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Dipeptide transport and hydrolysis in isolated loops of rat small intestine: effects of stereospecificity

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1. Isolated jejunal loops of rat small intestine were perfused by a single pass of bicarbonate Krebs–Ringer solution containing either D- or L-phenylalanine or one of eight dipeptides formed from D- or L-alanine plus D- or L-phenylalanine.

2. At 0·5 mM L-phenylalanyl-L-alanine increased serosal phenylalanine appearance to forty times the control rate giving a value similar to that found with 0·5 mM free L-phenylalanine. No serosal dipeptide could be detected.

3. Perfusions with the two mixed dipeptides with *N*-terminal D-amino acids (D-alanyl-L-phenylalanine and D-phenylalanyl-L-alanine) gave rise to the appearance of intact dipeptides in the serosal secretions although there were substantial differences in their rates of absorption and subsequent hydrolysis.

4. L-Alanyl-D-phenylalanine was absorbed from the lumen three to five times as fast as L-phenylalanyl-D-alanine. At 1 mM L-alanyl-D-phenylalanine transferred D-phenylalanine across the epithelial layer at more than seven times the rate found with the same concentration of the free D-amino acid.

5. Perfusions with D-alanyl-D-phenylalanine or D-phenylalanyl-D-alanine showed that these two dipeptides are poor substrates for both transport and hydrolysis by the rat small intestine.

6. Analysis of mucosal tissue extracts after perfusion with the two mixed dipeptides with *N*-terminal D-amino acids revealed that both dipeptides were accumulated within the mucosa and suggested that exit across the basolateral membrane was rate limiting for transepithelial dipeptide transport.

It is generally accepted that most di- and tripeptides are absorbed intact from the lumen by the mucosa of the small intestine even though it is normally only the constituent amino acids which appear in the vascular system (Gardner, 1988; Matthews, 1991; Meredith & Boyd, 1995). However, the presence of peptidases on the enterocyte brush border, as well as in the cytosol and the submucosal compartment, makes the site of hydrolysis for any particular peptide uncertain. Some small oligopeptides such as glycyl-proline are relatively resistant to hydrolysis (Cheeseman & Johnston, 1982), and their transport across the brush-border membrane has been shown to be proton dependent (Ganapathy & Leibach, 1985). More recently a proton-coupled oligopeptide transporter has been cloned from rabbit small intestine (Fei *et al.* 1994) and another quite different molecule which stimulates peptide uptake has been cloned from human Caco-2 cell cultures (Dantzig *et al.* 1994); the latter molecule may act as an activator rather than a transporter.

In a recent major review, Matthews (1991) pointed out that despite many years of research on peptide absorption, there have been no systematic investigations of the absorption of short peptides from the lumen of the small intestine. This has been largely due to a shortage of commercially available peptides and a tendency to rely on transport studies with labelled peptides. For the studies reported in this paper we have synthesized the complete set of eight dipeptides containing both alanine and phenylalanine. By using phenylalanine dipeptides we have been able to detect the peptides and free phenylalanine directly with high-performance liquid chromatography (HPLC) analytical systems, thus avoiding the need to use derivatization or radiolabelling.

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One of the problems with studies of peptide absorption with intact intestinal preparations is the rapid rate of hydrolysis by brush border and cytosolic peptidases. Since we synthesized our own peptides we were able to test the effects of including D-amino acids which have been shown to confer a degree of resistance to peptidase activity (Asatoor, Chada, Milne & Prosser, 1973; Boyd & Ward, 1982). Previous reports have also suggested that the inclusion of D-amino acids reduces the rate of absorption from the intestinal lumen (Asatoor *et al.* 1973; Bai & Amidon, 1992).

The aim of the study described in this paper was to investigate the transmural transfer of the eight phenylalanine dipeptides and/or their constituent phenylalanines from the lumen to the serosa. By employing the isolated loop preparation of rat small intestine (Bronk & Hastewell, 1988) with a single pass luminal perfusate we were able to look at transmural peptide transport at constant luminal concentrations. Examination of the peptide and phenylalanine concentrations in the mucosal tissue at the end of each perfusion made it possible to determine the extent to which these solutes were accumulated within the mucosa. Care was taken to follow the procedures recommended by Gardner & Plumb (1979) who reported that peptidases were rapidly released from *in vitro* preparations of intestine unless the preparation was set up while the animals were under anaesthesia.

A preliminary report of some of this work has been published (Bronk, Lister & Helliwell, 1993).

METHODS

Materials

All chemicals were of analytical grade. The reagents used for peptide synthesis were purchased from the Sigma Chemical Company Ltd, Poole, Dorset, UK. Sodium pentobarbitone was purchased from May and Baker Ltd, Dagenham, UK.

Animals

Male Wistar rats (250 g) were purchased from Harlan Olac Ltd, Bicester, UK and fed *ad libitum* on rat and mouse diet (Bantin & Kingham Ltd, Humberside, UK) until they reached a weight of 280 g and then deprived of food, except for free access to 0·5% (w/v) D-glucose solution for approximately 18 h prior to the experiment. Each animal was anaesthetized with an intraperitoneal injection of sodium pentobarbitone (10 mg (100 g body wt)$^{-1}$). After removal of the loop of small intestine (see below) the animal was killed by exsanguination.

Peptide synthesis

Peptide synthesis was by standard techniques (cf. Bailey, 1990). For the experiments described in this paper the following eight dipeptides were synthesized: L-alanyl-L-phenylalanine, L-alanyl-D-phenylalanine, D-alanyl-L-phenylalanine, D-alanyl-D-phenylalanine, L-phenylalanyl-L-alanine, L-phenylalanyl-D-alanine, D-phenylalanyl-L-alanine and D-phenylalanyl-D-alanine.

Each of the dipeptides was synthesized by condensation of *N*-tertiary-butyl-oxycarbonyl-derivative of the *N*-terminal amino acid with the carboxy-benzyl-L-derivative of the *C*-terminal amino acid. The products were subjected to a series of acid and alkali washes to eliminate the starting materials and by-products before removal of the protecting groups. Finally, the samples were freeze dried and the purity, which was checked by mass spectrophotometry, NMR and HPLC, was more than 95%.

Perfusion medium

The perfusion medium was bicarbonate Krebs–Ringer solution, containing (mM): 120 NaCl, 4·5 KCl, 1 $MgSO_4$, 1·8 Na_2HPO_4, 0·2 NaH_2PO_4, 25 $NaHCO_3$, 1·25 $CaCl_2$ and 28 D-glucose. It was gassed for at least 45 min at 37·5 °C with 95% O_2–5% CO_2 to give a pH of 7·42 and circulated through the lumen of the intestine at 7 ml min^{-1}. The flow was interrupted with bubbles of 95% O_2–5% CO_2 (Fisher & Gardner, 1974) to produce a stirring effect and facilitate oxygenation (Hanson & Parsons, 1976).

Perfusion technique

Isolated jejunal loops 15–20 cm in length were perfused by single pass for 2 h by the technique of Fisher & Gardner (1974) modified as described by Bronk & Hastewell (1988). The loops were suspended in liquid paraffin (specific gravity 0·83–0·86) at 37·5 °C. The first 50 min was a control period during which the perfusate contained 28 mM glucose but no other transport substrate. The serosal fluid dripped off the intestinal loop and collected at the bottom of the liquid paraffin; samples of the serosal secretions were collected every 10 min and after 50 min a second perfusate containing the relevant transport substrate in addition to glucose was used for a further 70 min. The viability of the preparation was assessed by its ability to maintain a steady water flow and to transport D-glucose actively. The rate of appearance of the serosal fluid was $0·263 \pm 0·007$ ml min^{-1} (g dry wt)$^{-1}$ ($n = 116$). The average serosal D-glucose concentration was $57·5 \pm 0·3$ mM, ($n = 116$), giving a serosal : luminal ratio of approximately 2. Rates of glucose and water transport were unaffected by the addition of peptides or amino acids to the luminal medium.

Preparation of serosal and luminal samples for analysis

The volumes of serosal samples were measured after centrifugation at 1800 *g* for 2 min to enable a meniscus between any paraffin and the secretion to form. Luminal perfusate samples were taken initially and at intervals of 20 min or less during the perfusion. Samples of the serosal secretions and the luminal perfusate were deproteinized with 6% perchloric acid and centrifuged at 1800 *g* for 2 min; an aliquot of the supernatant was neutralized with 0·6 M KOH, rapidly frozen in liquid nitrogen, thawed, and recentrifuged at 1800 *g*.

Preparation of tissue samples

A 4 cm portion of intestine was cut open lengthwise, gently blotted, the mucosa removed by scraping with a microscope slide, frozen in liquid nitrogen and weighed before being homogenized in 1 ml of 6% perchloric acid. The resulting homogenate was then centrifuged at 1800 *g* for 2 min. An aliquot of the supernatant was neutralized with potassium hydroxide (0·6 M) and rapidly frozen in liquid nitrogen before being recentrifuged at 1800 *g*. No correction was made for extracellular space so that the tissue concentrations in Fig. 5 which are above those in the lumen will be underestimates of the true tissue concentration, whereas the reverse will be true for those tissue concentrations below the luminal concentration.

HPLC analysis

The deproteinized, neutralized samples of the perfusate, the serosal secretions and the mucosal tissue samples were analysed for peptide and free phenylalanine by HPLC at 210 nm on a 5 μm ODS C18 column (Jones Chromatography, Hengoed, Glamorgan, UK). The mobile phase was 20% methanol–80% 21 mM KH_2PO_4 (pH 5).

Assay of D-glucose

The D-glucose concentrations in the serosal and luminal samples were determined by an automated method using a Cobas Mira autoanalyser (Roche, UK).

Measurement of dry weight

A measured segment of the intestine was blotted, weighed (to determine the wet weight) and dried to a constant weight in an air oven (105 °C). This gave a dry weight : wet weight ratio which was used to calculate the water content of each tissue sample.

Calculation and expression of results

All results are expressed as means ± standard error of the mean (S.E.M.); $n = 4$ for each mean except where noted. Transport data are presented as cumulative rates of serosal appearance (nmol min^{-1} (g dry wt jejunal segment)$^{-1}$) calculated by co-variance. Statistical comparisons were carried out using two-way analysis of variance to compare sets of concentration data or covariance analysis for cumulative serosal appearance.

RESULTS

Transmural transport of phenylalanine in isolated loops

The serosal appearance of phenylalanine was measured in controls perfused for 120 min and in experimental groups which were perfused from 70 to 120 min in single pass with 0·1–1·0 mM L-phenylalanine after an initial 50 min control period. Figure 1 shows the cumulative serosal appearance of phenylalanine and indicates that at each of the concentrations tested, the rate of transfer from lumen to serosa did not begin to increase until 10 min after the change to luminal perfusate containing phenylalanine, and that the increased rate became approximately linear 30 min after phenylalanine addition. The rate of phenylalanine transport increased linearly with luminal concentration over the range tested. The inset plot in Fig. 1, which repeats the control data on a larger scale, shows that the controls, in which no luminal phenylalanine was added, released phenylalanine into the serosal secretions at a constant rate of 14 ± 1·3 nmol min^{-1} (g dry wt)$^{-1}$ for the entire 120 min perfusion period.

Serosal appearance of phenylalanine and dipeptide in loops perfused with dipeptides

For each of the eight dipeptides, sets of four single-pass loop perfusions were carried out at luminal concentrations of 1·0, 0·5 and (except for D-phenylalanyl-D-alanine) 0·1 mM. From the data obtained for each perfusion the control rate of phenylalanine appearance was determined from the cumulative plot for the period from 20 to 60 min. These values were used to determine the cumulative net appearance of phenylalanine in each experiment. For the group of perfusions with each concentration of the various dipeptides the net rate of phenylalanine appearance was calculated by covariance analysis using the points in all four experiments over the time period from 80 to 120 min, after subtraction of the control rate in each case. Table 1 shows the mean rates of phenylalanine appearance ± S.E.M. for perfusions with each of the eight dipeptides. The table also gives the net rates of phenylalanine appearance calculated in the same way for the groups of experiments with the various concentrations of free D- and L-phenylalanine.

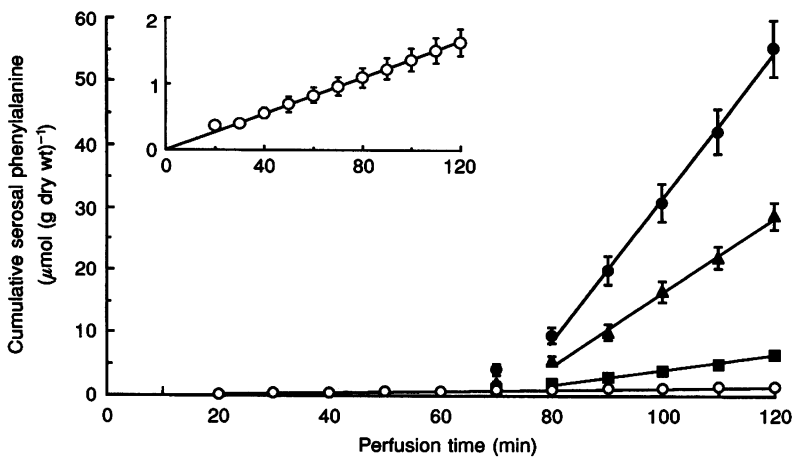

Figure 1. L-Phenylalanine transport in isolated loops of rat jejunum

Cumulative serosal appearance of L-phenylalanine in isolated loops perfused by a single pass from 50 to 120 min with 0 (○), 0·1 (■), 0·5 (▲) or 1·0 (●) mM L-phenylalanine. The inset plot shows the control perfusions, with no added phenylalanine, on a larger scale. Values are means ± S.E.M. for 4 perfusions. The regression lines were calculated from all the individual time points over the period shown.

Table 1 shows that, in addition to increasing the serosal appearance of phenylalanine, two of the dipeptides, D-alanyl-L-phenylalanine and D-phenylalanyl-L-alanine, were transported intact across the epithelial layer at substantial rates. At the highest luminal concentration there was some transmural transport of D-alanyl-D-phenylalanine, and trace amounts of D-phenylalanyl-D-alanine were also found in the serosal secretions. Although the isolated loop preparation is inappropriate for kinetic analysis, a measure of the total rate of uptake from the lumen can be obtained for D-alanyl-L-phenylalanine and D-phenylalanyl-L-alanine by adding

Table 1. Net rates of serosal appearance of phenylalanine and dipeptide in isolated loops of rat jejunum perfused by a single pass

Luminal solute (50–120 min)	Conc. (mM)	Rate of appearance (nmol min^{-1} (g dry wt)$^{-1}$)	
		Phenylalanine	Dipeptide
L-Phenylalanine	1·0	1136 ± 88	—
	0·5	582 ± 48	—
	0·1	111 ± 14	—
D-Phenylalanine	1·0	144 ± 20	—
	0·5	95 ± 14	—
L-Alanyl-L-phenylalanine	1·0	1136 ± 194	0
	0·5	355 ± 51	0
	0·1	121 ± 15	0
L-Alanyl-D-phenylalanine	1·0	1038 ± 118	0
	0·5	348 ± 31	0
	0·1	103 ± 30	0
D-Alanyl-L-phenylalanine	1·0	689 ± 10	124 ± 20
	0·5	228 ± 11	22 ± 5
	0·1	75 ± 15	2 ± 2
D-Alanyl-D-phenylalanine	1·0	16 ± 2	35 ± 3
	0·5	12 ± 2	0
	0·1	2 ± 0·1	0
L-Phenylalanyl-L-alanine	1·0	1230 ± 182	0
	0·5	521 ± 51	0
	0·1	111 ± 19	0
L-Phenylalanyl-D-alanine	1·0	247 ± 25	0
	0·5	126 ± 13	0
	0·1	21 ± 8	0
D-Phenylalanyl-L-alanine	1·0	17 ± 1·3	87 ± 3
	0·5	13 ± 2·7	46 ± 3
	0·1	6 ± 4	22 ± 2
D-Phenylalanyl-D-alanine	1·0	13 ± 7	9 ± 1
	0·5	0	4 ± 0·8

Mean rates (± S.E.M., $n = 4$) were calculated by covariance from all the time points between 80 and 120 min.

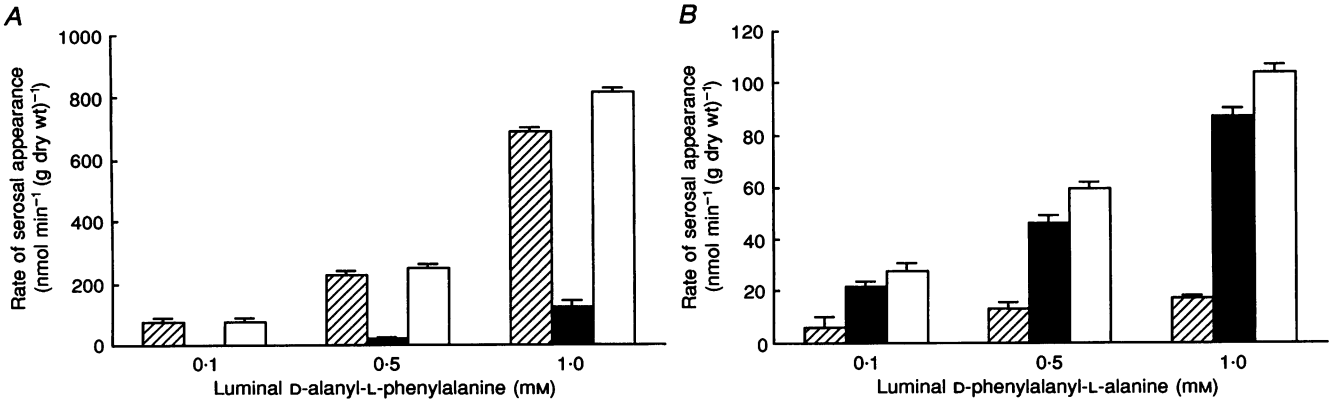

Figure 2. Serosal appearance rates of phenylalanine and dipeptide in loops perfused with dipeptides which have an *N*-terminal D-amino acid

Rates of serosal appearance of phenylalanine (▨), dipeptide (■) and the sum of phenylalanine + dipeptide (□) in isolated loops of rat jejunum perfused for 70 min with 0·1, 0·5 or 1·0 mM D-alanyl-L-phenylalanine (*A*) or D-phenylalanyl-L-alanine (*B*). Values are means ± S.E.M. ($n = 4$). Note the difference in scales in *A* and *B*.

together the net rates of serosal appearance of phenylalanine and dipeptide (see Fig. 2*A* and *B*). When these combined rates are compared with those for mixed peptides with *N*-terminal L-amino acids, it is clear the inclusion of an *N*-terminal D-phenylalanine reduces the uptake rate substantially whereas *N*-terminal D-alanine has little effect. Figure 3*A* and *B* shows the cumulative serosal appearance of D-alanyl-L-phenylalanine and D-phenylalanyl-L-alanine in the perfusions at peptide concentrations of 1·0 mM. Inset plots in Fig. 3*A* and *B* show the net cumulative serosal appearance of phenylalanine in each case and emphasize the fortyfold difference in rate of hydrolysis of these two dipeptides. Although there is a 10–15 min lag period before either of the dipeptides appears in the serosal effluent, the cumulative rates of appearance are approximately linear from 80 to 120 min indicating that the transmural dipeptide transfer has reached a steady state.

By contrast the presence of a *C*-terminal D-amino acid in a mixed dipeptide does not reduce peptidase activity sufficiently to permit transmural dipeptide transport. A comparison of the rates shown in Table 1 for the serosal phenylalanine appearance obtained with L-alanyl-D-

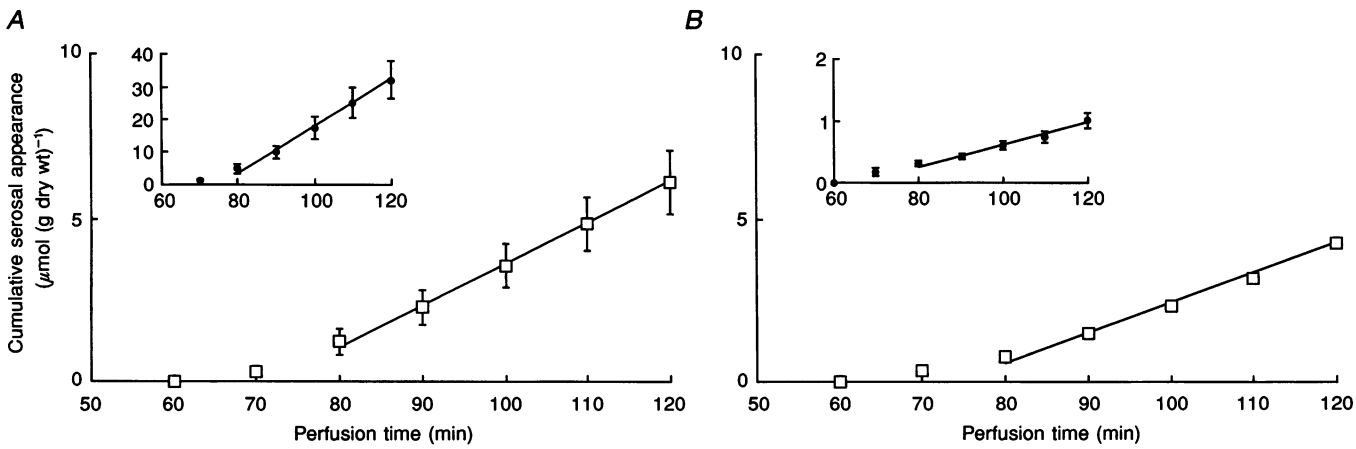

Figure 3. Transmural transport and hydrolysis

A, cumulative serosal appearance of dipeptide (□) in isolated loops perfused with 1·0 mM D-alanyl-L-phenylalanine from 50 to 120 min. Means ± S.E.M. ($n = 4$). Inset, cumulative serosal appearance of L-phenylalanine (●) on a smaller scale. *B*, cumulative serosal appearance of dipeptide (□) in isolated loops perfused with 1·0 mM D-phenylalanyl-L-alanine from 50 to 120 min. Means ± S.E.M. ($n = 4$). Inset, cumulative serosal appearance of D-phenylalanine (●) on a larger scale.

phenylalanine and L-phenylalanyl-D-alanine with those for the comparable peptides with a *C*-terminal L-amino acid indicates that the presence of *C*-terminal D-phenylalanine had no effect whereas *C*-terminal D-alanine reduced uptake and hydrolysis by 80%. The fact that we could not detect any serosal peptides in the perfusions with the four dipeptides with *N*-terminal L-amino acids suggests that these dipeptides do not cross the epithelial layer intact. However, Gardner & Plumb (1979) pointed out that some leakage of peptidases may occur even in physiologically viable preparations. In order to test this we carried out recovery experiments by adding 0·1 mM dipeptide to serosal samples as they were collected. The results indicated that approximately 50% of the dipeptide was hydrolysed when L-phenylalanyl-L-alanine was added whereas there was no measurable hydrolysis of dipeptides with *N*-terminal D-amino acids.

The groups of perfusions with D-alanyl-D-phenylalanine or D-phenylalanyl-D-alanine showed that these two dipeptides are poor substrates for both transport and hydrolysis by the rat small intestine. With D-alanyl-D-phenylalanine, dipeptide could not be detected in the serosal effluent until a luminal concentration of 1 mM was reached. At that concentration dipeptide appeared in the serosal secretions at only 28% of the rate found with D-alanyl-L-phenylalanine and the rate of D-phenylalanine appearance was only 1·5% of that from L-alanyl-D-phenylalanine. With D-phenylalanyl-D-alanine the rate at which the dipeptide appeared in the serosal secretions was about 10% of that for the same concentrations of D-phenylalanyl-L-alanine. The low level of this dipeptide detected in the serosal secretions also indicates that the capacity of this preparation for paracellular dipeptide transport is minimal.

Dipeptide absorption as a means of transferring D-phenylalanine across the epithelial layer

Comparison of the rates of D- and L-phenylalanine transfer from the lumen to the serosal secretions shows that the apical membrane of rat intestinal epithelium discriminates strongly between the two phenylalanine isomers (Table 1). However, Fig. 4 shows that at a luminal concentration of 1 mM L-alanyl-D-phenylalanine delivered serosal D-phenylalanine at a rate of $1{\cdot}04 \pm 0{\cdot}12$ μmol min^{-1} (g dry wt)$^{-1}$ whereas 1 mM luminal free D-phenylalanine only appeared in the serosal secretions at a rate of $0{\cdot}14 \pm 0{\cdot}02$ μmol min^{-1} (g dry wt)$^{-1}$. This comparison shows clearly that the dipeptide is absorbed prior to hydrolysis, and suggests that the basolateral membrane shows little selectivity toward the two phenylalanine isomers, although the serosal and/or mucosal exit ratios (Fig. 6) are lower for D-phenylalanine.

Accumulation of amino acids and dipeptides within the mucosa during the perfusions

Figure 5 shows the mean concentrations of D- and L-phenylalanine and dipeptide in the mucosa and serosal secretions at the end of the perfusions with each of the eight dipeptides and the two amino acids at a luminal concentration of 0·5 mM. Since D- and L-phenylalanine are not resolved by our HPLC technique, estimates of the D- and L-phenylalanine content of the tissue and serosal samples in Fig. 5 were calculated as follows for the perfusions with free D-phenylalanine and those with dipeptides containing D-phenylalanine. In each case the L-phenylalanine concentration in the tissue and in the final serosal sample as shown in Fig. 5 was assumed to be the same as that in the control group of perfusions to which no amino acid or dipeptide had been added. These values were subtracted from the measured phenylalanine

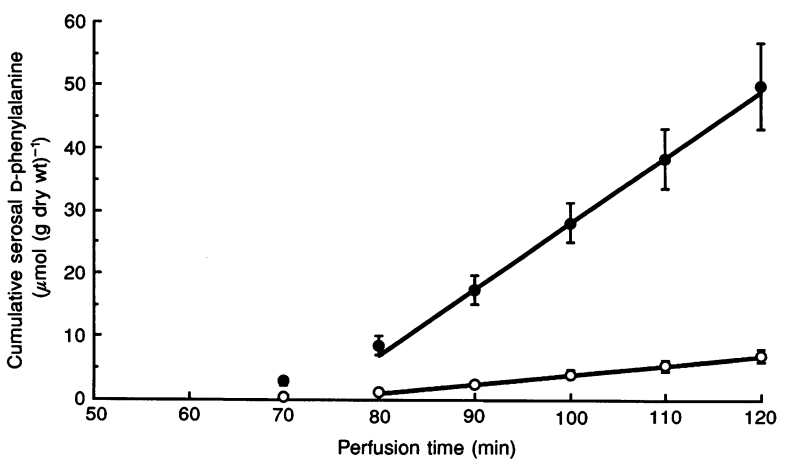

Figure 4. Transmural transport of D-phenylalanine in isolated loops

Cumulative serosal appearance of D-phenylalanine in perfusions with 1 mM L-alanyl-D-phenylalanine (●) and 1 mM D-phenylalanine (○). Means ± S.E.M. ($n = 4$).

concentrations in the tissue and serosal samples to provide the estimates of the D-phenylalanine concentration given in Fig. 5.

The tissue concentrations of both D-alanyl-L-phenylalanine (Fig. 5*B*) and D-phenylalanyl-L-alanine (Fig. 5*C*) were significantly above the luminal concentration ($P < 0.05$) indicating that these two peptides are absorbed against a concentration gradient. Only two other dipeptides could be detected in the mucosal tissue at the end of the perfusion, L-alanyl-D-phenylalanine and D-alanyl-D-phenylalanine, and the fact that neither of these dipeptides could be detected in the serosal secretions at a luminal concentration of 0·5 mM may indicate that they are poor substrates for the peptide transporter in the basolateral membrane. The phenylalanine concentration shown in the mucosa in Fig. 5 for those dipeptides that are absorbed from the lumen and hydrolysed completely within the tissue is substantially higher than the luminal peptide concentration, except for the perfusions with L-phenylalanyl-D-alanine.

The mucosal concentrations shown in Fig. 5 also reveal striking differences in the way that the intestine handles L-phenylalanine, D-phenylalanine and the two dipeptides which are transported most rapidly from lumen to serosa, D-alanyl-L-phenylalanine and D-phenylalanyl-L-alanine. These differences are illustrated in Fig. 6 which compares the entry and exit ratios for these four solutes at luminal concentrations of 0·5 and 1·0 mM. Only L-phenylalanine enters against a large concentration gradient and it is clear that the two dipeptides cross the basolateral membrane much less easily than the two amino acids.

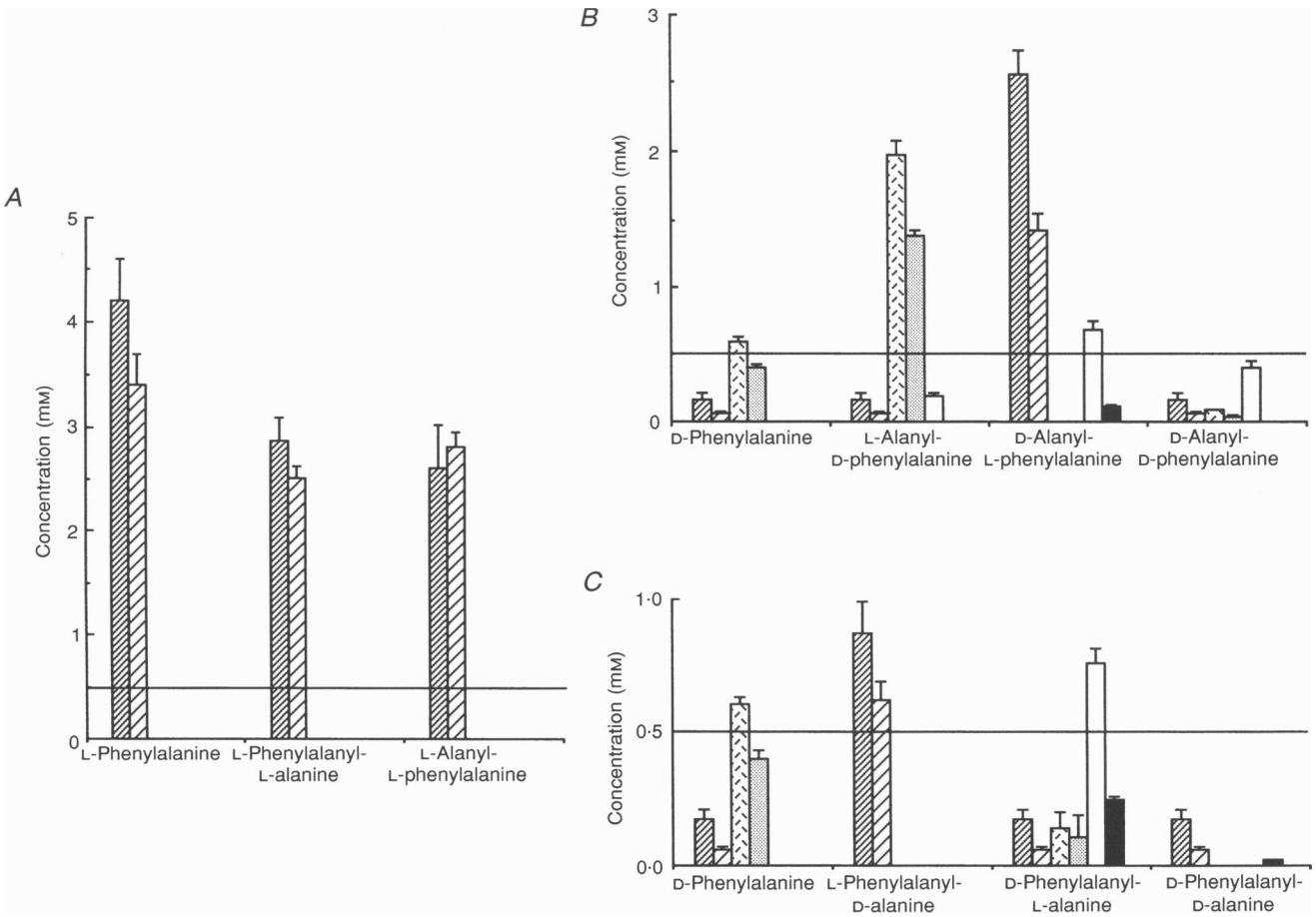

Figure 5. Mucosal and serosal concentrations of phenylalanine and dipeptide in loops perfused with dipeptides or free phenylalanine

Concentrations of phenylalanine and dipeptide in mucosal tissue and serosal secretions after perfusion of isolated loops for 70 min with the substrate at a luminal concentration of 0·5 mM (continuous line). *A*, L-phenylalanine and natural dipeptides; *B*, D-phenylalanine and dipeptides with *N*-terminal alanine; *C*, D-phenylalanine and dipeptides with *N*-terminal phenylalanine. Means ± S.E.M. ($n = 4$). ▨, tissue L-phenylalanine; ▨, serosal L-phenylalanine; ▩, tissue D-phenylalanine; ▩, serosal D-phenylalanine; □, tissue dipeptide; ■, serosal dipeptide.

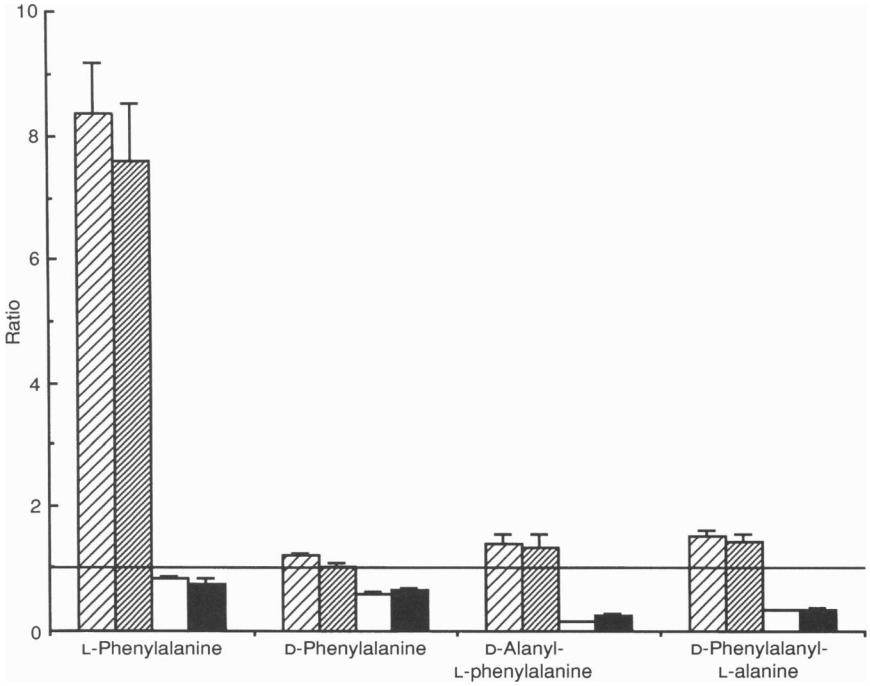

Figure 6. Entry and exit ratios for phenylalanine and dipeptides which have an *N*-terminal D-amino acid in isolated perfused loops

Entry (mucosal/luminal) concentration ratios at 0·5 mM (▨) and 1·0 mM (▨) and exit (serosal/mucosal) concentration ratios at 0·5 mM (□) and 1·0 mM (■) for L-phenylalanine, D-phenylalanine, D-alanyl-L-phenylalanine and D-phenylalanyl-L-alanine after perfusion through isolated jejunal loops for 70 min. Means ± S.E.M. ($n = 4$).

DISCUSSION

The recent publication of the sequences of two different proton-dependent peptide transporters from the intestine (Dantzig *et al.* 1994; Fei *et al.* 1994) resolves any remaining doubts about the capacity of intestinal epithelial cells to absorb peptides. However, it is important to recognize that the transport of peptides across the epithelial layer requires three distinct steps: uptake by the brush border, transfer to the basolateral pole of the enterocyte and exit across the basolateral membrane. The only alternative transepithelial route is the paracellular pathway which would not involve peptide transporters in the brush border or basolateral membrane. In the study reported in this paper we have used isolated loops of intestine because this enables us to look at transmural transfer from lumen to serosa, as well as providing us with some evidence concerning the three phases of the transfer process. The fact that we can measure the mucosal content of hydrolysis-resistant peptides at the end of the perfusion also enables us to obtain evidence on the relative importance of the transcellular and paracellular routes. However, it should be pointed out that single pass perfusions of isolated loops cannot yield a reliable picture of luminal disappearance.

This study is the first to use a preparation of intact epithelial tissue to investigate the transport of all eight dipeptide combinations of a single pair of amino acids. Our data give a clear picture of the fate of dipeptides of alanine plus phenylalanine perfused through the lumen of the rat small intestine. The two naturally occurring dipeptides were both completely hydrolysed within the mucosa after absorption from the lumen, and in neither case could we detect free phenylalanine in the luminal effluent. The dipeptide with L-phenylalanine in the *N*-terminal position gave the highest rate of transmural phenylalanine transfer which supports the view that a large lipophilic side chain at the *N*-terminus of the peptide enhances transport (Matthews, 1991). The rate of serosal phenylalanine appearance from this dipeptide did not differ from that of the same concentration of free L-phenylalanine but at 0·5 mM it was significantly faster ($P < 0·05$) than that from L-alanyl-L-phenylalanine.

Using all eight possible dipeptides of the D and L forms of the two amino acids has enabled us to examine the relative importance of the chiral form of each amino acid at both the *N*- and *C*-terminus of the dipeptide. The general conclusions from our results are summarized in Table 2. The four mixed dipeptides gave strikingly different results: *N*-terminal D-phenylalanine reduced the rate of transport by up to 90% whereas placing the D-phenylalanine at the *C*-terminus was virtually without effect; by contrast *N*-terminal D-alanine had relatively

Table 2. Fate of the eight dipeptides of alanine plus phenylalanine when perfused through the lumen of the rat small intestine

Peptide	Absorption	Hydrolysis	Serosal appearance
L-Alanyl-L-phenylalanine	++++	++++	0
L-Alanyl-D-phenylalanine	++++	++++	0
D-Alanyl-L-phenylalanine	+++	+++	+
D-Alanyl-D-phenylalanine	Minimal	Minimal	Minimal
L-Phenylalanyl-L-alanine	++++	++++	0
L-Phenylalanyl-D-alanine	++	++	0
D-Phenylalanyl-L-alanine	+	Minimal	+
D-Phenylalanyl-D-alanine	0	Minimal	Minimal

Relative rates (nmol min^{-1} (g dry wt)$^{-1}$) at a luminal substrate concentration of 1 mM: ++++, > 1000; +++, 500–1000; ++, 150–500; +, 50–150; Minimal, < 50.

little effect on transport, although when the D-alanine was placed at the *C*-terminus the rate was cut by 80%. These results suggest that earlier conclusions by other workers (Asatoor *et al.* 1973; Matthews, 1991; Bai & Amidon, 1992) on the influence that the inclusion of D-amino acids has on peptide transport need some qualification. For example, the natural dipeptide with an *N*-terminal L-phenylalanine showed the highest rate of transmural phenylalanine transfer and our data indicate that the rate was much lower for L-phenylalanyl-D-alanine with a *C*-terminal D-amino acid, whereas transmural phenylalanine transfer was somewhat less rapid with the natural dipeptide with an *N*-terminal alanine and yet virtually the same rate was obtained for L-alanyl-D-phenylalanine which has a *C*-terminal D-amino acid.

The results with L-alanyl-D-phenylalanine also show the power of employing a peptide with an *N*-terminal L-alanine to deliver a poorly transported molecule such as D-phenylalanine across the epithelial layer. It is also worth noting that L-alanyl-D-phenylalanine is particularly effective for the transmural transfer of D-phenylalanine because the peptide is rapidly hydrolysed within the mucosa. The rate of D-phenylalanine appearance in the serosal secretions in Fig. 4 indicates that the basolateral membrane is fairly permeable to the D-amino acid. However, it is worth noting in Fig. 6 that at 0·5 mM the exit (serosal : mucosal) concentration ratio for D-phenylalanine is significantly lower ($P < 0·05$) than that for L-phenylalanine.

Since dipeptides with an *N*-terminal D-amino acid are relatively resistant to hydrolysis by cytosolic peptidases, we have been able to show that these dipeptides are transported intact across the epithelial layer. However, whereas *N*-terminal D-phenylalanine confers strong resistance to hydrolysis, it was pointed out above that *N*-terminal D-alanine is less inhibitory. Both D-alanyl-L-phenylalanine and D-phenylalanyl-L-alanine accumulate within the mucosa and the substantial difference between the entry and exit ratios of these two dipeptides (Fig. 6) indicates that transport across the basolateral membrane may be limiting their rate of transepithelial transfer. The fact that we are able to see intact D-alanyl-L-phenylalanine in the serosal secretions is a consequence of a rapid rate of absorption from the lumen together with a more modest inhibition of hydrolysis. For the four dipeptides with *N*-terminal L-amino acids we found no evidence for the presence of intact peptide in the serosal secretions or in the mucosal samples taken at the end of the perfusion. However, the results of recovery experiments indicate that the serosal samples have sufficient peptidase activity to hydrolyse low concentrations of the dipeptides with *N*-terminal L-amino acids prior to analysis.

The experiments with the two dipeptides containing both D-amino acids indicated that neither peptide was absorbed or hydrolysed by the intestine to an appreciable extent. Our results with these two dipeptides are in agreement with the data obtained for the transport of D-leucyl-D-leucine by Boyd & Ward (1982) in *Necturus* intestine and by Asatoor *et al.* (1973) in rat jejunum. The fact that we could find no evidence for the presence of D-phenylalanyl-D-alanine in the mucosa when this peptide was perfused through the lumen means that it may provide a measure of the maximum capacity of paracellular transport of the dipeptides in our preparation. We only detected modest amounts of D-alanyl-D-phenylalanine in the serosal secretions at 1 mM and the rate of D-phenylalanyl-D-alanine appearance was less than 10% of that of D-phenylalanyl-L-alanine. These data make it clear that in this preparation of rat jejunum, paracellular transport of dipeptides is minimal, despite the fact that we used a luminal glucose concentration of 28 mM which gave a rate of transmural fluid transfer of

0·26 ml min^{-1} (g dry wt)$^{-1}$. Our results are contrary to those reported by Pappenheimer, Dahl, Karnovsky & Maggio (1994) who reported that an octapeptide composed of D-amino acids orally administered to intact rats in a solution of 278 mM glucose plus 88 mM creatinine was absorbed by a paracellular route. The fact that these results were obtained at ten times the glucose concentration used in our experiments suggests that high glucose concentrations may loosen the tight junctions. However, the results reported by Pappenheimer *et al.* (1994) are also at variance with those of Fricker *et al.* (1991) who used fluorescence derivatization to show that the octapeptide SMS 201-995 was absorbed by a transcellular route.

Although four of the eight dipeptides were completely hydrolysed following uptake from the lumen, we are confident that they were absorbed prior to hydrolysis because the perfusions were single-pass experiments and there was no detectable free phenylalanine in the luminal effluent. In addition, the results with L-alanyl-D-phenylalanine provide independent support for the conclusion that peptide uptake preceded hydrolysis since the rate of serosal appearance of D-phenylalanine from this dipeptide was up to seven times that from the same luminal concentration of free D-phenylalanine and the intact dipeptide was just detectable within the mucosa at the end of the perfusion (see Fig. 5*B*).

The results we have presented in this paper point to four general conclusions about intestinal peptide transport which should now be tested with other peptides. First, the transporter in the apical membrane shows some sensitivity to the chirality of both amino acids in the dipeptide. Second, peptides with *N*-terminal L-phenylalanine are more sensitive to the introduction of a *C*-terminal D-amino acid than the peptides with an *N*-terminal L-alanine. Third, the dipeptide transporter at the basolateral membrane has a very different specificity from the apical transporter. Finally, the low permeability of our preparation to dipeptides composed entirely of D-amino acids indicates that there is minimal paracellular transport of short peptides in the intact rat small intestine.

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