The Influence of Peptide Structure on Transport Across Caco-2 Cells. II. Peptide Bond Modification Which Results in Improved Permeability

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In order to study the influence of hydrogen bonding in the amide backbone of a peptide on permeability across a cell membrane, a series of tetrapeptide analogues was prepared from D-phenylalanine. The amide nitrogens in the parent oligomer were sequentially methylated to give a series containing from one to four methyl groups. The transport of these peptides was examined across confluent monolayers of Caco-2 cells as a model of the intestinal mucosa. The results of these studies showed a substantial increase in transport with each methyl group added. Only slight differences in the octanol–water partition coefficient accompanied this alkylation, suggesting that the increase in permeability is not due to lipophilicity considerations. These observations are, however, consistent with a model in which hydrogen bonding in the backbone is a principal determinant of transport. Methylation is seen to reduce the overall hydrogen bond potential of the peptide and increases flux by this mechanism. These results suggest that alkylation of the amides in the peptide chain is an effective way to improve the passive absorption potential for this class of compounds.

KEY WORDS: peptide; transport; permeability; lipophilicity; hydrogen bonding; cell culture.

INTRODUCTION

The successful development of peptides and peptide-like hormones as orally bioavailable therapeutic agents will clearly be aided by an understanding of the influence of structure on absorption across the intestinal mucosa. The development of such an understanding has been a goal of work in our laboratories for the last several years.

In a previous study, we showed that the permeability of a series of peptides and simple amides could be explained in the context of a model incorporating the total hydrogen bonding potential of the solute. It was argued that the rate limiting step in transport across the cell membrane was desolvation of the solute in order for it to enter the hydrophobic, non-hydrogen bonding interior of the membrane (1). Consistent with this model, then, is the prediction that peptides incorporating fewer hydrogen bonding sites should have greater permeability than similar peptides with more such bonds. One possibility for achieving this goal in a peptide structure might be to incorporate amino acids with little or no hydrogen bonding functionality in the side chains.

However, by its very nature, the amide backbone will have at least two hydrogen bonds for each amino acid in the primary sequence (1). It would seem desirable to establish techniques to reduce the hydrogen bonding potential of these amide bonds. One such method was suggested by early work of Wright and Diamond (2). It was shown that the permeability of a tertiary amide across rabbit gall bladder epithelium was substantially greater than for a structurally equivalent molecule containing a secondary amide (2). This observation suggested to us that alkylation of the nitrogen in the amide might result in improved transport across the intestinal mucosa.

In order to test this concept, a series of tetrapeptide analogues was prepared in which the amide nitrogens were sequentially methylated. The permeability of this series was then examined across monolayers of Caco-2 cells as an *in vitro* model of the intestinal mucosa (3–5).

EXPERIMENTAL

Materials. *N*-t-BOC-D-phenylalanine[3] and *N*-t-BOC-*N*-methyl-D-phenylalanine were obtained from Sigma. Acetic anhydride [1-^{14}C], specific activity 106–112 mCi/mmol, was purchased from Amersham. All other synthetic reagents were from Aldrich and were of reagent grade. Preparative silica gel 60 TLC plates were from EM Reagents. Caco-2 cells were obtained from ATCC at passage 13. Transwell inserts (3-μm pore) and six-well clusters were from Costar. All cell culture materials were from GIBCO.

General Synthetic Methods. Standard solution methodologies were used for the preparation of the nonradiolabeled peptides II–V (structures shown in Table I). After purification and characterization, the radiolabeled analogues were prepared and referenced to the authentic standards. Analytical HPLC was performed on a system consisting of a Beckman Model 110-A pump, Perkin-Elmer LC-55-B UV detector, and HP-3380-A integrator. Detector wavelength was typically 205 nm. For the radiolabeled analogues, detection was with a Flo-One HS Detector from Radiomatic fitted with a 2.5-ml flow cell and scintillant (Flo-Scint II)-to-column effluent ratio of 4:1. In both cases, the column was a Brownlee RP-18 Spheri-5, 4.6 mm × 10-cm column.

Acetamido-D-phenylalanyl-D-phenylalanyl-D-phenylalanylcarboxamide, [Acetyl-1-^{14}C] (I). This compound was prepared as described previously (1). The specific activity was 106 μCi/μmol.

Acetamido-D-phenylalanyl-D-phenylalanyl-N-methyl-D-phenylalanylcarboxamide, [Acetyl-1-^{14}C] (II). To a solution of Phe-Phe-*N*-methyl-PheNH$_2$ (10 mg, 0.022 mmol) in 5 ml chloroform was added triethylamine (5 μl, 0.035 mmol), followed by acetic anhydride, [1-^{14}C] (500 μCi, 0.0047 mmol, in 0.25 ml toluene). After stirring for 30 min at room temperature, BOP-Cl (2 mg, 0.008 mmol) was added and the resulting solution allowed to stir overnight. The crude product was washed with 5% HCl, 10% Na_2CO_3, and water, dried over

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[3] Abbreviations used: t-BOC, tertiarybutyloxycarbonyl; BOP-Cl, bis(2-oxo-3-oxazolidinyl)phosphinic chloride; DCC, dicyclohexylcarbodiimide; Et_3N, triethylamine; DMOA, *N,N*-dimethyloctylamine; HBSS, Hanks balanced salt solution; TLC, thin-layer chromatography.

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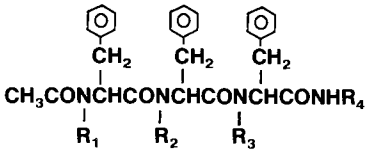
Table I. Structures of the Peptide Models



Compound	R_1	R_2	R_3	R_4
I	H	H	H	H
II	H	H	CH_3	H
III	H	CH_3	CH_3	H
IV	CH_3	CH_3	CH_3	H
V	CH_3	CH_3	CH_3	CH_3

Na_2SO_4, filtered, and concentrated under a nitrogen stream. The residue was applied to a preparative TLC plate and developed with CH_3OH–$CHCl_3$ (5:95). The product band was cut out and extracted with methanol–chloroform (50:50) to yield 45 μCi of the desired peptide with a specific activity of 106 μCi/μmol. Mass spec (nonradiolabeled peptide), $[M + H]^+$ at 515. RP-HPLC, 4.92-min retention time (40% CH_3CN, 0.02% CF_3CO_2H, 0.02% DMOA, at 1 ml/min).

*Acetamido-D-phenylalanyl-*N*-methyl-D-phenylalanyl-*N*-methyl-D-phenylalanylcarboxamide, [Acetyl-1-^{14}C] (III).* Preparation was as for II, starting with Phe-*N*-methyl-Phe-*N*-methyl-PheNH_2. Purification on preparative TLC eluting with methanol–chloroform–ammonium hydroxide (5:95:0.1) yielded 110 μCi of the product with a specific activity of 108 μCi/μmol. Mass spec (nonradioactive standard), $[M + H]^+$ at 529. RP-HPLC, 4.6-min retention time (40% CH_3CN, 0.02% CF_3CO_2H, 0.02% DMOA, 1 ml/min).

*Acetamido-*N*-methyl-D-phenylalanyl-*N*-methyl-D-phenylalanyl-*N*-methyl-D-phenylalanylcarboxamide, [Acetyl-1-^{14}C] (IV).* Preparation was as for II, starting with *N*-methyl-Phe-*N*-methyl-Phe-*N*-methyl-PheNH_2. Purification on preparative TLC eluting with isopropanol–chloroform–ammonium hydroxide (5:95:0.01) yielded 180 μCi of product with a specific activity of 108 μCi/μmol. Mass spec (nonradioactive standard), $[M + H]^+$ at 543. RP-HPLC, 6.7-min retention time (40% CH_3CN, 0.02% CF_3CO_2H, 0.02% DMOA, 1 ml/min).

*Acetamido-*N*-methyl-D-phenylalanyl-*N*-methyl-D-phenylalanyl-*N*-methyl-D-phenylalanyl-*N*-methylcarboxamide, [Acetyl-1-^{14}C] (V).* Preparation was as for II, starting from *N*-methyl-Phe-*N*-methyl-Phe-*N*-methyl-Phe-$NHCH_3$. Purification was by preparative TLC eluting with 20% isopropanol in ethylacetate to yield 95 μCi of the desired product with a specific activity of 112 μCi/μmol. Mass spec, $[M + H]^+$ at 557 for the nonradioactive standard. RP-HPLC retention time of 7.4 min (45% CH_3CN, 0.02% CF_3CO_2H, 0.02% DMOA, 1 ml/min).

Cell Culture. The preparation of confluent Caco-2 cell monolayers on Transwell polycarbonate filters has been described in detail previously (3). The cells used in these studies were between passage 26 and passage 40.

Transport Studies. The transport experiments were performed with monolayers of between 14 and 21 days in culture as previously described (3). Briefly, the monolayers were washed three times with HBSS before the solute of interest, in HBSS, was added to the donor compartment. Compounds II–V were found to be somewhat radiochemically unstable; therefore they were repurified immediately prior to preparation of the stock solutions. The donor concentrations ranged from 0.4 to 10 μ*M* in different experiments. In the case of peptides III and IV, transport studies were also done with donor solutions up to 200 μ*M*.

After a specified interval, the cup containing the monolayer and donor solution was transferred to a fresh receiver compartment. Permeability coefficients were calculated as described previously (1,3). At the end of the experiment, samples of the donor and receiver solutions were analyzed by HPLC to confirm that the radioactivity measured was that of the starting peptide.

Partition Coefficient Determinations. Partition coefficients were determined by overnight shaking of HBSS solutions of the radiolabeled peptides with a volume of HBSS-saturated octanol. Phase volumes were 1:50 and 1:100 (octanol:HBSS) for each peptide. After centrifugation to separate the phases, aliquots of both were taken for counting. The partition coefficients were calculated from the concentration of the radioactivity in the octanol divided by the concentration of radioactivity in the buffer.

Analytical Methods. Radioactivity was quantitated by liquid scintillation counting in a Beckman LS 3801 scintillation counter with ACS scintillant from Amersham. Mass spectral analysis of the nonradiolabeled peptide standards was provided by the Physical and Analytical Chemistry Department at Upjohn.

RESULTS

Flux of the Peptides Across Caco-2 Cell Monolayers. The appearance rates for the *N*-methyl peptides II–V across the Caco-2 cell monolayers is shown in Fig. 1. As can be seen, each introduction of a methyl group increases the relative flux of the peptide. For peptides III and IV, where flux was examined as a function of donor concentration, an increase in flux was observed when the donor peptide exceeded 50 μ*M*. Therefore only data from the donor solutions less than 10 μ*M* were used for this study. Effective permeability coefficients (P_{eff}) for each of the peptides were calculated from the steady-state rate of appearance as has been described before (3). These values, and that for compound I, which was previously reported, are summarized in Table I. As pointed out earlier (1), P_{eff} contains contributions from $P_{monolayer}$, the permeability coefficient for the peptide across Caco-2 cell monolayers, along with permeability through the aqueous boundary layer (P_{aq}) and through the filter (P_{filter}). Using the values for P_{aq} and P_{filter} previously determined (1,3), P_{eff} was corrected in order to obtain $P_{monolayer}$ for the peptides. These values are included in Table I.

To assure that radioactivity appearing in the receiver compartments was intact peptide and not metabolic fragments formed in the presence of the Caco-2 cells, samples of the donor and receiver solutions were assayed by HPLC with Radiomatic detection at the end of several experiments. No evidence of radioactive material could be seen other than that of the intact, parent peptide.

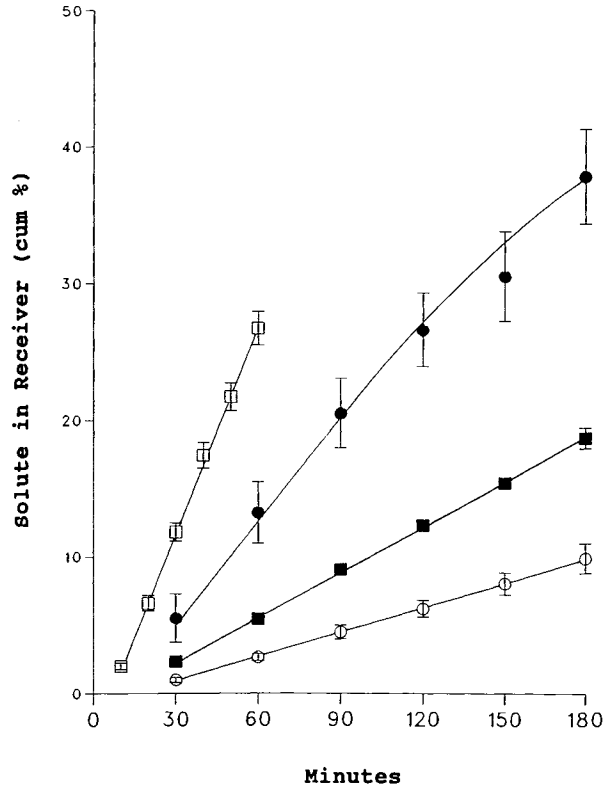


Fig. 1. Flux of the peptides II–V across confluent Caco-2 cell monolayers. Stock solutions of $AcPhe_2(NCH_3Phe)NH_2$ (○), $AcPhe(NCH_3Phe)_2NH_2$ (■), $Ac(NCH_3Phe)_3NH_2$ (●), or $Ac(NCH_3Phe)_3NHCH_3$ (□) were incubated in the Transwell system at 37°C. Each point represents the mean and standard deviation of at least six determinations.

For each of the peptides I–V, an initial lag time was seen before steady state for appearance of solute in the receiver was achieved. In the case of the tetramethylated derivative V, this lag was approximately 7–10 min and could be eliminated when the monolayer was preequilibrated with the peptide solution before starting the experiment (results not shown). These observations are consistent with an adsorption or binding of the peptide to the cell monolayer. Further, mass balance determination at the end of the experiment showed that about 2–10% of the peptide appeared to be associated with the cell monolayer.

Permeability of the Peptides as a Function of Lipophilicity. With the introduction of each methyl group in the series II–V, the carbon number of the peptide is increased. To determine whether the potential increase in lipophilicity due to this increase in carbon number might be contributing to the observed flux increases, the octanol–water partition coefficient for each of the peptides was determined. These values are shown in Table II, along with that for peptide I, which had been determined previously.

As can be seen from the results in Table II, the introduction of a methyl group has only a minor effect on the partition coefficient, while the permeability increases substantially. Thus, consistent with our earlier peptide study (1), we see no significant correlation between permeability and octanol–water partition coefficient for this series of structurally related peptides. This is in marked contrast to

Table II. Summary of Caco-2 Cell Permeability and Octanol–Buffer Partition Coefficients (PC) for the Peptides

Peptide	Permeability coefficient[a]		log PC[c]
	P_{eff}	$P_{monolayer}$[b]	
I	0.66 (0.03)[d]	0.67 (0.03)[d]	2.30 (0.02)[d]
II	2.78 (0.63)	2.88 (0.63)	2.63 (0.01)
III	5.68 (0.52)	6.11 (0.52)	2.53 (0.01)
IV	13.8 (1.7)	16.7 (1.7)	2.92 (0.01)
V	23.8 (2.8)	33.9 (2.9)	3.24 (0.02)

[a] cm/sec × 10^6 mean (±SD).
[b] Calculated from P_{eff} using $P_{aq} = 8 \times 10^{-5}$ cm/sec and $P_{filter} = 1 \times 10^{-3}$ cm/sec (1, 3).
[c] Mean (±SD).
[d] Data from Ref. 1.

earlier work with both Caco-2 cell monolayers and *in vivo* models employing nonpeptide model substrates, where a sigmoidal relationship was found between permeability and partition coefficient (3,6,7).

Permeability as a Function of Hydrogen Bond Number. A hydrogen bond number N was assigned to each of the peptides based on the earlier work of Stein (8). This is the expected number of such bonds each functionality in the peptide can make with water summed over the number of functionalities in the molecule. Consistent with our previous study (1), we assign two bonds to each secondary amide (CONH), three to a primary amide ($CONH_2$), and one to the methylated tertiary amides. This results in a hydrogen bond number reduction of one for each methyl group introduced into $AcPhe_3NH_2$. Figure 2 shows the relationship of permeability with hydrogen bond number for the series. Included in the figure are the permeability results from our earlier peptide study with the Caco-2 model (1). The good correlation seen again suggests that solute–solvent interaction via hydrogen bonding is a strong determinant of membrane or cellular permeability.

DISCUSSION

The results shown in these studies demonstrate that alkylation of the amide bond is an effective method for improving the permeability of a peptide across Caco-2 cells. This is consistent with observations from Wright and Diamond when measuring reflection coefficients of a simple series of amides in gallbladder epithelium (2) and our earlier results comparing transport of primary, secondary, and tertiary amides across Caco-2 cells (1).

A number of possible mechanisms can be conceived to explain how methylation could bring about this improvement in transport. In general, it is thought that for simple drug molecules, a strong correlation exists between octanol–water partition coefficient and absorption (7,9,10), and the same relationship has been shown with the Caco-2 model (3,6). Thus, one possibility is that methylation results in an increase in lipophilicity of the peptide.

However, as we also showed earlier, this relationship between partition coefficient and absorption does not seem to apply to the transport of simple peptides across Caco-2 cell monolayers (1). The results presented here further sub-

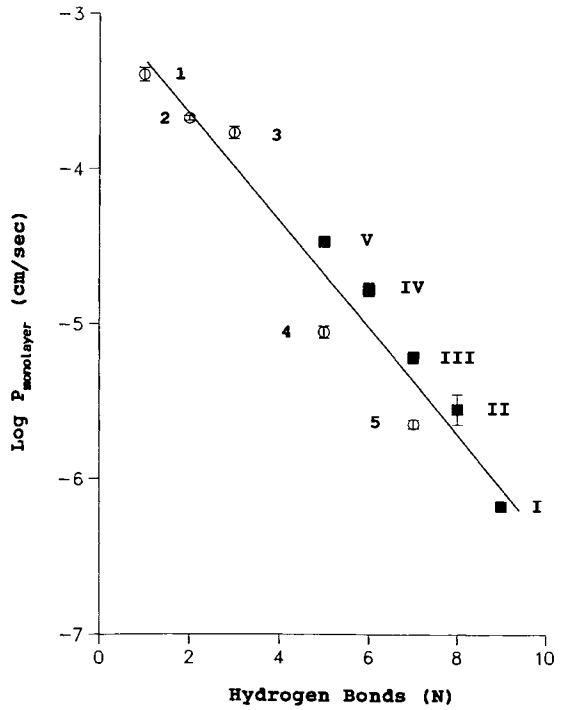


Fig. 2. Relationship of the permeability coefficients for peptides I–V (■) across Caco-2 cell monolayers and number of potential hydrogen bonding sites in the solute. Included are data for *N*-benzyl, *N*-methylacetamide (1), *N*-benzylacetamide (2), 3-phenylpropanamide (3), $AcPheNH_2$ (4), and $AcPhe_2NH_2$ (5) taken from our previous work (1). The correlation coefficient for the regression line shown is −0.971.

stantiate and extend these observations. Sequential alkylation of $AcPhe_3NH_2$ has little influence on the measured partition coefficient of these peptides in octanol. Yet with each alkyl group added, a substantial increase in flux is observed. These results are similar to earlier work reported by Diamond and Wright. In comparing cellular transport of structurally related solutes they found, in several cases, very similar partition coefficients but markedly different permeability coefficients. For example, the partition coefficient ratio for methyl acetate and glycerol in isobutanol is 26, while the ratio of permeability coefficients for the flux of the two solutes into the giant algae *Nitella* is 78,000 (11). These results suggest that the membrane displays a difference in "selectivity" relative to pure organic solvents and is much more sensitive with respect to distinguishing between similar chemical structures (11). Again, our results here are consistent with these early observations.

An alternative mechanism to that of lipophilicity to explain the absorption of peptides, which we proposed earlier, is that desolvation of the amide backbone and other hydrogen bonding groups in the molecule is the principal determinant (1). In the context of this model, alkylation of the peptide amide can be seen as effectively reducing the number of hydrogen bonds in this functionality. Thus, the sequential methylation of the pseudotetrapeptide, $AcPhe_3NH_2$, results in an apparent hydrogen bond number reduction from 9 to 5 with the introduction of the four methyl groups. Thus, the strong correlation shown in Fig. 2 is consistent with this reduction in hydrogen bond number as the cause of the improved permeability.

It might be interesting to speculate on the mechanism by which methylation could reduce hydrogen bonding. Since the N–H can act as a hydrogen bond donor, the effect of alkylation would be to remove the bond-forming proton. This is the most obvious explanation and is consistent with the known hydrogen bonding potential of the NH group from infrared and other types of studies (12,13). Further, the importance of the N–H was also cited by Jacobs and White in order to estimate a reduction in the free energy of desolvation of 4.2 kcal/mol for a model peptide upon replacement of an amino acid by proline in the sequence (14).

An alternative explanation is that the process of methylation of the peptide backbone induces some conformational change. The result of this imposition of a potentially restricted conformation on the peptide could, in principal, promote intramolecular hydrogen bonding between separated amide groups along the chain. It has been shown, for example, that the energy cost of desolvating an amide bond in order to transfer it from water to a non-hydrogen-bonding solvent is 6.12 kcal/mol. However, if the amide is intramolecularly hydrogen bonded, no solvent bonds need be broken and the free energy change involved in transfer is only 0.55 kcal (15). Clearly, then, promotion of intramolecular hydrogen bonding could have a profound effect on the transfer free energy and therefore the permeability coefficient.

While this conformational mechanism is an appealing one, it seems unlikely in the present case. The peptides utilized in this work are only four residues in length. Peptides of this size have been found not to possess any stable secondary structure in solution (16), although some structure could be induced at a phase boundary such as a cell membrane (17,18). A second reason for doubting this explanation is that it does not predict the cumulative effect of sequential alkylation which is seen in this study. It is not obvious how adding methyl groups to adjacent amide bonds could effect such a regular increase in permeability through a conformational perturbation.

Finally, it may be possible that alkylation contributes to improved permeability by reducing intermolecular hydrogen bonding. In this case, self-association of the peptides could result in the formation of large, nondiffusible aggregates in solution. Thus, by reducing the extent of aggregation, more free monomer will be available for transport. While we cannot rule out this contribution, the limited results obtained examining the effect of concentration on permeability do not seem to support this mechanism. In the case of $AcPhe_3NH_2$, which would be expected to be the most highly self-associated, no influence of concentration was seen over the range of 2–10 μM. Similarly, for peptides III and IV, a small increase in permeability was found with much higher concentrations. While the reason for these increases is presently unknown, the results are the opposite of what would be expected from a model in which self-association retards transport.

In summary, the exact mechanism responsible for the increase in permeability of $AcPhe_3NH_2$ across Caco-2 cell monolayers with methylation of the amide bonds remains to be determined. However, the results are clearly consistent with a model in which the total hydrogen bonding capacity of

the molecule has been reduced. From a design perspective, one might expect that the utilization of *N*-alkyl amino acids as synthons would result in peptides with greater absorption potential than the equivalent nonalkylated structure. The principal disadvantage to such an approach, however, is that such a modified structure may no longer possess the desired biological activity due to decreased receptor binding. Such a balance between absorption potential and intrinsic activity will have to be determined empirically. A possible method for overcoming this apparent limitation would be to design a reversible modification for an amide bond. This could allow for the desired reduction in hydrogen bonding to improve permeability, after which the modification would be removed, yielding the unsubstituted amide. Such a prodrug concept is currently being explored in our laboratories.

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