Size-Dependent Permeability of Hydrophilic Probes Across Rabbit Colonic Epithelium

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ABSTRACT

Colon-specific delivery of metabolically labile molecules, such as proteins and peptides, is of particular interest in pharmaceutical research. Among the factors that may influence the permeability of drug molecules across colonic mucosa are their molecular weight and geometry. The purpose of this study was to evaluate the influence of molecular geometry on in vitro permeability across rabbit distal colonic epithelia. Permeability of radiolabeled hydrophilic probes with different molecular weights and geometries across isolated rabbit distal colonic tissue was evaluated by means of the Ussing chamber technique. The hydrodynamic radii of the probes (an indicator of molecular geometry) were estimated by theoretical models as well as dynamic light scattering. We conducted the permeability studies in the presence and absence of the epithelial cells to evaluate the contribution of the underlying connective tissue to the overall in vitro permeability across the colonic mucosa. The rank order of the permeability of the markers was mannitol > lactulose > polyethylene glycol (PEG) 400 > PEG 900 > PEG 4000, which is consistent with their molecular weights and estimated hydrodynamic radii. The permeability of inulin, a polyfructose molecule with a molecular weight of about 5000, however, was approximately the same as that of PEG 900 (molecular weight about 900). When the epithelial cells were removed, for the homologous series of PEGs, the permeabilities were proportional to their free diffusion coefficients in water. It appears that for the PEG and lactulose probes, theoretical estimation of the hydrodynamic radii, which assumes the molecules to be spherical in shape, provides a good basis for the dependence of permeability on geometry. The relatively high permeability of inulin seems to be due to its compact structure. The PEG permeability values in the absence of epithelial cells, in combination with their diffusion coefficients, indicate that the underlying connective tissue does not contribute to the overall permeability of these molecules across colonic mucosa in vitro.

Site-specific drug delivery to the colon has received much attention in recent years as an approach to avoid metabolism within the small intestine and thereby increase systemic delivery (Rubinstein et al., 1992; Friend, 1992; Kopecˇek et al., 1992). A variety of p.o. drug delivery systems have been designed that release compounds in the colon (Brandsted and Kopecˇek, 1991; Friend and Chang, 1984). Passive diffusion across the colon can be either a transcellular or a paracellular pathway (Smith et al., 1992). Although passive transcellular transport is in general limited to relatively small hydrophobic compounds, paracellular transport of hydrophilic drugs is restricted by tight junctional complexes (Madar, 1989). Intestinal permeability of different hydrophilic probes has been related to factors such as the molecular weight and volume of the molecules evaluated (Hamilton et al., 1987) and their smallest cross-sectional diameter obtained by computer modeling (Hollander et al., 1988). Although these studies provide insight into the effect of molecular geometry on permeation, they are based on human absorption and urinary recovery data, so nonintestinal factors such as metabolism, distribution and renal clearance of the markers are potential sources of misinterpretation.

In an attempt to eliminate the pre- and postmucosal factors that may affect the assessment of intestinal permeability in in vivo studies, investigators have employed isolated tissues of the small and large intestine (Pantzar et al., 1994; Sawada et al., 1989) and intestinal cell cultures (Ma et al., 1992; Artursson et al., 1993; Conradi et al., 1991). Results from these studies suggest that in addition to molecular weight, properties such as molecular geometry, flexibility, hydrophobicity, charge and molecular stability influence permeability.

One of the differences between the absorption barrier presented by mucosal tissues in vitro and in vivo is the potential...
contribution of the lamina propria to the absorption barrier in vivo. In vitro, the absorbed compound is carried away by the subepithelial capillary network; in vitro, the compound has to diffuse across the lamina propria as well. The specific aims of this research are 1) to investigate the influence of the geometry of passively absorbed hydrophilic molecules on their permeability across rabbit distal colonic epithelia, and 2) to evaluate the contribution of the underlying connective tissue to the in vitro permeability across the colonic mucosa.

Materials and Methods

Marker molecules. [14C]-mannitol was used as received from New England Nuclear (NEN, Boston, MA). [3H]-PEG 400 from American Radiolabeled Chemicals (ARC, St. Louis, MO), [3H]-PEG 900, [3H]-PEG 4000, [3H]-inulin (NEN) and [3H]-lactulose (ARC) were purified using a PD-10 column (Pharmacia, Uppsala, Sweden). Briefly, 0.5 ml of the solution received from the manufacturer was applied on a PD-10 column with distilled water as the eluent, and 25 fractions (0.5 ml) were collected. [3H]-inulin, received in solid form, was solubilized in water before the purification process by heating the material to 50°C. For each fraction collected, 10 μl was mixed with 10 ml of scintillation liquid (Ready Safe, Beckman Instruments, Inc., Fullerton, CA), and radioactivity was determined in a Packard Tri-Carb 4640 scintillation counter. For [3H]-PEG 4000, [3H]-PEG 900 and [3H]-PEG 4000, four fractions with the highest radioactivity were mixed and used as the stock solution, and their purity was checked for the absence of low-molecular-weight fractions by mixing 10 μl of each solution with 0.49 ml of distilled water and eluted on a PD-10 column (Pharmacia) with distilled water as the eluent, followed by collection of 25 fractions of 0.5 ml, and their radioactivity was determined as described above. For [3H]-lactulose and [3H]-inulin, two fractions with the highest radioactivity were collected and used as the stock solution. The purity of [3H]-lactulose stock solution was checked by injecting 25 μl of the stock solution (4 μCi/ml) onto a 250 × 4-mm Dionex Carbopac PA1 anion exchange column (Dionex U.K., Camberley, Surrey, U.K.) and eluted with 0.15 mol/l of NaOH, at a flow rate of 1 ml/min at room temperature. Detection was by pulsed amperometric detection on an HPLC system (Dionex) equipped with a FLO-ONE/Beta series A-100 flow radio-chromatography detector (Radiomatic, Tampa, FL). By this purification method, the purity of [3H]-lactulose was improved from 85% (as received from the manufacturer) to 97% after purification. The purity of the [3H]-inulin stock solution was checked by the procedure described for the [3H]-PEG series. The unlabelled PEG molecules were purchased from Aldrich (Milwaukee, WI). Except where otherwise indicated, all compounds used in this study were purchased from Sigma Chemical Co. (St. Louis, MO).

DLS. DLS measurements for PEG 900 (concentration 15% w/v), PEG 4000 (5% w/v) and inulin (1% w/v) were performed in duplicate by means of a standard laser light multianceptometer with a He-Ne and argon-ion laser and a 78-channel BI 2030, multibit, multiaxial autocorrelator (Brookhaven Instruments, Holtsville, NY). The samples were maintained in toluene at 22°C to match the refractive index. Bicarbonate buffer was used as the solvent.

Tissue preparation. Fifteen to twenty-centimeter sections of distal colon from New Zealand White rabbits were cut along the mesenteric border and rinsed of luminal contents with ice-cold bicarbonate buffer containing 112 mM NaCl, 5 mM KCl, 1.2 mM CaCl2, 1.2 mM MgCl2, 0.4 mM Na2HPO4, 1.6 mM Na2HPO4 and 25 mM NaHCO3. The epithelium was stripped of the underlying mucus layer as described previously (Jett et al., 1991). Tissues were mounted in Ussing chambers exposing a surface area of 1.13 cm2 and bathed with 10 ml of bicarbonate buffer (37°C) and gassed with 95% O2/5% CO2. Under these conditions, the pH of the solution was 7.4.

Electrical measurements. Tissues were equilibrated for 90 min, after which the PD (mV), with reference to the serosal bathing solution, and the Isc (amp/cm2) were measured with automatic voltage clamps (Physiologic Instrument VCC600, Precision Instrument Design, Tahoe City, CA) at the desired time intervals, and R0 (ohm · cm2) was calculated from the open-circuit PD and Isc (R0 = PD/Isc). When the basal PD was less than 0.5 mV, R0 was calculated from the current required to clamp the PD briefly (less than 5 sec) to 5 mV.

Permeability measurements. Ten mM mannitol was added to the mucosal bathing solution, and 2 mM mannitol and 8 mM glucose to the serosal bathing solution. Lactulose, PEG 400, PEG 900 and PEG 4000, were added to both bathing solutions to a final concentration of 1 mM, and inulin to a final concentration of 0.1 mM, in order to avoid nonspecific binding of radioactive tracers. After the 90-min equilibration, 3 to 5 μCi of the compound under study and 3 μCi of [14C]-mannitol were added to the mucosal bathing solution, and the serosal chamber was sampled at 15, 30, 45, 60 and 75 min (control period) after the addition of the radiolabeled markers. Immediately after collection of the 75-min sample, 0.5 ml (final concentration 5% v/v) of CapMul MCM (Karlshamns, OH), a mixture of medium-chain glycerides, was added to the mucosal bathing solution, and the serosal chamber was sampled again at 85, 95, 105, 115 and 130 min (CapMul period). Fluxes were calculated from 1-ml samples taken from the receiver side (these volumes were replaced with 1 ml of bathing solution to maintain constant volume) and 50-μl samples from the donor side (these volumes were not replaced). Samples were mixed with 10 ml of scintillation liquid (Ready Safe, Beckman Instruments), and radioactivity was counted in a Packard Tri-Carb 4640 scintillation counter. Permeability (P, cm/hr) was calculated by dividing the transepithelial flux by the concentration of the transported molecule in the donor solution. In all experiments, the permeability of [14C]-mannitol was measured simultaneously with the permeability of [3H]-labeled markers. [14C]-mannitol transport served as an internal reference (Marks et al., 1991), and the permeability index was calculated by dividing the permeability of the compound by the permeability of mannitol in the same tissue. Isc and R0 were determined concurrently with fluxes as a measure of tissue viability and integrity. At the end of the [3H]-inulin permeability experiments, solutions in both the serosal and the mucosal reservoirs were collected and stored at ~20°C until further analysis.

Analysis of [3H]-inulin in mucosal and serosal solutions. Before analysis, all solutions were thawed at room temperature. The mucosal solution was then centrifuged at 30,000 × g to separate the phases, and the aqueous phase was analyzed. Samples were applied on a PD-10 column (Phexadex G-25, Pharmacia) with distilled water as the eluent, and 25 fractions of 0.5 ml were collected. Either 10 μl or the entire 0.5 ml was analyzed for radioactivity by the procedure described above (Marker molecules). Radioactivity was measured in terms of dpm.

Determination of retention times and Pd of markers. The retention times of unlabeled PEG 900, PEG 4000 and inulin were determined by size exclusion chromatography using a FPLC system with a Superose 6 HR 10/30 column (Pharmacia) eluted with a phosphate buffer system containing 1.136 g Na2HPO4, 0.292 g KH2PO4, 8.47 g NaCl and 0.5 g NaN3 per liter of distilled water, final pH 7.2. The Mw, Mn and Pd of inulin were estimated on the same column calibrated with dextrans of known molecular weight. The Mw, Mn and Pd of [14C]-mannitol were determined on a Superose 12 HR 10/30 column (Pharmacia) using PEG molecules of known molecular weight.

Oil/water partitioning. CapMul-saturated bicarbonate buffer containing the radiolabeled marker was mixed with an equal volume of bicarbonate buffer-saturated CapMul MCM. The mixture was equilibrated in triplicate for 2 hr at 37°C and centrifuged at 30,000 × g. Then, after separation of the phases, radioactivity in each phase was counted. The partition index is defined as the concentration ratio of radioactivity in the oil phase to that in the aqueous phase.
Results

The hydrodynamic radii and “basal” permeabilities of the markers evaluated in this study are presented in table 1, along with their molecular weights. Results of the hydrodynamic radius determinations and permeability measurements in the control period (“basal”) indicate that when an average number for permeability is considered, in the size range of mannitol, lactulose and PEG 400, no correlation is found between molecular weight or hydrodynamic radius on the one hand and permeability on the other. For the homologous PEG series with substantial differences in molecular weight, however, the permeability in the control period is in the rank order PEG 400 > PEG 900 > PEG 4000, which is consistent with previous reports in the literature (Yeh et al., 1991a; Ma et al., 1992). Despite its higher molecular weight (about 5000), the permeability of inulin is comparable to that of PEG 900.

When mannitol, lactulose, PEG 400, and PEG 900 are ranked on the basis of their permeability index, a more resolved picture emerges (fig. 1, A and B). In the calculation of permeability index, for each tissue the permeability of the [3H]-labeled compound is divided by the permeability of [14C]-mannitol in the same tissue, and then an average of the values is obtained by adding the indices for each compound and dividing that sum by the number of tissues involved for that compound. In this case, a correlation is found between the permeability index and molecular weight, and the rank order for permeability index is mannitol > lactulose > PEG 400 > PEG 900 (fig. 1, A and B).

The hydrodynamic radii of mannitol and lactulose were estimated from the Stokes-Einstein relationship with correction for small molecular weight (Steward, 1982), and those of the PEG series and inulin were estimated on the basis of PEG diffusion studies in polymer gels and solutions (Johansson et al., 1991a; Johansson et al., 1991b) and were also measured in our laboratory by DLS (table 1). For mannitol, lactulose and PEG 400, the hydrodynamic radii based on computer modeling data published in the literature (Hollander et al., 1988) are also recorded in table 1. With the exception of PEG 400 and inulin, the theoretical calculations are in close agreement with the experimental measurements (PEG 900 and PEG 4000) and the computer modeling data (mannitol and lactulose). In the case of PEG 400, Hollander et al. (1988) reported the smallest cross-sectional radius of the molecule as the important parameter in permeability measurements, whereas we calculated the radius of an equivalent solid sphere that under the same conditions would have the same diffusion coefficient. For inulin, the theoretical value of 10 Å is based on the radius of a cylinder as calculated by Middleton (1977), as compared with the hydrodynamic radius measurement by DLS, where the molecule is treated as a hard sphere.

To determine the effect of molecular geometry, expressed in terms of hydrodynamic radius, on permeability, for lower-

TABLE 1

<table>
<thead>
<tr>
<th>Marker</th>
<th>Mw</th>
<th>Radius (Å)</th>
<th>N′</th>
<th>P × 10⁻⁹ (cm/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[14C]-mannitol</td>
<td>184</td>
<td>3.9ᵃ, (3.4)ᵇ</td>
<td>40/13</td>
<td>4.1 ± 0.35</td>
</tr>
<tr>
<td>[3H]-lactulose</td>
<td>343</td>
<td>5.1ᵃ, (4.8)ᵇ</td>
<td>6/3</td>
<td>4.5 ± 0.79</td>
</tr>
<tr>
<td>[3H]-PEG 400</td>
<td>400</td>
<td>5.5ᵃ, (2.7)ᵇ</td>
<td>10/4</td>
<td>4.5 ± 0.87</td>
</tr>
<tr>
<td>[3H]-PEG 900</td>
<td>900</td>
<td>8.0ᵃ, (8.4)ᵈ</td>
<td>9/3</td>
<td>1.7 ± 0.31</td>
</tr>
<tr>
<td>[3H]-PEG 4000</td>
<td>4000</td>
<td>15.9ᵃ, (16.4)ᵈ</td>
<td>6/3</td>
<td>0.7 ± 0.07</td>
</tr>
<tr>
<td>[3H]-inulin</td>
<td>~5000</td>
<td>10.0ᵃ, (13.9)ᵈ</td>
<td>9/4</td>
<td>2.1 ± 0.24</td>
</tr>
</tbody>
</table>

ᵃ Estimated from Stokes-Einstein relationship with small-molecule correction reported by Steward, (1982), the value for lactulose is assumed to be similar to that for sucrose, a molecule similar in structure.
ᵇ Based on smallest cross-sectional diameter determined by computer modeling, reported by Hollander et al. (1988).
ᶜ Estimated from the relation \(D_0 = 7 \times 10^{-5} \times M_w^{-0.45} \times kT/6 \eta R_m\), where \(K\) is the Boltzmann constant, \(T = 298\) K, the viscosity of water at 298 K is \(\eta = 0.000891\) kg/msec (Handbook of Chemistry and Physics, 1988) and \(R_m\) is the hydrodynamic radius (Johansson et al., 1991a, b).
ᵈ Measured by DLS in our laboratory.
ᵉ Based on the radius of a cylindrical solvated molecule (Middleton, 1977).
ᶠ Average values ± standard errors × 10⁻⁵.

To determine the effect of molecular geometry, expressed in terms of hydrodynamic radius, on permeability, for lower-

![Fig. 1. Permeability index of probes as a function of probe molecular weight](https://jpet.aspetjournals.org/article-figures/749-Fig1.png)
molecular-weight compounds we plotted the permeability indices as a function of probe radii (fig. 1B). Results show that there is a correlation between the theoretically determined hydrodynamic radii and the permeability indices of these compounds.

To examine the reason(s) for the relatively high permeability value of inulin (Mw about 5000) compared with PEG 900 and PEG 4000 (table 1), we further analyzed the mucosal and serosal solutions of inulin experiments for the presence of low-molecular-weight impurities. Figure 2A, an elution profile of the mucosal solution, shows a [3H] peak around fraction 7 (inulin), a small [3H] peak around fraction 14 (impurity in inulin) and a [14C] peak around fraction 14 ([14C]-mannitol was present as an internal reference to check the tissue integrity). Results demonstrate the presence of small amounts of impurity (less than 3.70% based on [3H] dpm in peak 14 divided by [3H] dpm in peak 7) and confirm that the impurity is tritium-labeled, small-molecular-weight saccharides (it elutes in the same fractions as [14C]-mannitol). The analysis of the serosal solution (fig. 2B) of the same tissue revealed the presence of a higher proportion of tritium-labeled low-molecular-weight compounds than in the mucosal solution. Thus part of the reason for the apparently high inulin permeability may be the presence of low-molecular-weight impurities.

To evaluate the extent to which this phenomenon may have affected the permeability value of inulin, we calculated the areas under the curves for [3H] peaks in Figures 2A (mucosal) and 2B (serosal) by adding the dpm values involved in each area labeled A, B, A’ and C as shown. Then the radioactive counts used for permeability calculations were modified in accordance with the formula

$$[(C - A') \times (B/A') \times DPM_1 = DPM_2$$

where DPM1 is the original disintegrations per minute obtained from scintillation counting, and DPM2 is the corrected value used for calculation of permeability. When these corrected dpm values were used in permeability calculations, the permeability value was reduced by about 20%. The new permeability value excludes the permeability of low-molecular-weight compounds and is approximately equal to that of [3H]-PEG 900 ([3H]-inulin: 0.0021 × 0.8 = 0.0017 cm/hr, [3H]-PEG 900: 0.0017 cm/hr; table 1). Therefore, the relatively high permeability of inulin compared with PEG 900 and PEG 4000 on the basis of molecular-weight considerations remained unexplained.

To test the hypothesis that inulin is a more compact molecule than the PEGs, a phenomenon that might explain the relatively high permeability value of inulin, we determined the retention times of PEG 900, PEG 4000 and inulin by size exclusion chromatography using a Superose 6 column (Pharmacia). The results indicate the following rank order: PEG 4000 (44.8 min) < inulin (48.2 min) < PEG 900 (49.6 min), the last two molecules having closer retention times (table 2).

The Pd of inulin, another factor that might affect its permeability, was estimated using a Superose 6 column calibrated with dextrans of known molecular weight. The results indicate that the estimated Pd values for PEG 900, PEG 4000 and inulin are in the same range (table 2), which rules out the possibility that differences in Pd explain the permeability results.

To assess whether the lamina propria is a significant barrier to overall permeability in in vitro studies, we performed permeability studies in de-epithelialized tissues. Histopathological studies have shown that distal colonic tissue can be de-epithelialized upon incubation with 1% v/v CapMul (Yeh et al., 1995). De-epithelialization is associated with a decrease in permeability. Results demonstrate the presence of small amounts of impurity (less than 3.70% based on [3H] dpm in peak 14 divided by [3H] dpm in peak 7) and confirm that the impurity is tritium-labeled, small-molecular-weight saccharides (it elutes in the same fractions as [14C]-mannitol). The analysis of the serosal solution (fig. 2B) of the same tissue revealed the presence of a higher proportion of tritium-labeled low-molecular-weight compounds than in the mucosal solution. Thus part of the reason for the apparently high inulin permeability may be the presence of low-molecular-weight impurities.

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![Fig. 2. Radioactivity profiles of mucosal and serosal solutions of an inulin permeability study eluted on a PD-10 column (Sephadex G-25, Pharmacia) using water as eluent. Twenty-five fractions (0.5 ml) were collected and counted for radioactivity. A) Mucosal solution; 0.5 ml of the aqueous phase was fractionated, and 10 μl of each fraction was analyzed for radioactivity; [3H]-inulin (C), [4C]-mannitol (Δ). B) Serosal solution of the same permeability experiment as in panel A; 0.5 ml of the serosal solution was fractionated, and whole fractions were analyzed for radioactivity. Mannitol elution is not shown here.](image-url)

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Mn</th>
<th>Mn</th>
<th>Pd</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 900</td>
<td>1100</td>
<td>950</td>
<td>1.16</td>
<td>49.6</td>
</tr>
<tr>
<td>PEG 4000</td>
<td>4700</td>
<td>4100</td>
<td>1.15</td>
<td>44.8</td>
</tr>
<tr>
<td>Inulin</td>
<td>6000</td>
<td>5150</td>
<td>1.17</td>
<td>48.2</td>
</tr>
</tbody>
</table>

Table 2: Properties of macromolecular markers determined by size exclusion chromatography.
crease in resistance. To achieve a more rapid de-epithelialization, we added 5% v/v CapMul (fig. 3). Rabbit distal colon exhibited an average resistance of 200 ohm · cm² in the control period, with a reduction to around 10 ohm · cm² after the addition of 5% v/v CapMul (fig. 4A). The decrease in resistance is associated with an increase in permeability in a time-dependent manner, and the difference in the permeability of the markers in the CapMul period, where the tissues are de-epithelialized, is dependent on molecular weight (fig. 4B).

Molecules that show significant partitioning into the penetration enhancer phase do not show enhanced absorption (Sekine et al., 1985; Constantinides et al., 1994). To investigate whether partitioning of the PEGs into the CapMul phase might be responsible for the reduced enhancement of permeability, we measured the partitioning of mannitol and the PEGs in CapMul and calculated the effective concentration of the probes in the aqueous phase in the presence of the penetration enhancer (table 3). Results indicate that greater than 99% of the compounds are present in the aqueous phase during the CapMul period.

At the end of the CapMul incubation period, the maximal permeabilities of the compounds were measured, and the maximal permeabilities of the PEGs relative to mannitol (P_{PEG}/P_{man} at the end of CapMul period) were calculated and compared with the free diffusion coefficient of these compounds in water at 37°C relative to mannitol (D_{PEG}/D_{man}) (table 3). Results show that the permeability ratios are roughly the same as the free diffusion coefficient ratios.

**Discussion**

Various hydrophilic probes have been used clinically to assess the extent of intestinal permeability in the human for both the healthy and the diseased state (Philipsen et al., 1987; Chadwick et al., 1977; Nasrallah and Iber, 1969; Bjaranson, 1994). In general these studies focus on the permeability of the small intestine, and in addition to the aforementioned pre- and postintestinal factors, results are influenced by issues such as subject-to-subject variation in mucosal area, thickness of the unstirred water layer and intestinal transit (Travis and Menzies, 1992). The Ussing chamber technique provides an in vitro means to study the permeability across the epithelial cells of the specific regions of the intestine. Using this technique, it is possible to exclude nonintestinal factors and minimize intestinal factors such as variation in the mucosal area and the unstirred water layer.

In this study, we determined the permeability of six commonly used hydrophilic probes across rabbit distal colonic epithelia (table 1). These permeabilities were then correlated with molecular weight and the hydrodynamic radii of the
respective probes. In the range of molecules that are similar in molecular weight (mannitol, lactulose and PEG 400), no significant difference in permeability was observed (table 1). However, when the permeability index was considered, a correlation was found between molecular weight and the hydrodynamic radii on the one hand and permeability on the other (fig. 1). The reason for this observation seems to be the inherent presence of animal-to-animal and tissue-to-tissue variation in these types of studies, and for compounds that have close permeability values, it is not possible to differentiate their relative permeabilities unless a uniform membrane is used or a correction is applied. One approach to this problem is to measure the permeability of the compound of interest relative to mannitol, a known paracellular marker, in a given tissue and then correlate the resulting permeability index with probe properties such as molecular weight and geometry. The results shown in fig 1A indicate that as the molecular weight increases, the permeability index of the markers decreases. Although this correlation appears to be linear (r = 0.989), caution must be exercised because previous studies have shown that there is a sharp decrease in permeability across colonic tissue once the molecular weight passes a critical range between 300 and 400 Da (Kimura et al., 1994; Artursson et al., 1993). Similar observations were reported when the effect of PEG molecular weight on coloanal transport was studied; permeability was drastically reduced once the molecular weight passed a critical range of 370 to 500 (Liaw and Robinson, 1992). Therefore, in interpreting the dependence of permeability on molecular weight and radius, (fig. 1, A and B) more data points are needed to establish the pattern of permeability reduction as a function of size in rabbit distal colon.

The importance of probe geometry in intestinal permeability has been emphasized by various investigators (Hamilton et al., 1987; Hollander et al., 1988). Ma and Hollander have investigated the size-dependent permeability of a series of hydrophilic markers, including mannitol and PEG 400, by using small intestinal cell cultures (Ma et al., 1992) or analyzing published human permeability data (Hollander et al., 1988). In these studies, the intestinal permeability is correlated best with the smallest cross-sectional diameter of the probes (determined by computer modeling), and it is argued that mannitol and low-molecular-weight PEGs such as PEG 400 diffuse through the pores more readily than molecules with a higher smallest cross-sectional diameter such as Cr-EDTA (Cr-labeled ethylenediaminetetraacetate) and lactulose. To test this hypothesis with colonic permeability, we evaluated mannitol, lactulose, PEG 400, PEG 900, PEG 4000 and inulin as permeability probes. The estimated hydrodynamic radii (R_H) of the probes are reported in table 1. For mannitol and lactulose, the R_H values estimated from the Stokes-Einstein relationship with small-molecule correction (Steward, 1982) were in close agreement with computer modeling data obtained by Hollander et al., (1988). For the PEG series, the values were estimated from a model used to study the diffusion of monodisperse fractions of PEG (300 < M_w < 4000) in different polymer gels and solutions (Johansson et al., 1991a; 1991b). This model is based on three assumptions. 1) In the interaction between a solute and its surroundings, in addition to the Stokes-Einstein frictional term, a chemical term that reflects the retardation of solute diffusion that results from attractive forces between the solute and the membrane must be taken into account. 2) This retardation is due to the structure of a static network that is not distorted by the diffusing species. 3) The structure of the network is composed of a series of cylindrical pores. Independent measurements of R_H for PEG 900 and PEG 4000 in our laboratory using DLS techniques yielded results that were in agreement with the theoretical estimations (table 1). However, there is an almost 2-fold difference between the theoretically estimated radius for PEG 400 based on the above model (5.5 Å) and the smallest cross-sectional diameter estimated by computer modeling (2.7 Å from Hollander et al., 1988), where the molecule is assumed to have a linear structure. When permeability index was plotted against the theoretical estimate of hydrodynamic radii, there was a good correlation (r = 0.960) between permeability across colonic tissue and the hydrodynamic radii (fig. 3B). Our results suggest that in the assessment of permeability across intestinal membranes (specifically the distal colon), the permeability of PEG 400 correlates better with the hydrodynamic radius, where the molecule is assumed to have a spherical shape, and to have interactions with the membrane, than with the smallest cross-sectional diameter estimated by computer modeling, where the molecule is assumed to be linear. This may be due to factors such as hydration of the probe (Eagland et al., 1993), its conformation and diffusion behavior in solution (Liu and Parsons, 1969) and the propensity for hydrogen bonding between the probes containing hydroxyl groups and the membrane (Conradi et al., 1991).

The compactness of inulin may explain its higher permeability relative to PEG 900 and PEG 4000.
PEGs that have extended chains (Eagland et al., 1993; Liu and Parsons, 1969), based on physicochemical data derived using a space-filling model, it is suggested that inulin has a compact cylindrical configuration where fructofuranose units are (2−1) linked to form a helical structure with a semi-length of 25 Å and a radius of 10 Å for the hydrated molecule (Middleton, 1977). Results of size exclusion chromatography studies (retention times in the order of PEG 4000 (44.8 min) < inulin (48.2 min) < PEG 900 (49.6 min), table 2), suggest that despite its higher molecular weight, inulin has a molecular configuration that contributes to its retardation in size exclusion chromatography (i.e., later elution). This retention time being high relative to that of PEG 4000 and similar to that of PEG 900 may in part explain the permeability value obtained in our studies. On the other hand, we have found the hydrodynamic radius of inulin as measured by DLS to be 13.9 Å, closer to that of PEG 4000 (16.4 Å) and substantially higher than that of PEG 900 (8.4 Å), whereas the retention time of inulin is closer to that of PEG 900 in size exclusion chromatography (table 2). We conclude that in the correlation of colonic permeability with size, when different classes of molecules are considered, the Stokes-Einstein radius determined by hydrodynamic methods alone may not necessarily reflect the relative permeability of the compounds of interest, and other factors such as molecular configuration need to be considered.

In conclusion, the in vitro permeability of hydrophilic markers was shown generally to decrease with an increase in molecular weight and hydrodynamic radius. The relatively higher permeability of inulin appears to be due in part to the compactness of its structure. These results indicate that conclusions about protein and peptide permeability based on permeability measurements with “model” molecules need to be interpreted with caution. Finally, the underlying connective tissue does not contribute to the overall permeability of these molecules across colonic mucosa in vitro.

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References

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