sites of hydrolysis of dipeptides containing leucine and glycine by rat jejunum *in vitro*. *Biochem. J.* **114**, 855–861.

FUJITA, M., PARSONS, D. S. & WOJNAROWSKA, F. (1972). Oligopeptidases of brush border membranes of rat small intestinal mucosal cells. *J. Physiol.* **227**, 377–394.

HAMILTON, J. D. & MCMICHAEL, H. B. (1968). Role of the microvillus in the absorption of disaccharides. *Lancet* **ii**, 154–157.

HIMUKAI, M., SUZUKI, Y. & HOSHI, T. (1978). Difference in characteristics between glycine and glycylglycine transport in guinea pig small intestine. *Jap. J. Physiol.* **28**, 499–510.

HOSHI, T., SUZUKI, Y., KUSACHI, T. & IGARASHI, Y. (1976). Interrelationship between sugar-evoked increases in transmural potential difference and sugar influxes across the mucosal border in the small intestine. *Tohoku J. exp. Med.* **119**, 201–209.

IGARASHI, Y., SAITO, Y., HIMUKAI, M. & HOSHI, T. (1976). Interpretation of disaccharide-dependent electrical potential difference in the small intestine. *Jap. J. Physiol.* **26**, 79–92.

KOHN, P. G., SMYTH, D. H. & WRIGHT, E. M. (1968). Effects of amino acids, dipeptides and disaccharides on the electric potential across rat small intestine. *J. Physiol.* **196**, 723–746.

MATTHEWS, D. M. (1975). Intestinal absorption of peptides. *Physiol. Rev.* **55**, 537–608.

MATTHEWS, D. M., ADDISON, J. M. & BURSTON, D. (1974). Evidence for active transport of the dipeptide carnosine (β-alanyl-L-histidine) by hamster jejunum in vitro. *Clin. Sci.* **46**, 693–705.

NORÉN, O., DABELSTEEN, E., SJÖSTRÖM, H. & JOSEFSSON, L. (1977). Histological localization of two dipeptidases in the pig small intestine and liver, using immunofluorescence. *Gastroenterology* **72**, 87–92.

PARSONS, D. S. & PRICHARD, J. S. (1971). Relationships between disaccharide hydrolysis and sugar transport in amphibian small intestine. *J. Physiol.* **212**, 299–319.

PETERS, T. J. (1970). The subcellular localization of di- and tri-peptide hydrolase activity in guinea-pig small intestine. *Biochem. J.* **120**, 195–203.

PRICHARD, J. S. (1969). Role of the intestinal microvilli and glycocalyx in the absorption of disaccharides. *Nature, Lond.* **221**, 369–371.

ROBINSON, J. W. L. & ALVARADO, F. (1979). Interactions between tryptophan, phenylalanine and sugar transport in the small intestinal mucosa. *J. Neural. Transm.* suppl. **15**, 125–137.

RUBINO, A., FIELD, M. & SHWACHMAN, H. (1971). Intestinal transport of amino acid residues of dipeptides. *J. biol. Chem.* **246**, 3542–3548.

SEGEL, I. H. (1975). *Enzyme Kinetics*, pp. 170. New York, London, Sydney, Toronto: John Wiley.

SIGRIST-NELSON, K. (1975). Dipeptide transport in isolated intestinal brush border membrane. *Biochim. biophys. Acta* **394**, 220–226.

UGOLEV, A. M. (1974). Membrane (contact) digestion. In *Biomembranes, Intestinal Absorption*, vol. 4A, ed. SMYTH, D. H., pp. 285–362. London, New York: Plenum.