Oral Absorption of D-Oligopeptides in Rats via the Paracellular Route

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Purpose. This study was undertaken to examine the structural determinants of oral bioavailability in the rat of a set of oligopeptides comprising D-amino acids, which were taken to be absorbed paracellularly based on a pronounced sensitivity of permeability to electrical resistance in Caco-2 cell monolayers.

Methods. The study series comprised eleven D-oligopeptides, designed not to be recognised by peptidases or transport proteins, and to have molecular weights between 222 and 406 daltons with different net electrical charges and composition of D-amino acids. All the peptides were [^{3}H]-radiolabelled and analyzed by HPLC with radiometric detection. Bioavailability was estimated based on 24-hr urinary excretion of unchanged peptide after oral and intravenous administrations.

Results. As expected, the series proved metabolically stable. Bioavailability was independent of oral dose when varied by a factor of 10,000, suggesting passive absorption. Whereas bioavailability decreased sharply from 30% to 1% with increasing molecular weight, net charge showed little, if any, effect on bioavailabilty.

Conclusions. This D-oligopeptide model series served as a useful probe for the structural requirements for paracellular absorption *in vivo*. A critical determinant of bioavailability is molecular size, expressed as molecular weight in this study; net charged appeared of much lesser importance.

KEY WORDS: D-oligopeptides; intestine; oral bioavailability; paracellular absorption; rat.

INTRODUCTION

Despite the continuing popularity and importance of the oral route for drug delivery and the vast amount of experimental data generated, the gastrointestinal tract still presents many problems to those involved in drug design and development. Historically, solute transport across biological membranes has been dominated by the concept of diffusion through the lipid barrier of cell membranes. However, many small-to-medium size (200–600 Dalton) polar drugs are quite well absorbed from the intestinal tract (1–3). These hydrophilic molecules, which have difficulty in traversing lipophilic cell membrane surfaces are probably absorbed *via* the aqueous-filled paracellular spaces, through the tight junctions. Many commonly used probes of intestinal permeability, such as mannitol, PEG400, inulin and lactulose, are also presumed to be absorbed *via* paracellular pathways (4–6).

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The relationship between chemical structure and paracellular intestinal permeability is poorly understood. Molecular size is important with permeability decreasing with increasing size (3–5), but the role of shape is less clear. Reported data on the effect of net electrical charge on paracellular uptake have been taken to indicate that there is a charge specificity (7–8), but the role of charge distribution is uncertain. Adson *et al.* (1) reported that protonated amines permeate tight junctions in epithelial cell layers more rapidly than neutral compounds of similar size and that anions permeate more slowly and argue that the paracellular route is negatively charged. To better understand the structural requirements for paracellular absorption, we have synthesized a series of structurally related polar oligopeptides, based on D-amino acids, which are expected to be metabolically stable and absorbed *via* the paracellular route. Oligopeptides were chosen as they can be readily synthesized and permit systematic changes in shape, charge distribution, size and other molecular properties. All the peptides studied were [^{3}H]-radiolabelled. Most mechanistic studies are undertaken using *in vitro* systems such as isolated perfused loops, cell monolayer culture and intestinal epithelial. To clarify the physiological relevance of these observations, reliable comparative *in vivo* data are needed. The objective of this study was to characterise and examine the molecular determinants of oral bioavailability for D-oligopeptides in the rat.

MATERIALS AND METHODS

Materials

A series of 11 peptides, all derivatives of D-phenylalanine (Fig. 1), both unlabelled and [^{3}H]-labelled (in the 4-position of phenyl ring) were synthesised by standard solution methods (10).

Animal Experiments

Male Sprague-Dawley rats (200–300 g), housed at ambient temperature and humidity, were fed a standard rat chow and allowed free access to food and water before the experiment. The right jugular vein was cannulated for i.v. administration with PE tubing under light anaesthesia with halothane. Sodium heparin (10 U/ml) was administered *via* the cannula to prevent blood clotting. After recovery overnight rats were transferred into individual metabolic cages for separate and quantitative urine collection. Whenever possible based on the ability to adequately separate materials by HPLC (see assay), the D-oligopeptides were administered as multi-component mixtures, to maximise ability to detect differences among the compounds. D-oligopeptide solutions were prepared from the radiolabelled isotopes and the corresponding unlabelled compounds in saline to give final concentrations of 1.25 μg/ml for the tracer experiment and 1.25 mg/ml for the high-dose study. Using a crossover design, each rat received solutions orally (2 ml/kg), by gavage and intravenously (1 ml/kg) with total urine collection from 0 to 6 hr and 6 to 24 hr, following each administration. The 24-urine collection period was sufficient to ensure complete collection of excreted material, based on preliminary experiments. Urine samples were frozen at −20°C until analysed. Reported results for each compound are based on studies in 3–6 animals.

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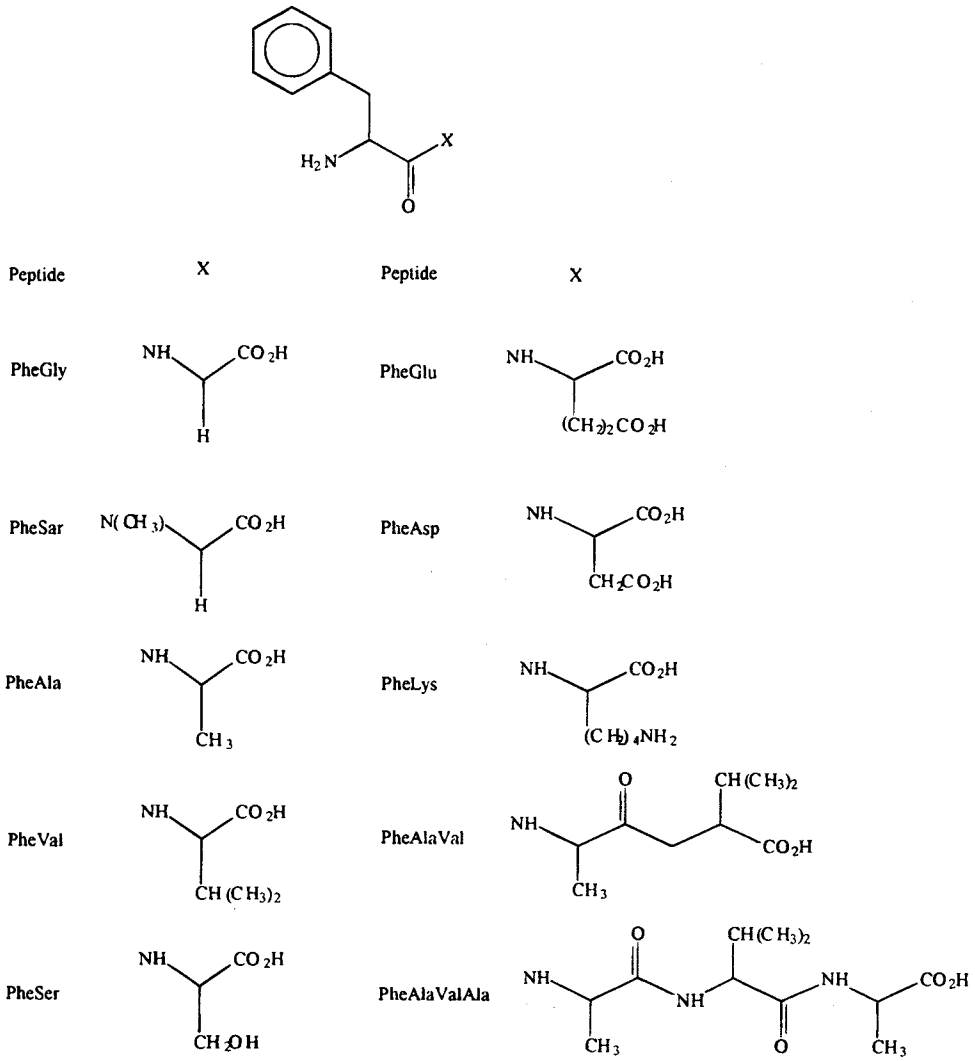

Fig. 1. Structures of peptides used, all based on D-amino acids.

HPLC Analysis

The chromatographic system used for analysis of the peptides consisted of a solvent degasser, gradient pumps (P4000), autosampler (AS300), UV detector (UV150), data jet integrator (SP4600), all from Spectra-Physics (Analytical Inc), and radiomatic detector (Flo-one\beta, Packard), with the column temperature maintained at 40°C. Isocratic and gradient conditions were used, as appropriate. Isocratic conditions:- Perisil ODS (25 cm × 4.6 mm, Thames Chromatography, Berkshire, UK) column, with mobile phase (acetonitrile: 50 mM phosphate (pH4.0) buffer, 13:87) at a flow rate of 1.0 ml/min. Gradient conditions:- Vydac C18 (25 cm × 4.6 mm, Hichrom Ltd, Berkshire, UK) column with mobile phase (acetonitrile: 50 mM phosphate buffer (pH4), increasing acetonitrile from 0% to 40% linearily over 15 min) at a flow rate of 1.5 ml/min. The concentration of radiolabelled compound was determined by reference to a standard calibration curve, obtained by injecting known amounts onto the HPLC.

Radiolabel purity was assessed from the percent of the radiolabelled material injected onto the HPLC that was associated with the peak eluting at the same retention time as unlabelled compound, having previously demonstrated total recovery of injected radiolabelled material based on the counts associated with all radiolabelled peaks. The standard solutions for iv and oral administration were prepared by diluting the original stock solutions 5- and 20-fold, respectively. The urine samples were passed through 0.22 μm Ultrafree-MC filters, (Millipore, UK) and injected directly onto the HPLC. An aliquot (50 μl) of standard solution or urine sample was injected onto the column at ambient temperature *via* an autosampler.

Calculation of Bioavailability

Oral bioavailability F, was estimated as:

$$F = \frac{[\text{Ae/Dose}]_{\text{oral}}}{[\text{Ae/Dose}]_{\text{iv}}} \quad (1)$$

where Ae is the total amount of drug excreted unchanged.

RESULTS

The eleven oligopeptides of varying molecular weight and molecular properties, all had D-phenylalanine as the N-terminal

amino acid. The attempted synthesis of non-radiolabelled Phe-Glu proved problematic, producing material which showed signs of racemisation (by NMR and HPLC). In this case the radiolabelled compound was prepared by a solid-phase technique.

Analysis

The inter-day coefficients of variation of HPLC analysis for each oligopeptide when injecting 0.05 μCi total radioactivity are summarized in Table 1. Although no internal standard was used for the HPLC assay, the coefficient of variance of HPLC analysis ranged from only 0.55% for PheAlaVal to 4.07% for PheAsp. Because of sufficient differences in their retention times, the following mixtures were analysed as combinations: PheGlu and PheVal; PheAlaVal and PheSer; PheLys and PheAlaVal; PheAsp and PheAla; PheSer and PheAlaValAla.

Radiolabeled Purity

Table I summarizes the molecular weight, purity at dosing and analytical reproducibility of all the radiolabelled compounds. The molecular weight varies from 226 to 406 Daltons, which covers a reasonable molecular weight range for commonly used drug molecules. [^{3}H]-PheGly and [^{3}H]-PheSar showed the lowest purity, probably due to instability. The remaining tritiated D-oligopeptides were much purer, with purity ranging from 92% for PheVal and PheGln to 99% for Phe-Glu; these peptides were also much more stable, with less than 1% decomposition per month.

In Vivo Findings

Table II lists the percentages recovered unchanged in urine over 24 hr for the oligopeptides following intravenous administration, together with oral bioavailabilities, associated with the administration of the radiolabelled tracer doses. For all compounds, over 95% of that recovered in urine was excreted within the first 6 hr, indicating that the 24-hr collection provides a reasonable estimate of the total amount to be excreted. As shown in Table II, the peptides were relatively stable following intravenous administration, with generally greater than 60% excreted unchanged.

Table I. Molecular Weights, Radiolabeled Purities, and Analytical Reproducibility by HPLC of D-Peptides

Compound	MW	Purity (%)	CV[a](%)
PheGly	222.2	35	NM
PheAla	236.0	94	2.05
PheVal	264.3	96	2.27
PheSar	236.0	84	NM
PheSer	252.3	99	2.32
PheAsp	280.3	96	4.07
PheGln	293.3	92	3.72
PheLys	293.4	97	3.67
PheGlu	290.0	99	2.97
PheAlaVal	335.4	94	0.55
PheAlaValAla	406.5	96	2.83

[a] The coefficient variance of HPLC analysis when injecting 0.05μCi total radioactivity. NM: Not measured due to instability.

Table II. Mean (±SEM) Oral Bioavailability (*F*) and Percentage Recovery Unchanged in the Urine Within 24 Hours Following IV Administration in the Rat

D-oligopeptide	Oral Bioavailability (%) (*F*)	% Unchanged in Urine after IV Administration
PheGly	28.6 (5.9)	61.9 (8.6)
PheAla	29.6 (2.9)	61.3 (8.8)
PheVal	26.6 (6.8)	57.3 (4.9)
PheSar	18.9 (3.3)	77.0 (4.1)
PheSer	8.6 (0.6)	79.2 (4.5)
PheAsp	7.3 (1.2)	64.4 (7.7)
PheGln	7.0 (0.8)	74.4 (2.6)
PheLys	5.9 (0.9)	79.5 (2.3)
PheGlu	4.4 (0.9)	59.6 (7.3)
PheAlaVal	2.8 (0.5)	66.8 (3.4)
PheAlaValAla	1.1 (0.1)	50.5 (3.6)

A large range of oral bioavailability of unchanged drug was observed varying from 1% for the PheAlaValAla to around 30% for PheAla, despite the narrow molecular weight range. The calculated oral bioavailabilities based upon unchanged compound (Table II) and total radioactivity are compared in Fig. 2. Although for many of the peptides similar values of bioavailability were observed based on total and unchanged compound, there were significant differences between the two measurements for some of them. This applied particularly to PheGly, PheSar, PheGln, PheGlu and PheAlaValAla. For PheGly and PheSar, it is difficult to speculate on the cause of the discrepancy between the unchanged compound and total radioactivity due to the significant radiolabelled impurities in the original stock solution. On the other hand, PheGln, PheGlu and PheAlaValAla were relatively pure (92%, 99%, 96%), and there were no metabolites detected following intravenous administration. This indicates that these last three peptides were partially metabolized in the gastrointestinal tract. Clearly, the only solution to obtaining reliable oral bioavailability values of these peptides is to measure the unchanged species.

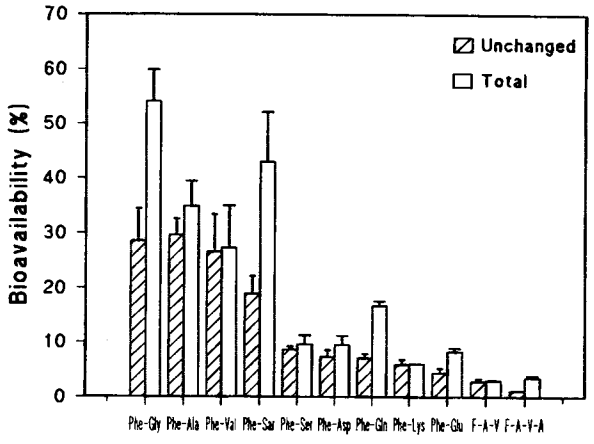

Fig. 2. Estimated oral bioavailabilities of D-oligopeptides calculated based on both total (□) and unchanged (▨) radiolabeled compound. Data are presented as mean ± SEM (N = 3 to 6). F-A-V and F-A-V-A represent Phe-Ala-Val and Phe-Ala-Val-Ala, respectively.

PheAla, PheVal, PheSer, PheAsp, PheLys and PheAlaVal were comparatively pure, and for these the same estimated bioavailability was obtained whether based on unchanged compound or total radioactivity, suggesting that these D-peptides are resistant to the extracellular peptidases anchored to the microvilli of absorptive cells. To confirm that the peptides synthesized are not transported *via* an active system, the dose-dependency of the oral absorption of some of the peptides was investigated. Figure 3 shows the results of the dose-dependency of bioavailability for PheAla, PheVal, PheSer, PheAp, PheLys, PheGlu, PheAlaVal and PheAlaValAla. Except for PheAlaValAla, the oral bioavailability remained constant when the oral dose was altered by a factor of 10,000 (from 2.5 μg/kg to 25 mg/kg), providing evidence that they are likely to be absorbed from the gastrointestinal tract *via* a passive transport system. A slightly higher bioavailability of PheAlaValAla (3.2 ± 2.1% vs 1.1 ± 0.10%) was observed following the higher dose suggesting that there is some metabolism of PheAlaValAla in the gastrointestinal tract and this may be saturable.

A clear, inverse and essentially log-linear decline in oral bioavailability with the molecular weights of the peptides was observed (Fig. 4). However, it is important to note that there was a large range in bioavailability from 1% (PheAlaValAla) to 30% (Phe-Ala), within a narrow molecular weight range between 250 and 300 Daltons. Using estimated pI values for the peptides gives the following net charge for major contributors under the pH conditions used: zero (zwitterionic), PheGly, PheAla, PheVal, PheSer, PheGln, PheAlaVal, PheAlaValAla; −1 (two negative, one positive charge), PheAsp, PheGlu; +1 (one negative, two positive charges) PheLys, PheGlu, PheGln and PheLys, which have similar molecular weights (around 290 Daltons), but net negative, neutral and positive charges at physiological pH respectively had similar bioavailabilities (4.4 ± 0.9, 7.0 ± 0.8, and 5.9 ± 0.9, respectively).

DISCUSSION

Drug design is complex. In certain circumstances, increasing hydrophilicity may be an advantage. Hydrophilic compounds tend to have reasonable aqueous solubilities and have restricted tissue distribution, often not readily entering the central nervous system. Also, many are relatively stable metabolically, with the majority excreted unchanged, thereby rendering their disposition kinetics reasonably predictable. Examples are such diverse compounds as atenolol, hydrochlorothiazide, ranitidine and many antibiotics. However, such compounds permeate cells poorly, especially those of the intestinal epithelium, such that oral absorption may be a problem, depending heavily on permeation *via* the paracellular route. However, the structural and physicochemical determinants of absorption of drugs *via* the paracellular route remain poorly defined.

The major restricting structure to paracellular permeation appears to be the junctional complexes (11–12). The importance of tight junction complexes in regulating intestinal permeability has been supported by studies showing a close relationship between transepithelial electrical resistance and the density of tight junctional strands (13) and between transepithelial resistance and permeability (14–15).

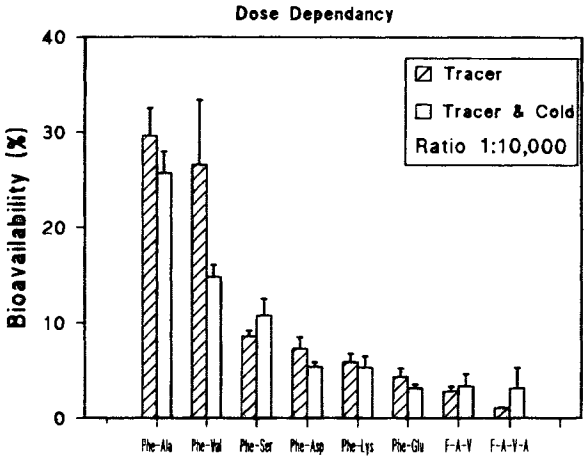

Fig. 3. Effect of dose on oral bioavailabilities of D-oligopeptides. The two treatments shown are (i) a radiolabeled tracer-dose alone (2.5 μg/kg; N = 3 to 6) ▨ and (ii) a tracer-dose together with unlabeled compound (25 mg/kg; N = 5 to 6)□.

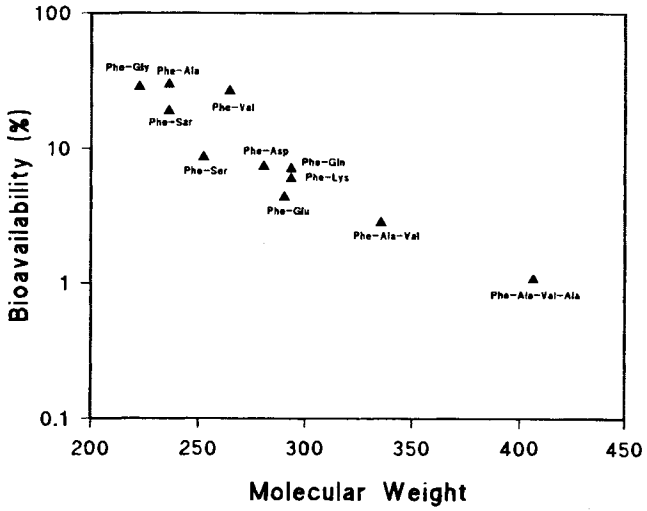

Fig. 4. Semi-logarithmic plot of oral bioavailability for D-oligopeptides *versus* molecular weight.

Compounds

The choice of peptides was guided by several principles. The series was based on the unnatural D-series of amino acids to minimise metabolism from intestinal peptidases (16). In constructing the larger peptides (PheAlaVal and PheAlaValAla) two contiguous D-Ala residues were avoided to minimise potential interaction with D,D-aminopeptidases of gut flora. D-Phenylalanine was chosen as a common residue at the N-terminus to facilitate radiolabelling the tracer compounds. The remaining peptides were chosen to provide a wide range of charge, polarity and size. We also believed that the peptides would be transported predominantly by the paracellular route, due to their zwitterionic nature, despite the presence of relatively hydrophobic residues in some cases.

Metabolism

In addition to intestinal stability, we hypothesized that the D-oligopeptides would be stable once absorbed and sufficiently polar and small to be predominantly eliminated by renal excre-

tion, thereby assisting in the interpretation of absorption from urinary excretion data. The high urinary recovery of unchanged compound following iv administration of all the D-oligopeptides supports this view. Furthermore, the finding that the vast majority was excreted within the first 6-hr urine collection implies that these compounds are rapidly eliminated from the body.

As predicted, the majority of the D-oligopeptides were also reasonably stable in the gastrointestinal tract. However, some did show evidence of limited gut metabolism, probably caused by aminopeptidases, endopeptidases or dipeptidylaminopeptidases that reside on the apical surface of the intestinal epithelium (17). We found that replacement of Gly by D-Ala markedly enhances stability, in keeping with the finding that replacement of Gly in [Met]-enkephalin by D-Ala made the resultant peptide more stable towards intestinal peptidases (18).

When metabolism occurs within the gastrointestinal tract, bioavailability based on unchanged compound underestimates the full potential of the compound to permeate the intestinal epithelium. However, for the current series of compounds, with relatively little intestinal metabolism occuring, differences in observed bioavailability probably do reflect differences in intestinal permeability.

Absorption

As with the iv dose, for all the D-oligopeptides, of that found in urine the majority was excreted within the first 6 hr collection period after oral administration, suggesting that these probe compounds are mainly absorbed within six hours, probably through the small intestine. Evidence in favour of their absorption occurring *via* the paracellular route is (i) the general lack of dose dependence in bioavailability, (ii) no evidence of competition in that bioavailability was similar whether the compound was given alone or as a multi-component mixture, and (iii) the pronounced sensitivity in the permeability across the human Caco-2 intestinal cell line with changes in the tightness of the tight junction for all compounds, achieved by changing the transepithelial electrical resistance with the calcium chelator, EGTA (data not given). Nonetheless, some transcellular absorption cannot be excluded. Even the more polar classical markers of paracellular absorption, mannitol and PEG 400, are reported to be partially absorbed from the small intestine *via* the transcellular pathway (19), although it is noted that these investigators based their analysis on total radioactivity, which does not lend itself to unambiguous and specific statements.

Structural Requirements

The most pronounced effect noted was the sharp decrease in oral bioavailability with increasing molecular weight, with the drop from 30% for PheAla to only 1% for PheAlaValAla. A clear molecular weight dependence has also been demonstrated for the oral absorption of PEG oligomers in the molecular weight range 282–1296 (20), and of hydrophilic drugs such as atenolol (Mwt 266) and teicoplanin (Mwt 1800), which have oral bioavailabilities in human of 50% (21) and 1% (22), respectively. Molecular weight is just one measure of molecular size, others being cross-sectional diameter, hydrodynamic volume, which can be obtained both experimentally and by computer techniques. Which of these parameters are the attributes or cluster of attributes of molecular size which best correlate with paracellular permeability has yet to be unambiguously defined.

The second potentially important factor influencing permeability is the charge properties of a molecule. Relatively small (212 Daltons), highly-charged anions, such as ferrocyanide ion ($Fe(CN)_6^{4+}$), are completely excluded from transport across the perfused rat intestine (4), while uncharged polar solutes, such as creatinine and inulin, are cleared from the perfused fluid (11). Contrary to expectation, we found that net charge had a negligible effect on the oral bioavailability of D-dipeptides *in vivo*, supporting the lack of effect of charge on the permeability of D-dipeptides through either Caco-2 cell monolayers or intestinal rat tissue *in vitro* (personal observations). Furthermore, the permeabilities of hexa- and penta-peptides were not influenced by negative charge (8) (9). The discrepancies observed as compared to the data from Pappenheimer and Reiss (11) may be due to the charge density, as the maximum net charges in the peptides we studied are only +1, −1 and zero. On the other hand, the net charge density in ferrocyanide is much higher. Both further experimental data and molecular modelling are needed to clarify this issue.

In conclusion, D-oligopeptides provide a useful series of model compounds to study the structural requirements for the paracellular absorption. Compounds with molecular weights up to around 400 Daltons can be reasonably absorbed *via* the paracellular route. It is clear that molecular size, which is expressed as molecular weight in this study, is a critical determinant for the absorption of paracellularly absorbed drug. The *in vivo* bioavailability is not influenced by net charge for peptides varying in charge from −1 through to +1. Further characterization of the molecular properties using molecular modelling techniques and other methods is essential to understand the details of paracellular absorption and are now in progress in our laboratories.

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