Structural Requirements for the Intestinal Mucosal-Cell Peptide Transporter: The Need for N-Terminal α-Amino Group

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The requirement for a free α-amino group for the intestinal peptide carrier-mediated transport was investigated. A series of dipeptide analogues without the N-terminal α-amino group [including phenylpropionylproline, phenylacetylproline, *N*-benzoylproline, phenylacetyl-α-methyldopa, and hippuric acid (*N*-benzoylglycine)] were studied in the perfused rat intestinal segment. The absorption of phenylpropionylproline, phenylacetyl-α-methyldopa, and *N*-benzoylproline was concentration dependent. The transport parameters (mean ± SD) of phenylpropionylproline and *N*-benzoylproline were as follows: J_{max*}, 0.037 (±0.019) m*M*; K_m, 0.045 (±0.027) m*M*; P_{c*}, 0.830 (±0.130); and P_{m*}, 0.673 ± 0.049; and J_{max*}, 1.34 (±0.24) m*M*; K_m, 1.31 (±0.30) m*M*; P_{c*}, 1.02 (±0.11); and P_{m*}, 0; respectively. The intestinal permeabilities of phenylpropionylproline, phenylacetylproline, *N*-benzoylproline, and hippuric acid (*N*-benzoylglycine) were significantly reduced by dipeptides and cephradine. These results strongly suggest that these dipeptide analogues, without an α-amino group, are transported by the peptide carrier and provide more direct evidence that a free α-amino group is not absolutely essential for the mucosal-cell peptide carrier-mediated transport.

KEY WORDS: dipeptide analogues; peptide carrier-mediated transport; α-amino group; phenylpropionylproline; phenylacetylproline; *N*-benzoylproline; phenylacetyl-α-methyldopa; hippuric acid (*N*-benzoylglycine).

INTRODUCTION

The transport of small peptides (di/tripeptides) in the intestinal mucosal cell has been shown to be carrier mediated via a common pathway (1–3). Peptide carrier-mediated transport seems to be not as structurally restrictive as that for amino acids (1–2,4,5). Recent discoveries further suggest the broad spectrum of peptide carrier-mediated transport in terms of structural specificity. The β-lactam antibiotics (6–9) with a free α-amino group, such as cefatrizine, cephalexin, cefaclor, amoxicillin, and 5 dipeptidyl derivatives of α-methyldopa, have been shown to share the intestinal peptide carrier system (10).

Recent studies indicate that peptide-like therapeutic agents without a free α-amino group, such as the ACE inhibitors, captopril (11), enalapril (12), and lisinopril (13), and the β-lactam antibiotics, cefixime and FK089 (14,15), are all transported by the peptide carrier system. Therefore, this mucosal-cell transport pathway could serve to improve the oral efficiency of poorly absorbed therapeutic agents. Hence the structural requirements of this carrier system must be determined in order to provide a basis for the oral delivery of peptide-like therapeutic agents and prodrugs.

Using dipeptide analogues as model compounds, the specific aim of this study is to investigate the significance of the N-terminal α-amino group in the mucosal-cell peptide carrier-mediated transport. Dipeptide analogues with proline at the C terminal were also selected to define the transport mechanism of dipeptide analogues, such as the ACE inhibitors.

EXPERIMENTAL

Materials

Phe-Pro was obtained from Chemical Dynamics Corporation (South Plainfield, NJ). ^{14}C PEG-4000 was obtained from Dupont NEN. PEG-4000, Gly-Pro, Gly-Gly, Met, Gly, Asp-Phe, Pro-Tyr, Gly-Phe, Pro-Phe, Phe-Phe, hippuric acid sodium salt, L-α-methyldopa, and hydrocinnamic acid were obtained from Sigma Chemical Co. (St. Louis, MO). *N*-Benzoyl-Pro was obtained from United States Biochemical Co. (Cleveland, OH). Cephradine was a gift from Squibb & Sons (Princeton, NJ). Phenylacetic acid, proline methyl ester hydrochloride, and *N*-methylmorpholine were obtained from Aldrich Chemical Co. (Milwaukee, WI). *N,N'*-Dicyclohexylcarbodiimide (DCC) was purchased from Pierce Chemical Co. (Rockford, IL). All the materials including buffer components were used as obtained. The amino acids and dipeptides used are all of L form and L-L form, respectively.

Stability Evaluation and Quenching Method

The stability of all dipeptide analogues was evaluated in the blank perfusate (obtained by perfusing buffer through the jejunum) at 37°C and room temperature. Quenching of each milliliter of phenylacetyl-α-methyldopa perfusate with 10 μl of 5 *N* HCl was used to stabilize phenylacetyl-α-methyldopa.

Single-Pass Perfusion Method

Animal. Male Sprague Dawley rats from Charles River, 230–380 g, were used.

Perfusion Solution. The perfusion solution consisted of a pH 5.5 Sorensen phosphate buffer, a substrate or a substrate and an inhibitor (or inhibitors), PEG-4000, and a trace amount of ^{14}C-PEG-4000 as a water flux marker. All the dipeptide analogues were studied at 0.1 m*M* in the inhibition experiments, except for phenylacetyl-α-methyldopa, which was used at 0.05 m*M*. Sodium chloride was used to adjust the osmolality of perfusion solution to about 300 ± 5 mosm/kg water.

Surgery and Perfusion Procedures. The experimental setup, surgery, and general procedure have been published previously (6) and were used without further modification, except for a 10-min wash step of perfusing blank buffer through the segment at 1.23 ml/min prior to the perfusion for

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the studies of Phe-Pro, phenylpropionylproline, phenylacetylproline, and *N*-benzoylproline to minimize the interference with the HPLC assay. The segment studied was the jejunum.

Assay Method. The water flux was checked by liquid scintillation counting using a Beckman LS-9000 counter (Beckman Instruments, Fullerton, CA) with automatic quenched correction. The HPLC system consisted of a Kratos Spectroflow 400 pump, a Kratos 783 absorbance detector (varied wavelength) (Kratos Analytic Instruments, Ramsey, NJ), an autoinjector (Model 710, Waters Associates, Milford, MA), and columns which were reversed-phase columns of either (a) octyl (C_8) (Altex Ultrasphere-ODS, 5 μm 4.6 mM × 15 cm) or (b) C_{18} (Altex Ultrasphere-ODS, 5 μm 4.6 mM × 15 cm) (Beckman Instrument, Berkley, CA). The HPLC assays (mobile phase, UV wavelength, flow rate, column) developed for all the compounds are summarized in Table I.

Detection of Degradation Products. The extent of hydrolysis, when perfused through the jejunum, was checked for phenylpropionylproline, *N*-benzoylproline, phenylacetylproline, and hippuric acid by quantifying their hydrolysis products in the perfusate samples. The HPLC assay methods (Table I) for phenylpropionic acid, phenylacetic acid, and benzoic acid were the same as those for their parent compounds, phenylpropionylproline, phenylacetylproline, *N*-benzoylproline, and hippuric acid (*N*-benzoylglycine), respectively.

Synthesis of Dipeptide Analogues

N-*Phenylpropionyl-L-proline.* A solution of L-proline methyl ester · HCl (2.5 g, 15.1 mmol) and 4-methyl morpholine (1.66 ml, 15.1 mmol) in anhydrous dichloromethane (10 ml) was added to a solution of phenylpropionic acid (hydrocinnamic acid) (2.3 g, 15.3 mmol) and *N,N'*-dicyclohexylcarbodiimide (3.15 g, 15.3 mmol) in anhydrous dichloromethane (50 ml) at 0°C. The resulting mixture was stirred at 0°C for 4 hr and then the bath was allowed to warm up slowly to room temperature. After stirring overnight, the dicyclohexylurea formed during the reaction was filtered off and the filtrate was concentrated. The residue was redissolved in ethyl acetate and washed successively with 1 *N* HCl, 5% $NaHCO_3$, and H_2O. The organic layer was then dried ($MgSO_4$) and concentrated to give 3.6 g (91%) of *N*-phenylpropionyl-L-proline methyl ester, which was purified by column chromatography (9:1 hexane/ethyl acetate as eluate).

The methyl ester group was hydrolyzed by treating with 1 equiv of 0.5 *N* ethanolic sodium hydroxide at 0°C for 60 min (monitored by HPLC). Then the reaction mixture was acidified, concentrated, and taken in ethyl acetate, which was subsequently extracted twice with saturated aqueous $NaHCO_3$. The combined aqueous extract was acidified to regenerate 2.7 g (72% yield) of *N*-phenylpropionyl-L-proline. It was further purified by recrystallization from ethyl acetate/hexane. Mp 105–106°C. NMR ($CDCl_3$): δ 1.90–2.00 (m, 3H, β-*H*, γ-H_2), 2.30–2.35 (m, 1H, β-*H*), 2.65, 3.00 (t, J = 7.83 Hz, 4H, CH_2CH_2), 3.30–3.36 (m, 1H, δ-*H*), 3.42–3.49 (m, 1H, δ-*H*), 4.57 (dd, J = 2.43 Hz, 7.56 Hz, 1H, α-*H*), 7.18–7.32 (m, 5H, Ar-*H*), 10.42 (s, 1H, COO*H*). FAB-MS, m/e 248 $(MH)^+$.

N-*Phenylacetyl-L-proline.* Phenylacetic acid (0.42 g, 3.1 mmol) was coupled with L-proline methyl ester using DCC and the resulting *N*-phenylacetyl-L-proline methyl ester was saponified, as described above. The yield was 65%. The title compound was recrystallized from ethyl acetate/hexane. Mp 133–134°C. NMR ($CDCl_3$): δ 1.95–2.05 (m, 3H, β-*H*, γ-H_2), 2.30–2.35 (m, 1H, β-*H*), 3.45–3.65 (m, 2H, δ-H_2), 3.74 (s, 2H, CH_2), 4.95 (dd, J = 2.30 Hz, 7.29 Hz, 1H, α-*H*), 7.25–7.36 (m, 5H, Ar-*H*), 10.25 (s, 1H, COO*H*). FAB-MS, m/e 234 $(MH)^+$.

N-*Phenylacetyl-L-α-methyldopa.* The synthesis of the title compound was accomplished, following the procedure described for *N*-phenylpropionyl-L-proline, starting from phenylacetic acid (0.27 g, 2.0 mmol) and L-α-methyldopa methyl ester · HCl using anhydrous dioxane as solvent. The crude product from saponification was purified by semipreparative HPLC (reversed-phase, C-18 column, 22 × 2.5 cm, Vydac), under isocratic conditions, using 80% H_2O and 20% CH_3CN (both containing 0.1% TFA, 280 nm), to yield 0.2 g (31%) of pure *N*-phenylacetyl-L-α-methyldopa. NMR

Table I. Summary of HPLC Assay Methods[a]

Compound	Column	Mobile phase	λ (nm) (UV)	Retention (min)
Phenylacetylproline	C_8	pH 2.5, 0.05 *M* $(NH_4)H_2PO_4$:(acetontrile:THF = 16.5:3.5) = 75:25	258	8.2
Phenylacetic acid	C_8	pH 2.5, 0.05 *M* $(NH_4)H_2PO_4$:(acetonitrile:THF = 16.5:3.5) = 75:25	258	9.4
Phenylacetyl-α-methyldopa	C_8	pH 3, 0.01 *M* NaH_2PO_4:methanol = 60:40	280	5.6
Phenylpropionylproline	C_8	pH 2.5, 0.05 *M* $(NH_4)H_2PO_4$:(acetonitrile:THF = 16.5:3.5) = 70:30	258	8.3
Phenylpropionic acid	C_8	pH 2.5, 0.05 *M* $(NH_4)H_2PO_4$:(acetonitrile:THF = 16.5:3.5) = 70:30	258	10
Phe-Pro	C_{18}	pH 2.5, 0.05 *M* $(NH_4)H_2PO_4$:(acetonitrile:THF = 16.5:3.5) = 78:22	258	3.8
Hippuric acid	C_8	pH 3, 0.01 *M* NaH_2PO_4:acetonitrile = 90:10	280	11.6
N-benzoyl proline	C_8	pH 3, 0.01 *M* NaH_2PO_4:acetonitrile = 75:25	280	7
Benzoic acid	C_8	pH 3, 0.01 *M* NaH_2PO_4:acetonitrile = 75:25	280	8.9

[a] All the flow rates are 1 ml/min.

($CDCl_3$): δ 1.57 (s, 3H, CH_3), 2.95, 3.03 (HB, α, J = 13.50 Hz, 2H, dopa CH_2), 3.57 (s, 2H, $C_6H_5H_2$), 5.96 (s, 1H, NH), 6.20–6.67 (m, 3H, dopa Ar-H), 7.16–7.36 (m, 5H, Ar-H). FAB-MS, m/e 330 $(MH)^+$.

The purity of the compounds synthesized was checked by analytical HPLC (reversed-phase, C-18, Vydac) at 230 nm. ^{1}H-NMR spectra were recorded on an IBM WP 270-MHZ spectrometer in $CDCl_3$ and reported as parts per million downfield from tetramethylsilane (TMS). FAB-MS were obtained on a VG analytical 70-2505 mass spectrometer.

DATA ANALYSIS

Permeability Estimation

A modified boundary layer model was used to estimate the wall permeability at steady state (6). The water flux was evaluated using Eq. (1) for each perfusate sample collected to insure that the assumption that no radial convection occurs during perfusion, was not violated:

$$\% \text{ of water flux} = [(A_i - A_f)/(A_f \times L)] \times 100\% \quad (1)$$

where A_i and A_f are the initial and final counts per minute of ^{14}C-labeled PEG 4000, respectively, and L is the length of the jejunum perfused. The perfusate samples used to estimate P_{w^*} were confined to those with water flux less than 0.5%/cm.

The dimensionless effective permeability (P_{eff^*}) and aqueous permeability (P_{aq^*}) were estimated using Eqs. (2) and (3), respectively.

$$P_{eff^*} = (1 - C_m/C_o)/4Gz \quad (2)$$

$$P_{aq^*} = (AGz^{1/3})^{-1} \quad (3)$$

where C_m and C_o are the outlet and inlet concentrations, respectively. Gz, the Graetz number, is defined in Eq. (4) and A is obtained by Eq. (5):

$$Gz = \pi DL/2Q \quad (4)$$

in which Q is the perfusion flow rate and D is the diffusion coefficient.

$$0.004 < Gz < 0.01 \qquad A = 10\,Gz + 1.01$$

$$0.01 \leq Gz < 0.03 \qquad A = 4.5\,Gz + 1.065$$

$$0.03 \leq Gz \qquad A = 2.5\,Gz + 1.125$$

P_{w^*} was estimated by the following equation:

$$P_{w^*} = P_{eff^*}/[1 - (P_{eff^*}/P_{aq^*})] \quad (6)$$

Under the assumption of normal distribution of the data in different groups with unequal variance, the statistical differences between the P_{w^*}'s of the control and the experimental groups were tested using two-sample tests with unequal variances (Behrens-Fisher).

Estimation of Carrier Kinetic Parameters

The membrane surface concentration is defined as follows:

$$C_w = C_o[1 - (P_{eff^*}/P_{aq^*})] \quad (7)$$

The overall flux through the intestinal wall with simultaneous saturable carrier-mediated transport and passive diffusion is given by

$$J = J_{max}C_w/(K_m + C_w) + P_mC_w \quad (8)$$

while the dimensionless wall permeability for the overall process is as follows:

$$P_{w^*} = J_{max^*}/(K_m + C_w) + P_{m^*} \quad (9)$$

where P_{m^*} is the unbiased dimensionless passive membrane permeability, J_{max^*} is the dimensionless maximal flux, and K_m is the apparent Michaelis Menten constant.

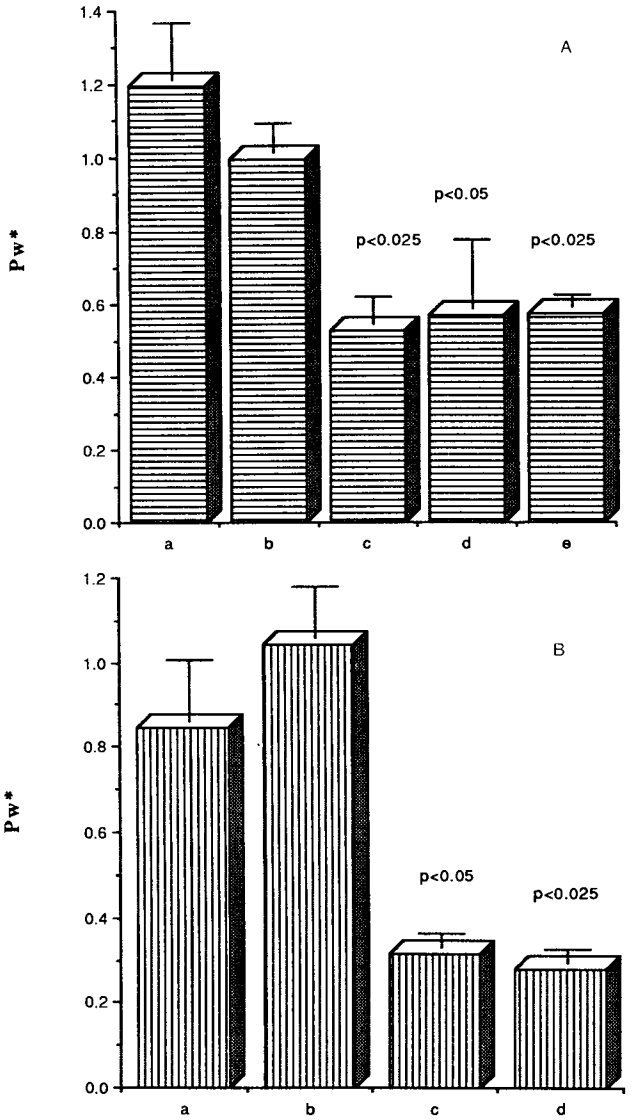


Fig. 1. (A) Inhibition of 0.1 m*M* phenylpropionylproline permeability: (a) control; (b) 27 m*M* Met; (c) mixed dipeptides (60 m*M* Gly-Gly, 2 m*M* Gly-Phe, 2 m*M* Pro-Phe); (d) 1 m*M* cephradine; (e) 27 m*M* Gly-Pro. (B) Inhibition of 0.1 m*M* phenylacetylproline permeability: (a) control; (b) 23 m*M* Met; (c) 23 m*M* Gly-Pro; (d) 2 m*M* cephradine. The values reported are mean ± SE.

The first-order carrier permeability is defined as

$$P_{c^*} = J_{max^*}/K_m \quad (10)$$

Hence P_{w^*} can be converted to the following form:

$$P_{w^*} = P_{c^*}/[1 + (C_w/K_m)] + P_{m^*} \quad (11)$$

RESULTS AND DISCUSSION

Stability Evaluation

All the dipeptide analogues except phenylacetyl-α-methyldopa were very stable in the blank perfusate and in the buffer at either 37°C or room temperature. The instability of phenylacetyl-α-methyldopa was due to the oxidation of α-methyldopa, thus the perfusate of this compound was quenched when collected (10). The quenched samples were stable at room temperature for at least 4 hr (less than 1.43 ± 0.76% loss after 4 hr), which is 2.5 times longer than the assay period for all the perfusate samples from one rat, together with the samples of the original perfusion solution. During assay, the loss of phenylacetyl-α-methyldopa after quenching in both buffer and perfusate was similar and insignificant. The percentage of 0.025 m*M* phenylacetyl-α-methyl-dopa remaining in the blank perfusate after a 15-min incubation at 37°C was 99.0 ± 0.65. Since the mean residence time of phenylacetyl-α-methyldopa in the jejunum during perfusion was about 10 min, the loss as a result of oxidation is negligible. There was no significant loss of phenylacetyl-α-methyldopa due to oxidation with or without coperfusing the inhibitors during the perfusion or assay.

Lability to Degradation by the Brush Border Membrane Enzymes

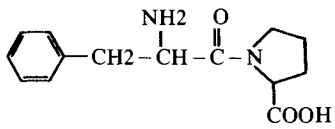
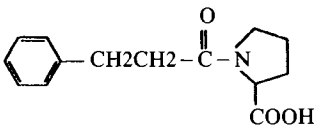
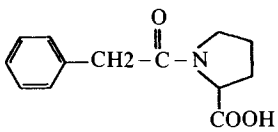
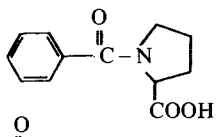
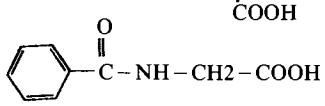
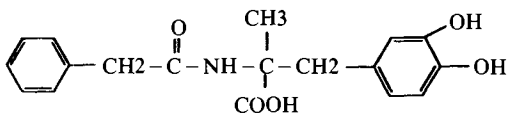
To interpret correctly the permeability results, the possibility of enzymatic hydrolysis needs to be examined. A free α-amino group is required for the enzyme activity of aminopeptidases (16,17), while carboxypeptidase P releases the C-terminal amino acid when proline is in the penultimate position (18–20). Since there are no α-amino groups in the dipeptide analogues studied and they contain proline at the C terminal, they are expected to be poor substrates for these enzymes.

This conclusion is supported by the following results. (a) No detectable amount of hydrolysis products from these dipeptide analogue was found in the perfusate samples or in the blank perfusate, such as phenylpropionic acid from phenylpropionylproline, phenylacetic acid from phenylacetylproline, and benzoic acid from both *N*-benzoylproline and *N*-benzoylglycine (hippuric acid). (b) High concentrations of Met had no significant inhibitory effect on the disappearance of 0.1 m*M* phenylpropionylproline (Fig. 1A) and 0.1 m*M* phenylacetylproline (Fig. 1B) in the intestinal lumen. Met has been shown to be an effective inhibitor of aminopeptidases isolated from the rat intestine (16,21).

Intestinal Permeability

The dimensionless intestinal wall permeabilities are summarized in Table II. The wall permeabilities fall in the following order: Phe-Pro > phenylpropionylproline > phe-

Table 2. Permeabilities of Dipeptide Analogues Investigated for the Need of N-Terminal α-Amino Group

Compound	Chemical structure	P_{w^*}[a]
Phe-Pro		2.01 ± 0.54
Phenylpropionylproline		1.19 ± 0.16
Phenylacetylproline		0.84 ± 0.15
N-Benzoylproline		0.90 ± 0.06
Hippuric acid		0.18 ± 0.03
Phenylacetyl-α-methyldopa		0.61 ± 0.07

[a] All compunds were studied at 0.1 m*M*, while phenylacetyl-α-methyldopa was at 0.05 m*M*.

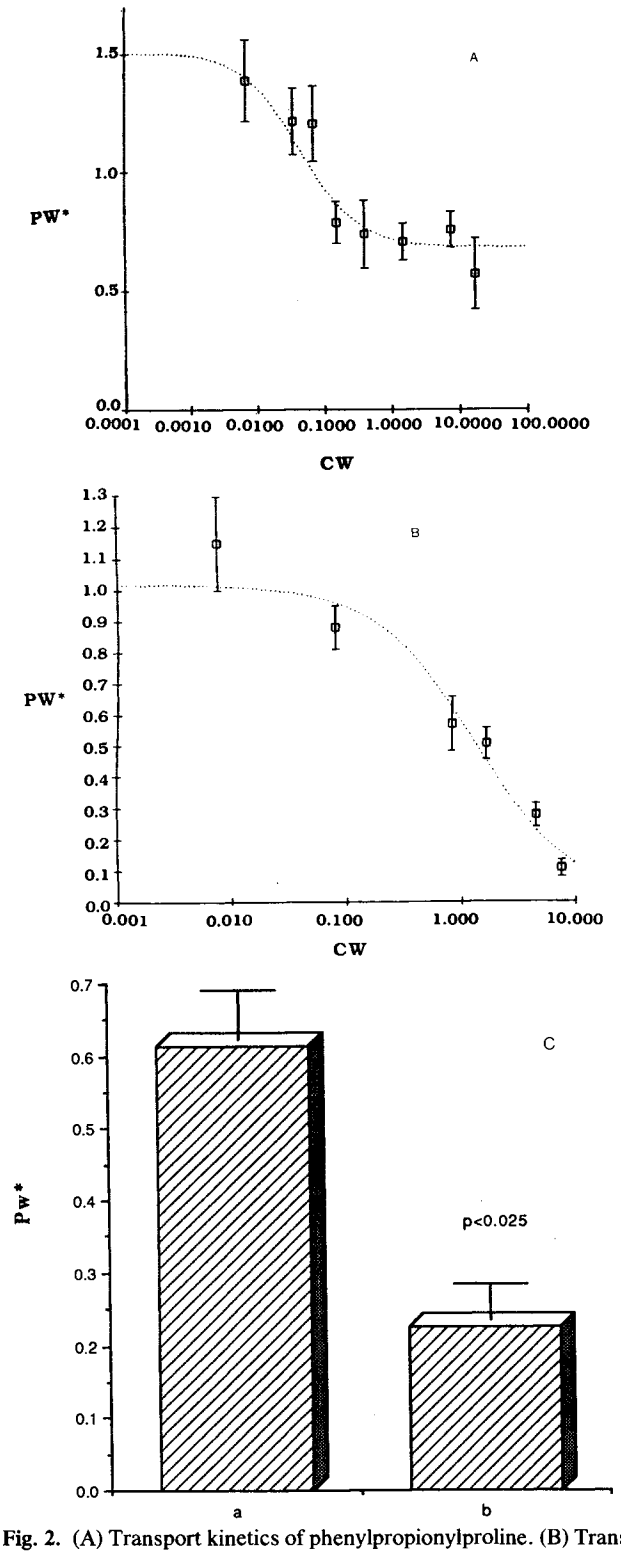


Fig. 2. (A) Transport kinetics of phenylpropionylproline. (B) Transport kinetics of *N*-benzoylproline. (Transport parameters, K_m, J_{max*}, P_{m*}, and P_{c*}, are listed in Table III). (C) Permeability of phenylacetyl-α-methyldopa at two concentrations: (a) 0.05 m*M* and (b) 0.5 m*M*. The values of P_{w*} shown are mean ± SE.

nylacetylproline ~ *N*-benzoylproline > phenylacetyl-α-methyldopa > hippuric acid.

Even though the structure of *N*-benzoylproline differs from that of Phe-Pro to a greater extent than those of phenylpropionylproline and phenylacetylproline, its wall permeability was still substantial. However with glycine at the C terminal, hippuric acid showed the lowest wall permeability among the compounds studied. Since the hydrophobicity of *N*-benzoylproline is higher than that of hippuric acid, it is possible that the hydrophobic interaction between the ligand and the carrier improves the transport efficiency and permeability.

Table III. The Transport Kinetics of Phenylpropionylproline and *N*-Benzoylproline

	Phenylpropionylproline	*N*-Benzoylproline
J_{max*}	0.037 ± 0.019[a] m*M*	1.340 ± 0.240[a] m*M*
K_m	0.045 ± 0.027 m*M*	1.310 ± 0.300 m*M*
P_{m*}	0.673 ± 0.049	~0
P_{c*}	0.830 ± 0.130	1.020 ± 0.110

[a] Estimated mean ± SD of the parameters.

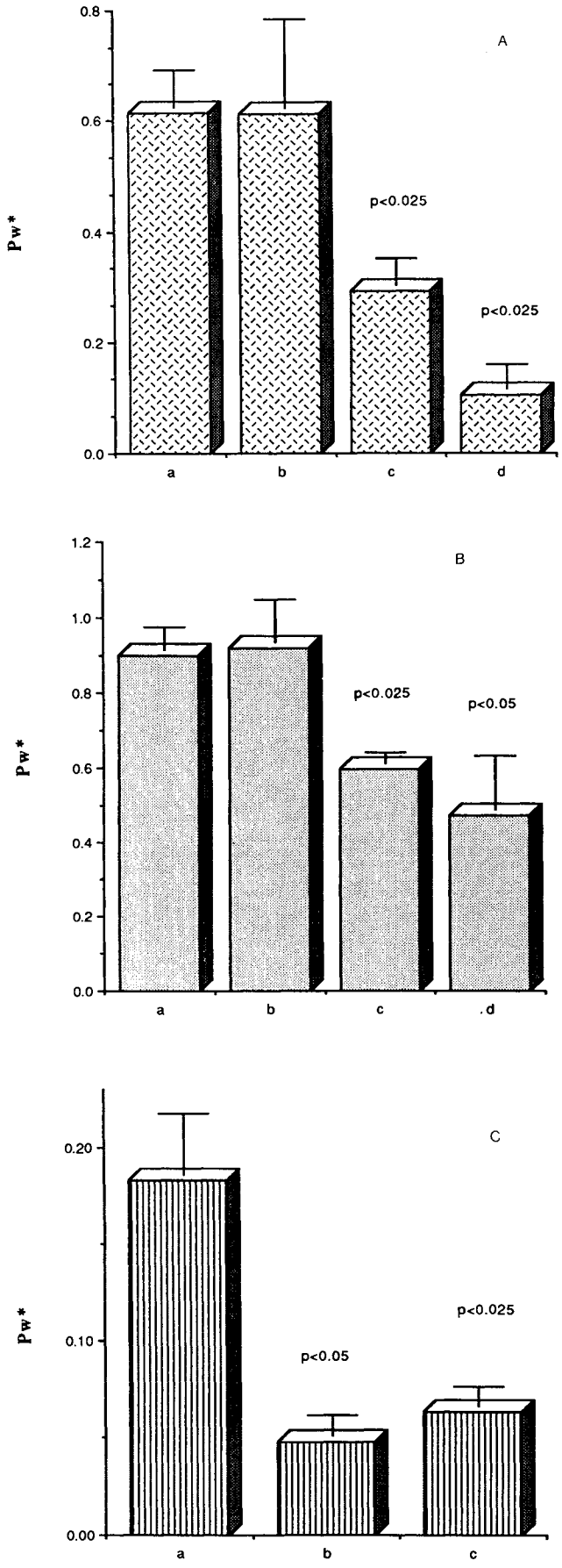
Transport Kinetics

The permeabilities of phenylpropionylproline (Fig. 2A), *N*-benzoylproline (Fig. 2B), and phenylacetyl-α-methyldopa (Fig. 2C) were concentration dependent. Using nonlinear regression, the transport parameters (mean ± SD) of phenylpropionylproline and *N*-benzoylproline (Table III) were determined to be as follows: J_{max*}, 0.037 (±0.019) m*M*; K_m, 0.045 (±0.027) m*M*; P_{c*}, 0.830 (±0.130); and P_{m*}, 0.673 ± 0.049; and J_{max*}, 1.34 (±0.24) m*M*; K_m, 1.31 (±0.30) m*M*; P_{c*}, 1.02 (±0.11); and P_{m*}, 0; respectively. Because of the high passive permeability observed for phenylpropionylproline, its J_{max*} and K_m obtained from the nonlinear regression are not well defined (high standard deviations). At low concentrations, the contribution to its overall absorption from the carrier-mediated transport is, however, significant (>50%). *N*-Benzoylproline possessed negligible passive permeability and its transport was mainly carrier mediated. Phenylpropionylproline demonstrated a much higher passive permeability than *N*-benzoylproline as might be expected since hydrophobicity is important for passive transport. The dimensionless wall permeability of phenylacetyl-α-methyldopa was also significantly reduced at a 10-fold higher concentration 0.5 m*M* ($P < 0.025$).

Inhibition Studies

The intestinal absorption of 0.05 m*M* phenylacetyl-α-methyldopa was significantly reduced by 80 m*M* Gly-Gly ($P < 0.025$) and by 10 m*M* mixed dipeptides (4 m*M* Gly-Gly, 2 m*M* Gly-Phe, 2 m*M* Gly-Pro, 2 m*M* Asp-Phe) ($P < 0.025$) (Fig. 3A), while 10 m*M* Gly had no inhibition on its intestinal absorption. The inhibition by the mixed dipeptides was due to competition for the peptide carrier with phenylacetyl-α-methyldopa. Based on the previous discussion, phenylacetyl-α-methyldopa is a poor substrate of the brush border membrane enzymes. Thus the inhibition by the dipeptides on its wall permeability indicates that phenylacetyl-α-methyldopa shares the common carrier system with small peptides.

The results of inhibition studies of phenylpropionylpro-



line, phenylacetylproline, *N*-benzoylproline, and hippuric acid are shown in Figs. 1A and B and Figs. 3B and C, respectively. The intestinal permeabilities of 0.1 m*M* phenylpropionylproline (Fig. 1A), 0.1 m*M* phenylacetylproline (Fig. 1B), and 0.1 m*M* *N*-benzoylproline (Fig. 3B) were significantly reduced in the presence of 27 m*M* Gly-Pro ($P < 0.025$), 23 m*M* Gly-Pro ($P < 0.05$), and 10 m*M* Gly-Pro ($P < 0.025$), respectively. Their permeabilities were also significantly reduced by 1 m*M* ($P < 0.05$), 2 m*M* ($P < 0.025$), and 2.5 m*M* ($P < 0.05$) cephradine, respectively. Cephradine, a β-lactam antibiotic, has been shown to be transported by the peptide carrier with a K_m of 1.5 m*M* (7). The mixed dipeptides with 60 m*M* Gly-Gly, 2 m*M* Gly-Phe, and 2 m*M* Pro-Phe also inhibited the intestinal absorption of 0.1 m*M* phenylpropionylproline ($P < 0.025$). Based on the fact that phenylpropionylproline, phenylacetylproline, and *N*-benzoylproline are poor substrates for the brush border enzymes, the inhibition of their intestinal wall permeabilities by dipeptides and cephradine strongly suggests that the dipeptides and cephradine compete for the same carrier with these dipeptide analogues.

Peptide carrier-mediated transport has been shown to be independent of amino acid carrier-mediated transport (1,5). To rule out the possibility that *N*-benzoylproline was absorbed by an amino acid carrier, 10 m*M* proline was coperfused with *N*-benzoylproline. The result indicates that 10 m*M* proline did not reduce the permeability of 0.1 m*M* *N*-benzoylproline. The inhibition by 10 m*M* Gly-Pro and lack of inhibition of 10 m*M* proline on the intestinal absorption of *N*-benzoylproline indicate that *N*-benzoylproline is transported by the small peptide carrier system, instead of the amino acid carrier system. The wall permeability of 0.1 m*M* hippuric acid was significantly decreased in the presence of 2 m*M* cephradine ($P < 0.025$) and 22.66 m*M* mixed dipeptides ($P < 0.05$) with 12.66 m*M* Phe-Gly and 10 m*M* Gly-Phe (Fig. 3C). This demonstrates that hippuric acid is transported by the peptide carrier as well, although less efficiently than *N*-benzoylproline.

These inhibition results for phenylacetyl-α-methyldopa, phenylpropionylproline, phenylacetylproline, *N*-benzoylproline, and hippuric acid strongly indicate that these dipeptide analogues without an α-amino group with either α-methyldopa, proline, or glycine at the C terminal are all transported by the small peptide transporter.

While recent results (11–15) suggest that the free *N*-terminal α-amino group may not be required for the transport of peptide-like therapeutic agents via the peptide carrier, this study directly provides conclusive evidence that the role of the N-terminal α-amino group on the substrate for the intestinal mucosal-cell peptide carrier-mediated transport is not critical.

Fig. 3. (A) Inhibition of 0.05 m*M* phenylacetyl-α-methyldopa permeability: (a) control; (b) 10 m*M* Gly; (c) 80 m*M* Gly-Gly; (d) 10 m*M* mixed dipeptides (4 m*M* Gly-Gly and 2 m*M* Gly-Phe, Gly-Pro, and Asp-Phe each). (B) Inhibition of 0.1 m*M* *N*-benzoylproline permeability: (a) control; (b) 10 m*M* Pro; (c) 10 m*M* Gly-Pro; (d) 2.5 m*M* cephradine. (C) Inhibition of 0.1 m*M* hippuric acid permeability: (a) control; (b) 12.66 m*M* Phe-Gly and 10 m*M* Gly-Phe; (c) 2 m*M* cephradine. The values reported are mean ± SE.

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